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PIM SIGNALING IN CANCER – REGULATION OF CELL MOTILITY AND METABOLISM

Niina Santio

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♡ Perheelleni ja ystävilleni ♡

ABSTRACT

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Breast and prostate cancer belong to the top four most common cancers. Even though several effective therapy methods are available, cancer still remains one of the leading causes of death all over the world. PIM kinases have been reported to be upregulated in various cancers, and to promote tumorigenesis there. Furthermore, PIM kinases have become novel targets for anti-cancer drug development, especially because of their specific structure, which enables the design of selective inhibitors.

While the ability of PIM kinases to support cell survival is well known, only recently have the effects on cell motility raised interest. In addition, very little is known about the importance of PIM kinases in cell metabolism, which is often altered in cancer cells. This thesis focuses on studying the role of PIM kinases in prostate cancer cell motility and breast cancer cell metabolism as well as breast and prostate tumorigenesis.

PIM upregulation was shown to increase prostate cancer cell motility and tumor progression, while a novel PIM inhibitor DHPCC-9 decreased tumor size and the number of metastases in an orthotopic mouse model for prostate cancer. Similar results were also gained after culturing prostate cancer cells on a chicken embryo chorioallantoic membrane. Thereafter the study concentrated more precisely on the PIM-dependent signaling pathways. Interestingly, it was found that the formation of prostate cancer metastases seems to depend on PIM-mediated activation of the CXCR4/CXCL12 chemokine pathway as well as the NOTCH1 receptor intracellular domain. In addition, PIM kinases inactivate FOXP3 as well as GSK3B and support prostaglandin and integrin-mediated cell motility in prostate cancer. In breast cancer, cell energy metabolism depends on the interaction of PIM kinases with the NOTCH1 intracellular domain. NOTCH1 activation by PIM also promotes hormone-dependent breast tumor growth in the chorioallantoic membrane model.

Keywords: breast cancer, metabolism, metastasis, motility, PIM inhibitors, PIM kinases, prostate cancer

TIIVISTELMÄ

Niina Santio o.s. Tiensuu
PIM-KINAASIT SYÖVÄSSÄ – SOLUN LIIKKUVUUDEN JA AINEENVAIHDUNNAN SÄÄTELY
Turun yliopisto, Matemaattis-luonnontieteellinen tiedekunta, Biologian laitos, Genetiikan ja fysiologian osasto sekä Turun yliopiston tutkijakoulu, Lääke-tutkimuksen tohtoriohjelma
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Eturauhas- ja rintasyöpä kuuluvat yleisimpiin syöpätyyppeihin. Huolimatta useista tehokkaista syövänhoitokeinoista, syöpä on edelleenkin yksi johtavista kuolinsyistä maailmassa. PIM-kinaasien on havaittu ilmentyvän voimakkaasti useissa syöpätyypeissä ja edistävän sekä syövän syntyä että kehittymistä. PIM-kinaaseista on tullut myös mielenkiintoinen syöpälääkekehityksen kohde, mikä johtuu osittain siitä, että niiden rakenne mahdollistaa hyvin valikoivan PIM-kinaaseihin kohdistettavan lääkekehityksen.

PIM-kinaasien kyky edistää solun elinkykyä tunnetaan hyvin, kun taas vaikutukset solun liikkuvuuteen ovat heikosti tunnettuja. PIM-kinaasien vaikutuksista solun aineenvaihduntaan ei myöskään tiedetä paljon, vaikka aineenvaihdunnan muutokset ovatkin erittäin tärkeitä syövän etenemisen kannalta. Tässä työssä on tutkittu PIM-kinaasien vaikutuksia eturauhassyöpäsolujen liikkuvuuteen ja rintasyöpäsolujen aineenvaihduntaan sekä eturauhas- ja rintasyöpäkasvainten kehittymiseen.

PIM-kinaasien yli-ilmentämisen havaittiin edistävän eturauhassyöpäsolujen liikkuvuutta ja eturauhassyövän kehittymistä, kun taas PIM-inhibiittori DHPCC-9 hidasti kasvaimen kasvua ja vähensi etäpesäkkeiden määrää ortooppisessa eturauhassyöpämallissa hiirillä. Samankaltaisia tuloksia saatiin myös, kun eturauhassyöpäsoluja viljeltiin kanan alkiokalvolla. Näiden havaintojen jälkeen tutkimukset kohdennettiin PIM-kinaasien viestintäreitteihin. Tulokset viittaavat PIM-kinaasien edistävän eturauhassyöpäkasvainten kehittymistä CXCR4/CXCL12-kemokiinivälitteisen signaalireitin sekä NOTCH1-reseptoriproteiinin aktivaation kautta. Osa PIM-kinaasien liikkuvuusvaikutuksista liittyi proteiinien FOXP3 ja GSK3B inaktivoimiseen sekä prostaglandiini- ja integriini-välitteiseen soluviestintään. Lisäksi havaittiin, että PIM-välitteinen NOTCH1-proteiinin aktivaatio säätelee rintasyöpäsolujen aineenvaihduntaa ja edistää hormoni-riippuvaista rintasyöpäkasvainten kasvua kanan alkiokalvomallissa.

Avainsanat: aineenvaihdunta, eturauhassyöpä, etäpesäkkeet, liikkuminen, PIM-inhibiittorit, PIM-kinaasit, rintasyöpä

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ABBREVIATIONS

Commonly approved abbreviations for units or chemical symbols are not described here.

ADP	adenosine diphosphate
ACTB	actin beta
AKT	v-akt murine thymoma viral oncogene homolog
AKT1S1	AKT1 substrate 1
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
ATP	adenosine triphosphate
BA	benzo [*cd*] azulene
BAD	BCL2-associated agonist of cell death
BCL2	B-cell lymphoma 2
BRCA	breast cancer
BSA	bovine serum albumin
Cd34	Cd34 molecule/ antigen
CTAK	CDC25C associated protein kinase
CAM	chorioallantoic membrane
CBF1	C promoter binding factor
CCL	chemokine (C-C motif) ligand
CDC	cell division cycle
CDKN	cyclin-dependent kinase inhibitor
COXII	cytochrome c oxidase II
CSL	C promoter binding factor/ suppressor of hairless/ *lin-12* and *glp-1*
CTAK	CDC25C associated protein kinase
CXCR	chemokine (C-X-C motif) receptor
CXCL	chemokine (C-X.C motif) ligand
DAPT	N-{N-[2-(3,5-difluorophenyl)acetyl]-(S)-alanyl}-(S)-phenylglycine tert-butyl ester
DHPCC-9	dihydropyrrolocarbazole compound 9
DMA	N,N-dimethylacetamide
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EBNA	Epstein-Barr virus nuclear antigen
ERBB	erythroblastic leukemia viral oncogene
ERK	extracellular signal-regulated kinase
FDC-P	factor-dependent cell progenitor
ETS	v-ets avian erythroblastosis virus E26 oncogene homolog
FLT3	FMS-like tyrosine kinase 3
FOX	forkhead box
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GSK	glycogen synthase kinase
GST	glutathione S-transferase
GTP	guanosine triphosphate
H3F3	H3 histone, family 3
HeLa	Henrietta Lacks
HER	human epidermal growth factor receptor
HSC70	heat shock 70kD protein 8
HSPA8	heat shock protein family A (Hsp70) member 8
IF	immunofluorescence
IFN	interferon
IHC	immunohistochemistry
ITG	integrin
JAK	Janus kinase
KD	kinase-deficient
KID	kinase induced by depolarization
KLF	Kruppel-like factor
Lyve1	lymphatic vessel endothelial hyaluronan receptor 1
MAPK	mitogen-activated protein kinase
MDA-MB	M.D. Anderson - metastatic breast
MCF	Michigan Cancer Foundation
MT-CO2	mitochondrially encoded cytochrome c oxidase II
MTOR	mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
MYB	v-myb avian myeloblastosis viral oncogene homolog
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NFATC	nuclear factor of activated T-cells
NFKB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NICD	NOTCH intracellular domain
p21, 27, 100	proteins 21, 27, 100 kDa
PC	prostate cancer
PCC-13	pyrrolocarbazole compound 13
PI3K	phosphoinositide 3-kinase
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIM	proviral integration site for Moloney murine leukemia virus
PLA	proximity ligation assay
PRAS40	proline-rich Akt substrate, 40 kDa
PRKAA	protein kinase AMP-activated catalytic subunit alpha 1
PSA	prostate-specific antigen
PTGS	prostaglandin-endoperoxide synthase
PTEN	phosphatase and tensin homolog
RAS	rat sarcoma
RBPJ	recombination signal binding protein for immunoglobulin kappa J region
RFP	red fluorescent protein
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RUNX	Runt-related transcription factor
Saos	sarcoma osteogenic
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	short interfering ribonucleic acid
SND	staphylococcal nuclease and tudor domain containing
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
TNM	tumor, node, metastases
TP53	tumor protein 53
UT-SCC	University of Turku - squamous cell carcinoma
WB	Western blotting

LIST OF ORIGINAL PUBLICATIONS

I. Santio NM*, Vahakoski RL*, Rainio EM, Virtanen SS, Sandholm JA, Prudhomme M, Anizon F, Moreau P & Koskinen PJ. PIM-selective inhibitor DHPCC-9 reveals Pim kinases as potent stimulators of cancer cell migration and invasion. Molecular Cancer 2010; 9: 279

II. Santio NM, Eerola SK, Paatero I, Yli-Kauhaluoma J, Anizon F, Moreau P, Tuomela J, Härkönen P & Koskinen PJ. Pim kinases promote migration and metastatic growth of prostate cancer xenografts. PLoS One 2015; 10: e0130340

III. Santio NM, Salmela M, Arola H, Eerola SK, Heino J, Rainio EM & Koskinen PJ. The PIM1 kinase promotes prostate cancer cell migration and adhesion via multiple signalling pathways. Experimental Cell Research 2016; 342: 113-124

IV. Santio NM*, Landor SK-J*, Vahtera L, Ylä-Pelto J, Paloniemi E, Imanishi SY, Corthals G, Varjosalo M, Manoharan Gb, Uri A, Lendahl U, Sahlgren C, Koskinen PJ. Pim kinases regulate oncogenic Notch1 signaling. Oncotarget 2016.

*equal contribution

The Roman numerals I, II, III and IV are used later on in the Materials and Methods, Results and Discussion to refer to the original publications. The original publications are attached at the end of the thesis.

1 INTRODUCTION

Cell survival and proliferation are strictly regulated functions, but in certain cases this regulation can be altered leading to uncontrolled cell division and formation of a malignant tumor, also known as cancer. Breast and prostate cancers are typical examples of common cancers and they are also targets of intense research. Both of these cancers consist of a wide variety of tumor types, and therefore different kinds of therapeutical approaches are needed.

Chemical reactions are catalyzed by enzymes such as kinases, which are extremely important regulators of cellular functions. Kinases phosphorylate their substrates and thereby influence their structure and functions. There are various different kinases, of which one interesting group is serine/threonine kinases including PIM kinases. These are oncoproteins, which enhance cell survival, proliferation and motility as well as regulate cell energy metabolism. PIM kinases have been shown to support tumorigenesis by preventing apoptosis and promoting cell division. Furthermore, they can interact with several known pro-tumorigenic factors. In addition, upregulated PIM expression has been detected in both hematopoietic malignancies as well as solid tumors such as breast and prostate cancer. The influence of PIM kinases on cell motility and metabolism has to date received little attention in medical studies.

Cell motility is regulated by changes in the adhesion of cells to extracellular matrix and other cells. These adhesions are mainly regulated by cell surface proteins such as integrins. Tumor growth is highly dependent on angiogenesis and the availability of nutrients and oxygen, even though tumors typically survive surprisingly well in low-oxygen conditions e.g. by utilizing anaerobic metabolism. For invasion of cancer cells from the primary site to other tissues and organs, connections to blood vessels and the lymphatic system are needed. Through the vessels cancer cells are able to migrate throughout the body to form metastases in various target organs. This is regulated not only by the capabilities of the tumor cells to stay alive in the small vessels, but also by the factors present in the target organs.

This study focused on the role of PIM kinases and PIM inhibitors in breast and prostate tumorigenesis. PIM-mediated cell motility was mainly studied in different cell and animal-based prostate cancer models, while the PIM-dependent changes in cell metabolism were measured in breast cancer models.

2 REVIEW OF THE LITERATURE

2.1 Cancer in general

2.1.1 What is cancer?

Cellular functions are regulated by a complex network of protein interactions often referred to as cell signaling. There are several special proteins needed for proper decision making during the life of a cell, while the protein signaling also works in a close proximity with other cellular components such as lipids, sugars, DNA (deoxyribonucleic acid) or RNA (ribonucleic acid). Cell survival is regulated by various mechanisms, which can sense defects in DNA structure during DNA duplication. Cell viability also depends on the production and content of important components needed for example for the formation of intracellular organs and their function as well as cell-cell and cell-matrix interactions. The cell has multiple ways to monitor its well-being and to send signals to and receive signals from the surrounding environment. When there is an error, the cell usually detects it, and if the cell is unable to correct the error, the cell commits suicide also known as apoptosis. However, on some occasions, the cell signaling might be out of order and the cell may survive even though it should have ended up in apoptosis. Therefore, it is possible that damaged cells may start proliferating at an increasing speed. The cells can form a benign tumor, but if the circumstances are favorable, the cells can continue proliferating in an uncontrolled manner to form a malignant tumor, from where the tumor cells can spread to form metastases. The general description of cancer cells involves genome instability and mutations, the ability to avoid growth suppression, immunedestruction and cell death, increased proliferative signaling, immortality, decreased energy consumption as well as induction of tumor-promoting inflammation, angiogenesis and invasion (Hanahan and Weinberg 2011).

2.1.2 History of cancer and cancer treatment

It may be accurate to say that humans have suffered from cancer throughout their whole existence, since tumors have been found even in the bones of ancient mummies (Prates et al. 2011). Even though therapy methods and awareness have developed enormously during the past decades, there are still several cancer types that are highly lethal, and cancer continues to be one of the leading causes of death in the world. In addition, many therapy methods can only slow down tumor growth and thereby postpone death for few months or years without a complete recovery of the patient.

Different kind of factors may contribute to the formation of cancer, for example exposure to carcinogenic compounds in food and air, genetic susceptibility, ultraviolet radiation, tobacco carcinogens or radioactive radiation. The most common ways to treat cancer are surgery of the primary tumor, radiation therapy and chemotherapy. In radiation therapy, high energy atomic particle waves are used to cause damage to the DNA of cancer cells to induce apoptosis

(CancerTreatment.net 2015). Chemotherapy includes a wide variety of compounds that can be used more or less selectively to promote apoptosis of rapidly proliferating cells such as cancer cells. However, the more that is known about the regulation of cancer cell behavior, the more selective the treatments that can be designed for the patients. Therefore, it is extremely important to search for information on cancer cell signaling networks as well as look for and design novel therapeutic compounds against cancer.

2.1.3 Breast cancer

Breast cancer is the second most common cancer worldwide and the most common cancer in women in Finland (Cancer Research UK 2014a, Finnish Cancer Registry 2016a). Breast cancer is often found by mammography and ultrasound followed by biopsy, while magnetic resonance imaging has also proven to be useful (Cancer Research UK 2014b; Shah et al. 2014).

There are several known risk factors for breast cancer including age, radiation to the chest, the age of first pregnancy and family history, especially the possibility of hereditary mutations in the genes *BRCA1* and *BRCA2* (*breast cancer 1 and 2*) (Hulka & Moorman 2001). In addition, it has been suggested that hormonal contraceptives or hormone replacement therapy in prevention of menopausal symptoms can be risk factors along with lifestyle factors such as obesity or high alcohol consumption (Hulka & Moorman 2001).

Breast tissue is located in both the chest and the armpits and it consists of mammary glands or lobes, in which breast cancer usually begins (Breakthrough UK 2012). Clinically, breast cancers are divided into three main subsets according to presence of receptors for estrogen and progesterone, presence of HER2 (human epidermal growth factor receptor 2) or absence of those receptors (triple-negative breast cancer) (Tang et al. 2016). However, more complicated genomic profiles have been identified for different breast cancers (Perou & Borresen-Dale 2011).

Prognosis and therapy methods are defined according to the breast cancer stage (tumor structure, tumor size and the amount of metastases) and the expression of the previously mentioned receptors (Shah et al. 2014). The common treatments for breast cancer are surgery and radiotherapy, while both chemotherapy and hormonal therapy are also often needed. Common chemotherapy agents are antracycline antibiotics, even though their possible cardiotoxicity as well as hematological abnormalities such as leukemia as side effects are slightly problematic (Crozier et al. 2014). In addition, estrogen receptor antagonists or HER2-targeted antibodies can be used in hormone receptor or HER2-positive cases (Gucalp et al. 2014; Zhang et al. 2014a).

The main target organs for breast cancer metastases are the lymph nodes, bones, lungs, liver and brain (Berman et al. 2013; Shah et al. 2014). After detection of metastases, the treatment options are in principle similar to those for the primary tumor, but the prognosis is often poor (Shah et al. 2014). However, lymph node metastases may be surgically removed and the liver lesions may be treated e.g.

with radiofrequence ablation (Shah et al. 2014; Covey & Sofocleous 2008). Osteoporosis-preventing drugs such as denosumab and bisphosphonates are used to treat bone metastases (Van Poznak et al. 2011).

2.1.4 Prostate cancer

Prostate cancer is the fourth most common cancer diagnosed yearly in the world and the most common cancer in men in Finland (Cancer Research UK 2014a, Finnish Cancer Registry 2016b). In the case of prostate cancer, the most common way of detection is analysis of PSA (prostate-specific antigen) levels. However, the connection of PSA levels and prostate cancer is not straightforward. While higher PSA levels can predict cancer, patients with more severe metastatic prostate cancer can also show relatively low levels of PSA (Esfahani et al. 2015). Therefore, biopsy or rectal ultrasound are also needed for detection of prostate cancer (Cancer Research UK 2014c).

The risk factors for prostate cancer are not as easily defined as for breast cancer, but some endogenous and exogenous risk factors have been determined. Certain hormonal and dietary factors as well as environmental agents may increase the risk (Bostwick et al. 2004). Furthermore, inherited genetic alterations have also been connected to prostate cancer (Visakorpi 2003; Edwards & Eeles 2004).

The prostate gland is located below the bladder and it surrounds the urethra (Cancer Research UK 2014d). Similar to the breasts, hormones are important in the regulation of the prostate function and hormones also contribute to prostate tumorigenesis. Prostate cancer can be classified by a TNM (tumor, node, metastases) staging system according to the size and location of the tumor and existence of lymph node and distant metastases (Cancer.net 2016a). In addition, Gleason scores can be used to grade prostate cancer according to the differentiation stage of the cells (Gleason 1966). The prognostic markers for prostate cancer are the patient's PSA level, the size of the prostate, the results of the biopsy and the cancer stage (Cancer.net 2016a). The Gleason score can also be used as a prognostic marker (Egevad et al. 2002; Humphrey et al. 2004).

Similar to breast cancer, surgery, radiotherapy and chemotherapeutic agents can be used to treat prostate cancer (Damber & Aus 2008). Hormone production can be reduced e.g. by anti-androgens or surgical (orchiectomy) or chemical castration (Damber & Aus 2008). A relapse due to a loss of hormone dependency can follow after a certain amount of time and result in a castration-resistant cancer, which often leads to the death of the patient (Liu et al. 2014a). Luckily, several novel agents have been recently approved for treating castration-resistant prostate cancers (Suzman & Antonarakis 2014). Prostate cancer often metastasizes into the bones, even though metastases can also be found in other organs such as lymph nodes, lungs, liver, pleura and adrenal glands (Bubendorf et al. 2000). For treatment of prostate cancer bone metastases, osteoporosis-preventing drugs can be used in somewhat similar way as in breast cancer (Iranikhah et al. 2014).

2.1.5 Overview of basic cancer research and drug discovery

The biology of cancer is being widely studied all over the world using various cell and animal models. The most basic research is performed *in vitro,* which often refers to a test tube, but it can also refer to a cell culture. Cell-based models include both cancer cell lines as well as non-malignant cell lines immortalized e.g. by genetically altered viral vectors. The development of novel drugs often starts with *in vitro* high-throughput screens containing hundreds or thousands of samples on one microtiter plate. Then more specific cell culture and animal -based experiments follow, after which clinical trials may be planned.

Cell culture-based experiments can be used to study cellular functions such as survival, proliferation, metabolism and motility, while several methods have been developed e.g. for studies of protein-protein interactions, transcription factor activity or gene expression. Changes in cell proliferation can be measured by counting the cell numbers or for example by analysis of active metabolism by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MTT assay is a colorimetric assay, where a tetrazolium salt is converted to formazan in the mitochondria, and this can be detected by spectrophotometry (Twentyman & Luscombe 1987). Cell metabolism can be similarly studied by detecting metabolic products e.g. by spectrophotometry. Cell migration can be followed on cell culture plates by scratch wound assays (Figure 1A), while changes in cell invasion can be detected by Boyden Chamber assays (Figure 1B) (Boyden 1962; Augustin 2004).

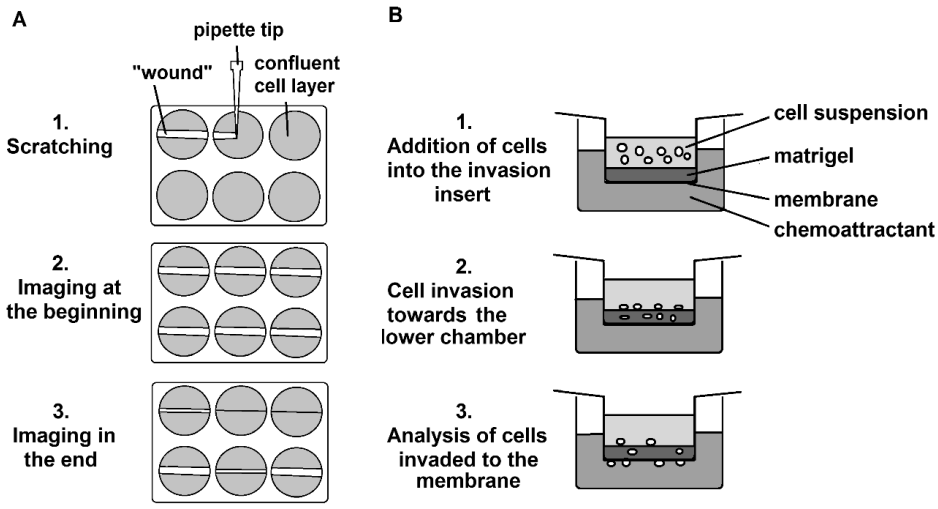

Figure 1 Methods for studying cell motility
A, Wound healing assay shows cell migration on the surface of a microtiter plate. **B,** Boyden Chamber assay is used to assess the invasion of cells through a matrigel towards a chemoattractant, which mimics cancer cell invasion (Boyden 1962).

Direct modification of proteins such as phosphorylation can be detected e.g. by using radioactively labelled phosphorus in the reaction or analysis of digested proteins by mass spectrometry. Fluorescent tags can be used for detection of proteins or other compounds by fluorescence microscopy. Fluorescence resonance energy transfer and fluorescence-lifetime imaging can be utilized for analysis of interactions between fluorescently-tagged proteins. Following protein-protein interaction a decrease can be seen in the donor fluorophore lifetime in the presence of the acceptor (Wallrabe & Periasamy 2005). On the other hand, plasmid constructs containing a transcription factor specific promoter and a detectable protein such as a luciferase, can be used for measuring transcriptional activity. Gene expression can be detected directly by multiplication of messenger RNA by polymerase chain reaction or by detection of protein levels by specific antibodies.

In principle, animal experiments should be avoided when possible, and if an animal model is needed, then the research should be performed in as low-level organisms as possible. For instance, tumor growth can be measured e.g. by 3D models, where cancer cells are embedded in a gel containing extracellular matrix components (Antoni et al. 2015). 3D cultures can also include co-cultures of cancer cells such as breast or prostate cancer cells with other cells such as endothelial cells for mimicking the natural microenvironment inside an organism (Hsiao et al. 2009; Weigelt et al. 2014). Tumor growth can also be studied *in vivo* e.g. by utilizing chicken embryos during their early development (Figure 2A-C), when the experiments are not considered as actual animal experiments (Vargas et al. 2007).

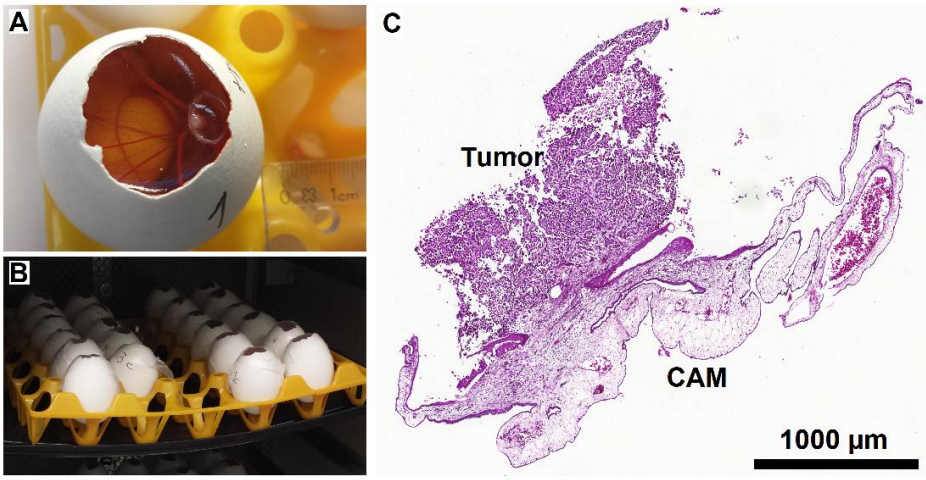

Figure 2 Chicken embryo chorioallantoic membrane model
A, Tumors are grown on top of the CAM (chorioallantoic membrane) in the chicken eggs. **B,** The eggs are cultured at 37 °C and 60 % humidity, while the whole in the egg shell is covered by an adhesive tape. **C,** Hematoxylin and eosin staining shows a tumor formed by prostate cancer cells on top of the CAM. (Photographs by S. Landor and tumor scanning by N. Santio)

Tumor growth can be followed for few days on the CAM (chorioallantoic membrane), which supports tumor growth very well (Deryugina & Quigley 2008). In addition, despite the short time-period of the experiment, tumor vascularization

and even metastases can be studied efficiently using this model (Deryugina & Quigley 2008). Similar to chicken embryos, zebrafish embryos can also be used for several purposes during their early embryonal development without animal experiment authorization (Strähle et al. 2012). On the other hand, microorganisms, such as yeasts or invertebrates, such as fruit flies or nematodes are also suitable for instance for testing of gene or protein inactivation or drug compound toxicity (Doke & Dhawale 2015). These methods are extremely useful due to the low costs and the ability to use large sample numbers and get results relatively fast as compared to experiments performed with adult organisms or for example mammals.

Different types of animal models can be used for studying tumor progression and drug efficacy *in vivo*. The basic rule for planning an animal experiment is the 3Rs principle (Replacement, Reduction and Refinement), which aims to as humane animal experimentation as possible (Flecknell 2002). This principle is also supported by the laws, which strictly regulate the permissions to perform animal experiments. The major problems in animal experiments are high costs and the controversy between benefits and animal suffering. Still, animal experiments are extremely important e.g. for drug development and various animal models have been developed for research purposes. One way to test the function of proteins is to generate knock-out or knock-in animals by genetic modification leading to complete or partial inactivation or activation of certain signaling pathways (Cheon & Orsulic 2011). In addition, tumor growth can be followed after injection of cancer cells e.g. to immunodeficient nude mice (Figure 3A). For instance, cancer cells can be injected to the veins, subcutaneously under the skin or orthotopically into a certain tissue, while several methods can also be used to induce spontaneous cancer (Ruggeri et al. 2014). The efficacy of drug compounds can be tested with various types of animal models depending on the drug targets, while manual or computer-based methods such as fluorescence imaging can be used for following tumor progression in animals (Figure 3B-C). In addition, important research materials can also be obtained from human samples. For example, analysis of surgically removed tumor tissues gives valuable data directly from patients.

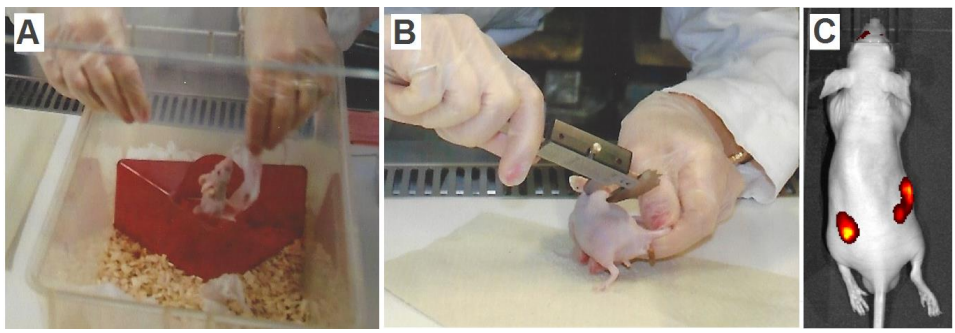

Figure 3 Mouse as an *in vivo* research model
A, Immunodeficient nude mice are often used for following tumor growth *in vivo*. **B,** Subcutaneous tumors can be palpated manually. **C,** Fluorescence-based imaging can be easily used to visualize fluorescently labelled subcutaneous tumor cells. (Photographs by J. Korhonen and fluorescence imaging by N. Santio)

After promising results from animal-based experiments, clinical trials can be planned for testing of drug compounds. While the costs for animal experiments are already high, the costs for clinical trials are much more. Clinical trials are long lasting and they are divided into various phases. Phase I usually involves safety analysis in healthy volunteers, while Phase II and III are performed with patients (Meinert 1986). After the drug has been approved for market, it will still be evaluated, which is often referred as the phase IV (Meinert 1986).

2.2 Regulation of cancer cell survival

2.2.1 Oncogenes and oncoproteins

Proto-oncogenes are genes that encode proteins, which promote cell proliferation and survival. When a gain-of-function mutation occurs leading to an abnormally high expression of the proto-oncogene, it can be referred to as an oncogene and the protein product as an oncoprotein. Oncoproteins may especially in a co-operative manner induce uncontrolled cell proliferation and cause cancer. The regulation of proto-oncogenes can be altered in eukaryotic cells by several types of mutations e.g. by a chromosomal rearrangement. However, a proto-oncogene can also become integrated into a genome of a retrovirus, where the expression of the gene can be upregulated e.g. by a hyperactive promoter leading to formation of an oncogene. When these retroviral oncogenes are integrated back into the eukaryotic cell genome, these genes may lead to enhanced cell proliferation. Interestingly, several known cellular proto-oncogenes have originally been identied as genes captured by retroviruses. Examples of these proto-oncogenes are i.a. the transcription factor *MYC* (v-myc avian myelocytomatosis viral oncogene homolog), *ERBB* (erythroblastic leukemia viral oncogene) family tyrosine kinases or *RAS* (rat sarcoma) family GTPases. MYC affects through various signaling pathways to promote cell cycle progression, tumor expansion as well as genomic instability (Soucek & Evan 2002; Vafa et al. 2002). *ERBB* genes encode four different types of epidermal growth factor receptors. For instance, ERBB2 (erb-b2 receptor tyrosine kinase 2) a.k.a HER2 (human epidermal growth factor receptor 2) is often upregulated in breast cancer and supports cell cycle progression (Scott et al. 1999). RAS family members induce continuous cell growth, while the MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) pathway positively regulates cell cycle, survival and migration as well as angiogenesis downstream of RAS (Croce 2008; Friday & Adjei 2008).

Several other oncoproteins can also support the formation of cancer such as the transcription factors NFATC (nuclear factor of activated T cells), NOTCH (neurogenic locus notch protein homolog), STAT (signal transducer and activator of transcription) or NFKB (nuclear factor of kappa light polypeptide gene enhancer in B-cells). NFATC family members promote e.g. cancer cell migration and tumor angiogenesis (Pan et al. 2013), while NOTCH1 is an important regulator of breast cancer cell metabolism and enhances breast tumor growth (Landor et al 2011). However, NOTCH family members NOTCH2 and NOTCH3 may rather function as tumor suppressors (O'Neill et al. 2007; Cui et al. 2013). In addition, JAK-mediated (Janus kinase) activation of STAT transcription factors induces cytokine

and hormone production, which plays an important role in normal tissue development as well as in cancer (Wagner & Schmidt 2011). Different STAT family members can have either tumor suppressor or oncogenic functions (Wagner & Schmidt 2011). NFKB protein complex regulates the expression of several genes important for cell cycle, apoptosis, extracellular matrix degradation etc. thereby supporting cancer progression (Luqman & Pezzuto 2010). In addition, the family of BCL2 (B-cell lymphoma 2) proteins contain both tumor suppressors and oncogenes, which regulate cell behavior by influencing the function of mitochondria (Adams & Cory 2007).

PIM (proviral integration site for Moloney murine leukemia virus) kinases are also encoded by proto-oncogenes, which were originally identified as retrovirally activated genes. Furthermore, PIM kinases have been shown to interact and co-operate with many other oncoproteins such as MYC and BCL2 (van Lohuizen et al. 1989; Möröy et al. 1991; Acton et al. 1992). On the other hand, the main activator of PIM expression is the JAK/STAT signaling pathway (Narlik-Grassow et al. 2014). NFKB and PIM signaling seems to also form a positive regulatory loop. NFKB leads to an increase in PIM expression and PIM kinases directly and indirectly lead to NFKB complex activation (Li et al. 2001; Zhu et al. 2002; Hammerman et al. 2004; Nihira et al. 2010).

2.2.2 Tumor suppressors

Tumor suppressor proteins are often responsible for the control of cell division e.g by acting in cell cycle checkpoints, but they can also be responsible for DNA repair. When a loss-of-function mutation occurs leading to inactivation of these genes, they cannot perform their tasks properly, and cells may divide despite serious mutations in DNA, chromosome number etc. Thereafter the progeny of these mutated cells may reproduce even faster and in more uncontrolled fashion leading to cancer. While several types of mutations can cause changes in the activity of a tumor suppressor protein, there are also viral proteins which can inactivate tumor suppressors and promote cancer formation.

Some well-known tumor suppressor proteins are the previously mentioned BRCA1 and BRCA2, TP53 (tumor protein p53, protein 53 kDa) as well as PTEN (phosphatase and tensin homolog). Unfortunately, mutations in the genes encoding these proteins are also inheritable. BRCA1 and BRCA2 are needed for DNA repair and they are often mutated in breast cancer (Foulkes & Shuen 2013). In addition, *BRCA* mutations can increase the risk of prostate cancer (Edwards & Eeles 2004). TP53 is a broadly mutated transcription factor in cancer, while normally it prevents cell cycle progression, if there is a DNA damage (Vogelstein et al 2000). PTEN is a phosphatase, which is able to dephosphorylate both proteins as well as lipids. Loss of PTEN is very common e.g. in prostate cancer, while active PTEN can efficiently decrease cell survival by inhibiting the PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) a.k.a PI3K (phosphoinositide 3-kinase) –mediated signaling pathway, which is described in more detail later on (Phin et al. 2013). As previously stated, there are also protein families, which contain both oncoproteins and tumor suppressors such as the BCL2 family. For

example, PIM kinases have been shown to inactivate the BCL2 family member BAD (BCL2 associated agonist of cell death), which is a proapoptotic protein (Aho et al. 2004). On the other hand, PIM kinases can support the expression of TP53, which counteracts the pro-tumorigenic behavior of the PIM kinases (Zemskova et al. 2010).

2.2.3 Metabolism in tumors

Normal as well as cancer cells use glucose as their main source of energy and the energy metabolism takes place in two parts of the cell, cytoplasm and mitochondria (Figure 4). First glucose is processed into pyruvate during an oxygen-independent process called glycolysis in the cytoplasm. Even though glucose is the main source for energy metabolism, lipids or amino acids can also be utilized to produce energy. The end product of glycolysis is pyruvate, which is further processed either in aerobic or anaerobic conditions. The most redundant energy gain is achieved by processing pyruvate into acetyl coenzyme A in the mitochondria and continuing into the citric acid cycle also called the Krebs cycle followed by electron transport system called the oxidative phosphorylation. The anaerobic energy production by lactic acid fermentation is a lot less efficient method for gaining energy, but it is extremely important e.g. during low oxygen conditions.

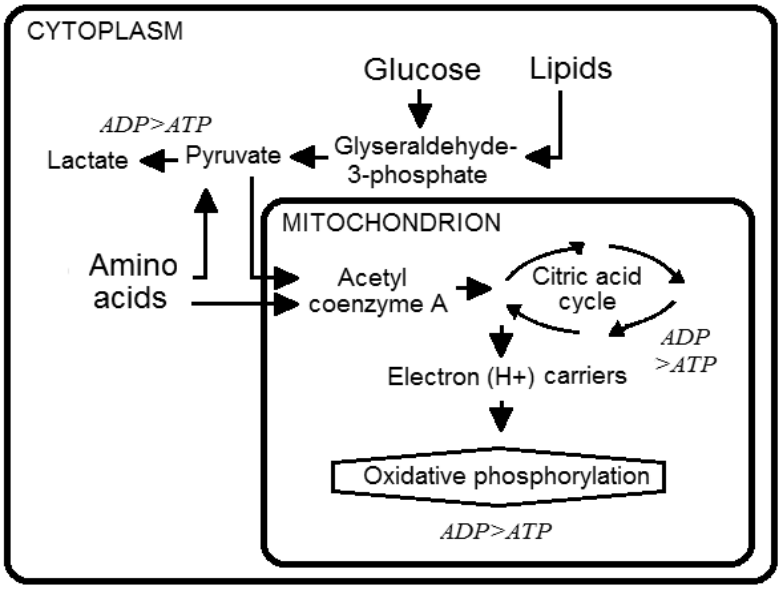

Figure 4 Eukaryotic cell energy metabolism
The anaerobic energy metabolism in the cytoplasm is based on glycolysis. The most efficient way to produce energy under normal oxygen conditions is to continue the processing of pyruvate inside the mitochondria, where great amounts of ATP (adenosine triphosphate) are produced during oxidative phosphorylation.

Tumor cells often have to survive under low oxygen conditions also called hypoxia. Therefore, it is beneficial for them to be able to produce energy also by anaerobic means. It has been known for decades that cancer cells may prefer

fermentation as a way to produce energy even under normoxic conditions (Warburg et al. 1927). Thus, other reasons than hypoxia such as activation of certain oncogenes are also likely to drive this so called "glycolytic switch" in cancer cells (Hsu & Sabatini 2008). In addition, increased use of glucose through glycolysis leads to an increase in the intermediates, which are important building blocks for cell growth and division (DeBerardinis et al. 2008). On the other hand, toxic metabolites such as lactate may also cause problems for cancer cells (Hsu & Sabatini 2008).

Since anaerobic metabolism is connected to enhanced survival of cancer cells, it has become an interesting target for cancer research and drug discovery. The main research targets are processes such as consumption of glucose, production of lactate and expression of important proteins such as glucose transporters. However, there is quite a lot of variation between the glycolytic potential of different cancer cells. For example, the highly invasive breast cancer cell line MDA-MB-231 (M.D. Anderson - metastatic breast 231) shows much higher amounts of solute carrier family 2 member 1 a.k.a glucose transporter 1 as compared to the less invasive MCF-7 (Michigan Cancer Foundation 7) cell line (Grover-McKay et al. 1998). A massive variation is also found in the metabolism of cancer cells at distinct breast tumor xenograft areas (Xu et al. 2013).

In addition to glucose, cancer cells can also use lipids and amino acids in their energy metabolism. For instance, glutamine is a major energy source for some cancer cells such as the HeLa (Henrietta Lacks) cervical cancer cells (Reitzer et al. 1979). Glutamine is first processed into glutamate, after which it is processed into malate in the citric acid cycle. While malate is normally further processed in the mitochondria, it can also be converted into pyruvate and fermented into lactate (Reitzer et al. 1979). Furthermore, glutamate has been shown to play an important role in the metabolism of cultured prostate cancer cells such as PC-3 (prostate cancer 3) cells (Panov & Orynbayeva 2013). The survival of cultured PC-3 cells is also highly dependent on glutamine supply. Though, there are also results indicating a great dependency on lipid synthesis in prostate cancer (Zadra et al. 2013).

2.2.4 Tumor angiogenesis

Oxygen and nutrients are essential for cancer cell division and growth inside a tumor. Therefore, the formation of vasculature also known as angiogenesis is needed during tumor progression. If there were no connection between the tumor tissue and the blood circulation, the survival and proliferation of tumor cells would be highly unlikely. However, tumor cells have several ways to induce endothelial cells to form vasculature to support the survival of the tumor. Vasculature also serves as a gateway for tumor cells to invade to the circulation, which the cells can leave to form metastases in distant organs.

Tumor angiogenesis is regulated by different inducers such as the fibroblast and vascular endothelial growth factors, while e.g. thrombospondin and angiostatin have been shown to inhibit angiogenesis (Hanahan & Folkman 1996). There are

also other important regulators such as interleukins, platelet-derived growth factors as well as hypoxia-inducible factors which influence angiogenesis (Gacche & Meshram 2013). The signals regulating angiogenesis are secreted from several sources such as tumor-associated macrophages. These cells are influenced by tumor-derived factors and they interact with tumor cells in a complex manner. Macrophages also secrete PTGS2 (prostaglandin-endoperoxide synthase 2), which causes tumor cells to release growth factors (Riabov et al. 2014). In addition, tumor cells may have elevated levels of the chemokine ligand CXCL12 (chemokine (C-X-C motif) ligand 12), of which receptor CXCR4 (chemokine (C-X-C motif) receptor 4) is expressed in certain macrophages, and this CXCL12/CXCR4 route can be used to attract the macrophages towards tumors (Welford et al. 2011).

In general, angiogenesis starts by degradation of the basement membrane (extracellular matrix) and the blood vessel (Figure 5), after which the endothelial cells can escape towards the tumor tissue and start forming new vessels (Gacche & Meshram 2013). However, there are also other possibilities for angiogenesis and sustaining the vasculature of tumors. The recruitment of bone-marrow-derived endothelial precursor cells can also be used for angiogenesis (Lyden et al. 2001).

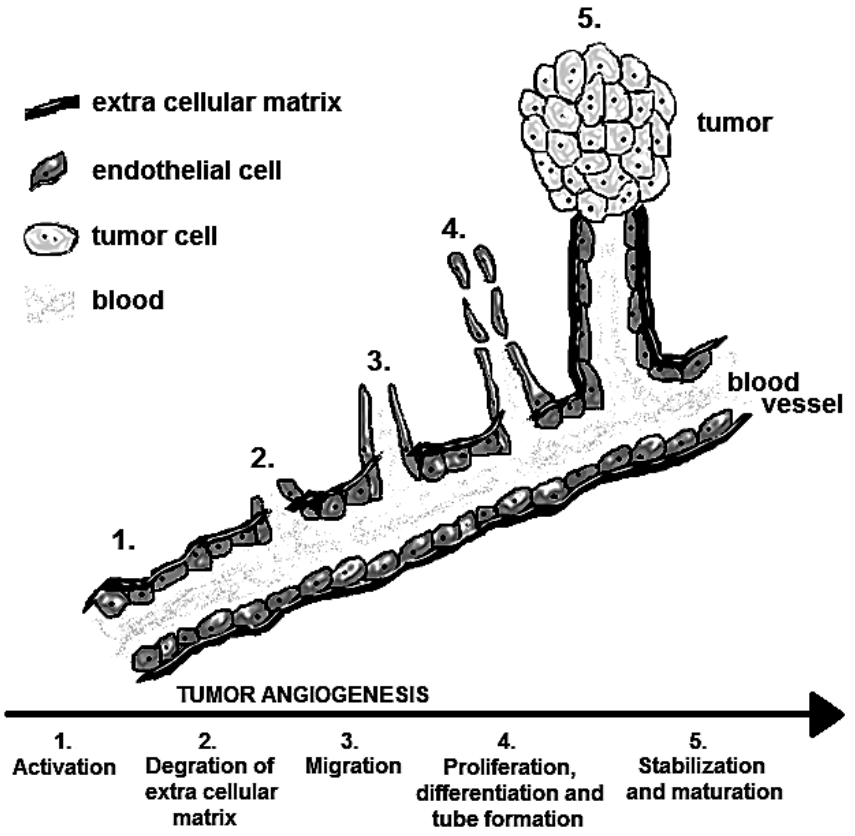

Figure 5 Tumor angiogenesis
Summary of different steps in connecting tumor cells to the blood vasculature (modified from Gacche & Meshram 2013).

In addition, tumor cells may have the ability to form tubular structures by themselves without endothelial cells, which is called vasculogenic mimicry. In the highly vascularized tissues the tumor cells can incorporate directly into the vessel walls, which is called vascular co-option (Auguste et al. 2005). On the other hand, pre-existing capillaries can also form new capillaries by re-organizing the endothelial cell layer (Ribatti & Djonov 2012). Finally, when vessels are formed, blood can flow inside the tumor, giving the tumor boost for growth.

Tumor cells do not only escape to surrounding blood vessels, but they can also invade lymphatic vessels after lymphangiogenesis (Figure 6). Both angiogenesis and lymphangiogenesis of tumors are regulated in a highly similar way by factors secreted by tumor cells and the surrounding cells such as the tumor-associated macrophages (Riabov et al. 2014). Though, there are differences in angiogenesis and lymphangiogenesis e.g. in the endothelial cell behavior (Stacker et al. 2014). Furthermore, increased expression of the CXCL12 ligand has been detected in tumor-associated lymphatic epithelium, while invading tumor cells have been shown to express high levels of the receptor CXCR4, which might promote tumor cell invasion into the lymphatic system (Hirakawa et al. 2009).

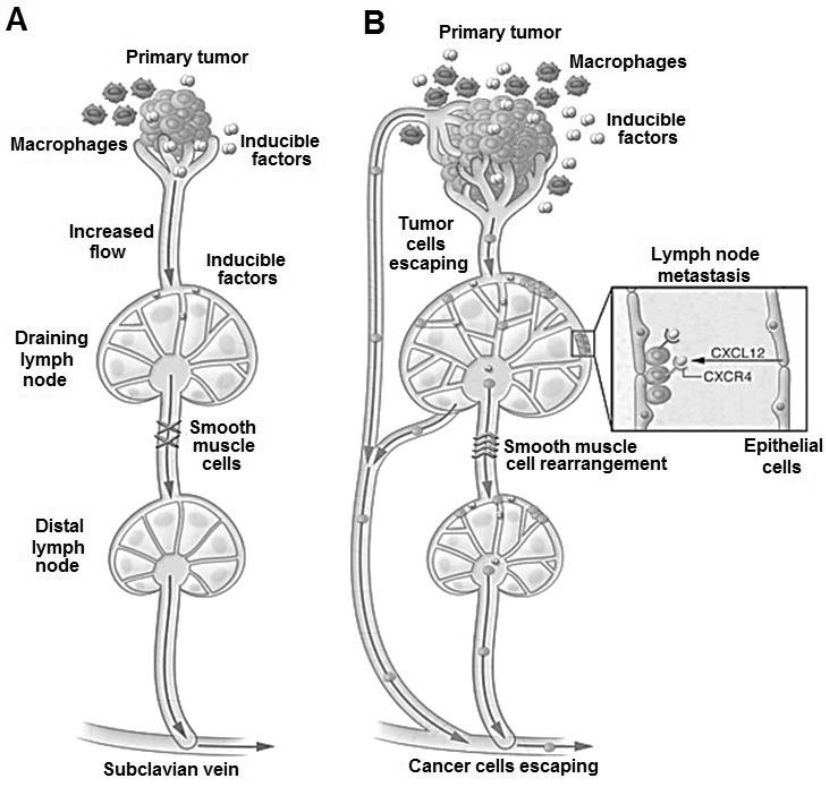

Figure 6 Tumor angiogenesis and lymphangiogenesis
A, The lymphatic system can be connected to the tumor, after which cancer cells can migrate to the lymphatic vessels. **B**, When tumor cells are connected to both blood and lymphatic vessels, they can efficiently escape to lymph nodes as well as other organs to form metastases. (Modified from Karaman & Detmar 2014)

While the function of blood vessels is highly different to lymphatic vessels, they both serve as an important gateway for tumor cells to escape to the surrounding tissues and the body. The lymphatic vessels are connected to the lymph nodes and they are an important route for cancer cells to migrate not only into the lymph nodes, but also further to form metastases.

2.3 Cancer cell motility and formation of metastases

2.3.1 Cell adhesion, migration and invasion

Cell adhesion is regulated by different types of cell-cell and cell-matrix-contacts via cell surface proteins such as cadherins, immunoglobulins, integrins and selectins. Cadherins are proteins involved in intercellular adherens junctions and they are able to connect two cells by recruiting other proteins such as catenins to bind cytoplasmic proteins such as actin filaments (Le Bras et al. 2012). While immunoglobulins play a major role in antibody response, there are also family members involved in cell adhesion (Wai Wong et al. 2012). Integrins are receptor complexes formed by alpha and beta subunits and their main function is to serve as a link between the extracellular matrix components and the cell cytoskeleton (Takada et al. 2007). Selectins are vascular cell adhesion molecules that function in the blood circulation mediating the contacts between leukocytes, platelets and the endothelium (Crockett-Torabi 1998). Furthermore, the proteins that degrade extracellular matrix are called metalloproteinases and their roles in cell adhesion and motility as well as in angiogenesis are very important (Sternlicht & Werb 2001).

Migration means both the movement of an individual cell and the transition of a whole organism. Cell movement is regulated by the intracellular and extracellular compartments as well as the previously mentioned transmembrane proteins regulating adhesion. The extracellular matrix is composed of different compounds such as collagen, laminin and fibronectin (Teti 1992). The cell cytoskeleton is mainly formed by three types of filaments called actin filaments, microtubules and intermediate filaments, such as keratin or vimentin (Huber et al. 2015). These filaments play an important role during cell movement and cell division. The movement of a whole cell as well as its intracellular compartments is driven by motor proteins that utilize the filaments. The intermediate filaments mostly exploit the microtubule and actin motor proteins such as kinesin and myosin (Huber et al. 2015).

Cell movement is usually initiated by formation of protrusions to the leading edge. These protrusions contain actin filament structures called lamellipodia and filopodia (Figure 7A), while the adhesions are predominantly mediated by the integrins (Mattila & Lappalainen 2008; Arjonen et al. 2011). While the integrins are bound to their regulatory proteins and the actin filaments inside the cell, they are simultaneously connected to the extracellular matrix components outside the cell. For movement, both the cell cytoskeleton as well as the adhesions needs to be reorganized. While the leading edge forms new adhesions to the extracellular matrix, the adhesions at the lagging edge are dissembled (Figure 7A-B).

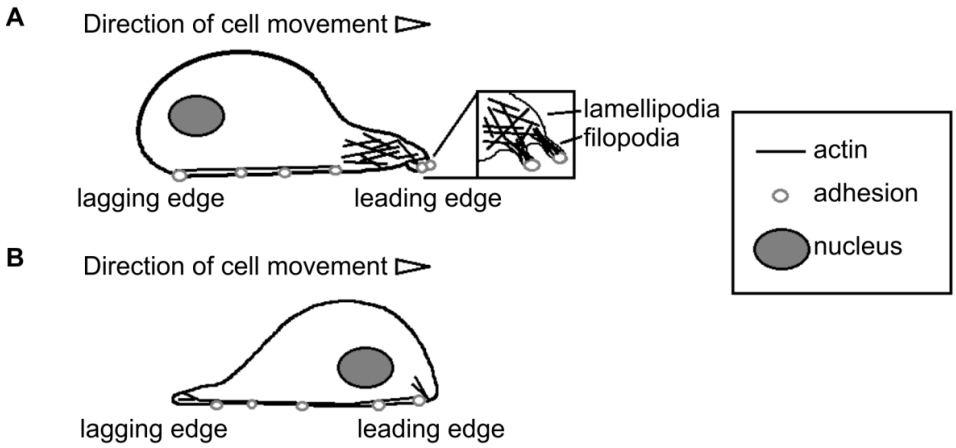

Figure 7 Cell migration
A, During cell movement, actin filament and adhesion site reorganization must take place first at the front side of the cell. **B**, Thereafter the retracting of the rear end is driven by actin and adhesion site reshaping. (Modified from Mattila & Lappalainen 2008)

Solid tumors are formed mainly by epithelial cells, which normally are flat and have a lot of protrusions and contacts to the surrounding environment. The term epithelial-mesenchymal transition has originally been used for describing the changes in cell structure and behavior during embryogenesis (Le Bras et al. 2012). During embryonal development it is highly important for the cells at certain steps to be able to lose their contact and to be more motile. By contrast in cancer, the epithelial-mesenchymal transition is a far more negative term, used for describing the changes in the behavior of an epithelial cell towards a more mesenchymal-like easy-moving cell (Le Bras et al. 2012). As previously mentioned, cadherins mediate the cell-cell interactions, which are also very important for cell movement. In cancer cells, the main regulator of intercellular junctions, cadherin 1 a.k.a. E-cadherin, is usually less abundant, while cadherin 2 a.k.a. N-cadherin expression gets higher leading to less stable junctions (Le Bras et al. 2012).

2.3.2 Cancer metastases

Before the formation of metastases, cancer cells need to overcome several difficulties, as reviewed by Langley & Fidler 2007. First they need to escape their primary site and find their way to the circulation. Since tumors usually have a vasculature system, it gives the cancer cells a chance to migrate to the blood or the lymphatic vessels. Secondly, tumor cells need to survive independently in the circulation without attachment to other cells or the surrounding environment and they need to be able to escape from the immune response. Finally, prior to the formation of a metastasis, cancer cells need to find a way to adhere to a certain spot in the vessel or in a host organ and to proliferate. However, attachment, survival and proliferation in the new environment are challenging for cancer cells and only a minority of them manages to form a secondary tumor also known as a metastasis.

It was suggested already as early as 1880s that metastases are not formed by chance to various organs, but the target organ qualities may support the formation of metastases into certain organs depending on the cancer type (Paget 1889). Nowadays, it is known that there are a wide variety of metastases and different target organs are more common in different cancer types (Nguyen et al. 2009). In addition, there can be different types of metastases in the same organ depending on the origin of the primary tumor. For instance, both breast and prostate cancer cells form bone metastases, but the metastases originated from breast cancer usually promote osteoclastogenesis or bone resorption, while prostate cancer metastases usually increase bone deposition (Weinberg 2007). Several factors are known to regulate the formation of metastases. The changes in cell adhesion as well as the formation of blood and lymphatic vessels are the main factors, which enable the invasion of cancer cells. However, the lymphatic system can also further support cancer cell spreading by different means such as increased pumping and lymph flow (Karaman & Detmar 2014). The previously mentioned cytokine ligand-receptor interactions also play an important role in the formation of metastases (Müller et al. 2001; Singh et al. 2004; Shanmugam et al. 2011; Chatterjee et al. 2014). In some cases the close proximity of a certain tissue to the tumor tissue and the direction of blood flow can explain the formation of metastases. For instance, the direction of the blood flow in the veins connecting tumor tissue and the spine may partly explain the formation of spinal metastases in certain cancers such as prostate and breast cancer (Maccauro et al. 2011).

There is a lot of variation in when and how the metastases are detected, while sometimes they can even be found before the primary tumor. The treatment of metastases is difficult, especially since the primary and secondary tumors may react to the same drug in a very different way (Steeg & Theodorescu 2008). Unfortunatelly, in most cases the therapy-resistant metastases are the main reason for patient death, and therefore it is important to search for anti-cancer therapies targeting especially the metastases (Sleeman & Steeg 2010).

2.4 PIM kinases and cancer

2.4.1 Identification and regulation of PIM kinases

PIM kinases are serine/threonine kinases, which promote cell survival and proliferation (Nawjin et al. 2011). The *PIM1* gene was originally identified when retroviral sites were cloned from murine Moloney leukemia virus-induced lymphomas (Cuypers et al. 1984). Later on also *PIM2* and *PIM3* a.k.a. *KID1* (kinase induced by depolarization) were identified (Breuer et al. 1989; Feldman et al. 1998). These genes are located in different choromosomes (Figure 8A), while each family member is encoded by six exons (Figure 8B) (Nawjin et al. 2011). The human PIM proteins are highly homologous and sequences are known for two PIM1 splice variants (34 kDa and 45 kDa) as well as one PIM2 (34 kDa) and PIM3 (36 kDa) isoform (Figure 8C) (UniProt Consortium 2015). However, PIM2 is likely to have three splice variants (34, 37 and 40 kDa) (Nawjin et al. 2011).

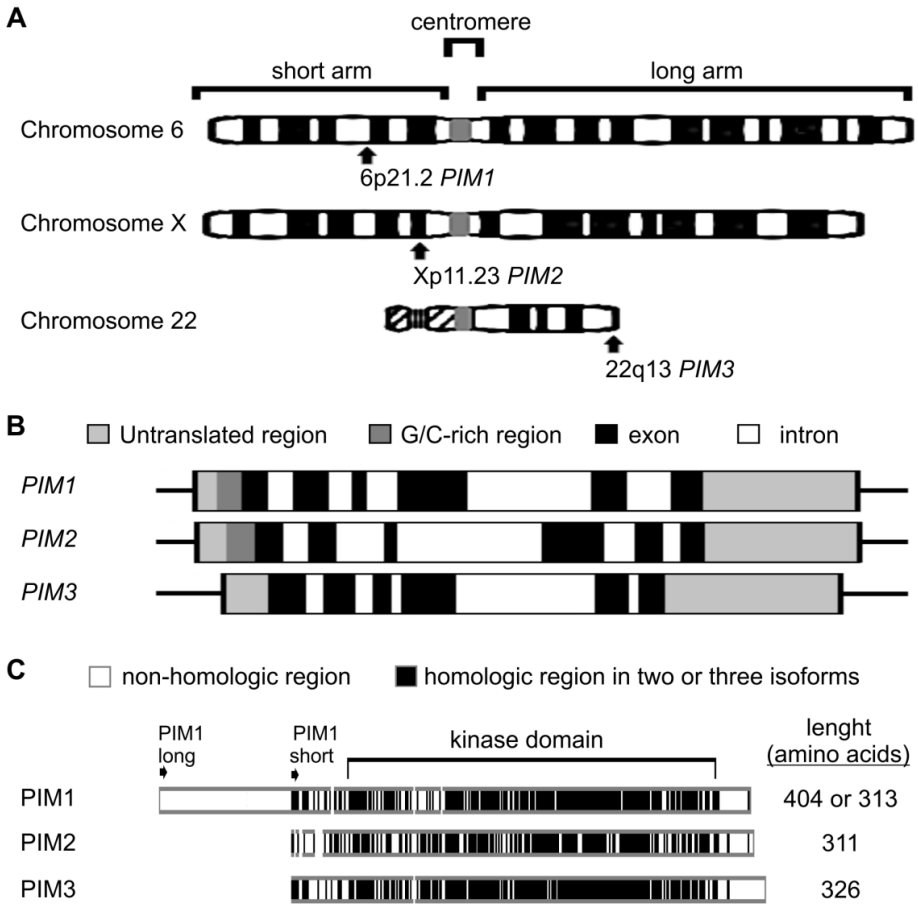

Figure 8 PIM kinase encoding genes and primary protein structures
A, Chromosomal location of *PIM* genes (NCBI 2016). **B**, *PIM* gene structure (Nawjin et al. 2011). **C**, Primary structure and homology of PIM proteins (Uniprot Consortium 2015).

The structure of PIM kinases is unique as compared to other kinases. PIM kinases contain a distinct hinge region, which affect the binding to ATP. While kinases generally form two hydrogens bonds with ATP, the PIM kinases only form one (Johnson et al. 1996; Qian et al. 2005). In addition, the crystallized PIM structure shows two types of loops (A-loop and C-loop), which interact in a highly specific way and are likely to lead to the constitutive activity of the protein (Figure 9) (Qian et al. 2005). Thereby, there is no need for translational modification such as phosphorylation for activation of PIM kinases. However, the stability and degradation of the PIM kinases are regulated e.g. by heat shock proteins (Mizuno et al. 2001; Shay et al. 2005).

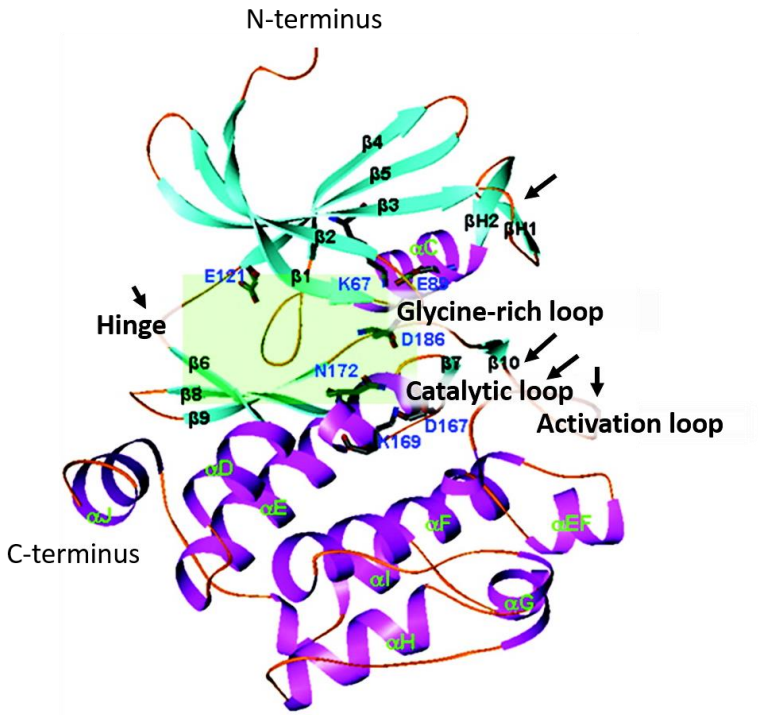

Figure 9 PIM1 kinase tertiary structure
PIM1 kinase contains unike structures (marked by arrows) which are only known in PIM family members or a couple of other kinases. The ATP binding site (green background) is close to the hinge region, while the ATP-binding amino acid residues are marked in blue (modified from Qian et al. 2005).

The three PIM family members have partially overlapping functions and expression patterns (van der Lugt 1995; Eichmann et al. 2000; Mikkers et al. 2002; Saurabh et al. 2014). Their expression is regulated by several factors such as growth factors (e.g. the granulocyte-macrophage colony-stimulating factor and interleukins), hormones (e.g. erythropoietin, thrombopoietin and prolactin), phorbol 12-myristate 13-acetate as well as interferons (Lilly et al. 1992; Dautry et al. 1988; Miura et al. 1994; Nagata & Tokodoro 1995; Buckley et al. 1995; Borg et al. 1999; Wingett et al. 1991; Yip-Schneider et al. 1995; Matikainen et al. 1999). The main upregulator of PIM expression is activation of the JAK/STAT signaling pathway (Narlik-Grassow et al. 2014). A negative regulatory loop has also been identified to keep PIM expression under control. PIM kinases can phosphorylate SOCS1 and SOCS3 (suppressor of cytokine signaling 1 and 3) leading to inactivation of STAT5 and thereby inhibition of JAK/STAT-upregulated *PIM* expression (Peltola et al. 2004). Direct upregulation of PIM expression is induced by binding of several transcription factors such as STAT, KLF (Kruppel-like factor) as well as ETS transcription factors ETS1 and ERG (v-ets avian erythroblastosis virus E26 oncogene homolog) to *PIM* promoter (Matikainen et al. 1999; Zhao et al. 2008; Li et al. 2009; Magistroni et al. 2011). On the other hand, tumor suppressor FOXN3 represses expression of PIM2, thereby inhibiting cell proliferation (Huot et al. 2014).

PIM kinases are normally expressed in several tissues, and their increased expression has been detected in many types of malignancies. Even though PIM kinases were originally identified in lymphomas, quite soon after that surfaced the first evidence supporting the importance of PIM signaling also in normal hematopoietic tissue (Amson et al. 1989; Domen et al.1993). Outside hematopoietic system, PIM kinases have been detected in different tissues such as testis, small intestine, colon, liver, spleen, lungs and the nervous sytem (Sorrentino et al 1988; Baytel et al. 1998; Amson et al. 1989; Feldman et al 1998; Konietzko et al. 1999; Eichmann et al. 2000).

When overexpressed in mice, PIM kinases are oncogenic and can enhance lymphomagenesis, especially in collaboration with other oncoproteins such as MYC and BCL2 (van Lohuizen et al. 1989; Möröy et al. 1991; Acton et al. 1992). In addition, there is evidence of PIM1 and MYC co-operation in epithelial-derived tumors such as prostate cancer (Wang et al. 2010a; Wang et al. 2012). High expression levels of PIM kinases have been detected in human leukemias and lymphomas as well as in several types of solid tumors such as prostate, colon, oral, hepatic and pancreatic cancers (Narlik-Grassow et al. 2014).

2.4.2 Main downstream targets of PIM kinases

The functional roles of PIM kinases have been thoroughly studied in the hematopoietic system, and their downstream signaling regulating cell survival, proliferation and apoptosis in hematopoietic malignancies is well known. PIM kinases regulate their substrates through phosphorylation of serine (Ser) or threonine (Thr) residues leading to inactivation or activation of the substrate. The consensus sequence for PIM1-mediated phosphorylation of the substrates was first reported to be K/R-K/R-K/R-X-S/T-X, where X represents an amino acid residue that is neither large and hydrophobic nor basic (Friedmann et al.1992). Other highly similar consensus sequences have also been described later for PIM kinases: K/R-K/R-R-K/R-L-S/T-X, where X represents a small chain amino acid residue and R-X-R-H-X-S, where X represents any amino acid (Palaty et al. 1997; Peng et al. 2007).

One of the first results on how PIM kinases affect oncogenesis came from studies where PIM interaction and co-operation with the BCL2 protein family members were studied. In general, the BCL2 family has several different functions mediating multiple cellular processes related e.g. to neuronal signaling, calcium responses and mitochondrial functions such as energetics (Hardwick & Soane 2013). However, they are also known to regulate cell survival by either promoting or preventing cell death via affecting the mitochondrial processes leading to apoptosis (Adams & Cory 2007). Since apoptosis plays a critical role to restrict the survival and proliferation of malfunctioning and genetically mutated cells, it also protects the human body from the development of cancer. Interestingly, PIM kinases can support the expression of the antiapoptotic protein BCL2 (Lilly et al. 1999) as well as to phosphorylate and thereby inactivate the proapoptotic protein BAD (Yan et al. 2003; Aho et al. 2004). In addition, by phosphorylating BAD, PIM kinases prevent its binding to another family member, the antiapoptotic

protein BCL2 like 1 a.k.a. BCL2 extra large, leading to increased cell survival (MacDonald et al. 2006). PIM kinases have been reported to directly phosphorylate and thereby stabilize MYC (Zhang et al. 2008). PIM activity has also been connected to TP53-mediated regulation of cell survival in solid tumors and hematopoietic malignancies. Surprisingly, PIM kinases can support the expression of TP53 in prostate cancer cells (Zemskova et al. 2010). This may counteract PIM-promoted cell survival, if TP53 is active. However, as previously stated, TP53 is often inactivated in cancer cells such as PC-3 prostate cancer cells (Rubin et al. 1991). In lymphomas, combined PIM inhibition and TP53 activation efficiently induces cell death (Sarek et al. 2013).

PIM kinases phosphorylate and activate several transcription factors such as the previously mentioned NFATC1 and NFKB as well as RUNX (runt related transcription factor), thereby promoting cancer progression (Rainio et al. 2002; Nihira et al. 2010; Aho et al. 2006). PIM1 can also physically interact with the transcriptional co-activator SND1 (staphylococcal nuclease and tudor domain containing 1) a.k.a. p100 (protein 100 kDa), leading to an increase in the activity of the transcriptional regulator of hematopoietic and lymphatic cells called MYB (v-myb avian myeloblastosis viral oncogene homolog) (Leverson et al. 1998). Furthermore, PIM1 can promote cell cycle transition to mitosis by phosphorylating and inactivating CCL27 (chemokine (C-C motif) ligand 27) a.k.a. CTAK (CDC25C associated protein kinase), which mediates inactivation of CDC25C (cell division cycle 25C), a phosphatase needed for activation of cyclin-dependent kinases and entering of cells to mitosis (Bachmann et al. 2004). CDC25C can also be directly phosphorylated and activated by PIM1 (Bachmann et al. 2006). In addition, the tumor suppressors CDKN1A and CDKN1B (cyclin-dependent kinase inhibitor 1A and 1B) a.k.a. p21 and p27 (protein 21 kDa and protein 27 kDa) are inactivated by PIM kinases (Banerjee et al. 2014; Morishita et al. 2008).

While the role of PIM kinases in cell survival is well known, there are only few studies implicating them in cancer cell motility. Firstly, higher PIM1 levels have been connected to increased squamocellular carcinoma cell migration (Tanaka et al. 2009). Secondly, PIM3 can support endothelial cell migration, cell spreading and vasculogenesis (Zhang et al. 2009a; Liu et al. 2014b), referring to a possible role for PIM3 in tumor angiogenesis. Finally, all PIM kinases have been connected to sarcoma cell bone invasion (Narlik-Grassow et al. 2012). Some of the PIM kinase substrates have also been connected to the regulation of cell migration. For instance, the transcription factors NFATC1 and FOXP3 (forkhead box P3) as well as the chemokine receptor CXCR4 are phosphorylated by PIM1 (Rainio et al. 2002; Li et al. 2014; Grundler et al. 2009). All these three substrates have been shown to be involved in the regulation of cell migration in breast or prostate cancer cells (Singh et al. 2004; Kukreja et al. 2005; Seifert et al. 2009; Douglass et al. 2014). In addition, the CXCR4/CXCL12 chemokine pathway may play an important role in promoting the formation of metastases (Chatterjee et al. 2014).

While changes in cell metabolism have recently become a topic of great interest in cancer research, there is not much information about the metabolic influences of PIM kinases. However, cells derived from *PIM* triple knock-out mice have low

levels of glycolytic enzymes, which suggests that PIM activity may be important for proper ATP production by oxidative phosphorylation (Din et al. 2014; Song et al. 2014). PIM kinases have been reported to influence glycolysis and mitochondrial biogenesis through inhibition of PRKAA1 (protein kinase AMP-activated catalytic subunit alpha 1) a.k.a. AMPK (adenosine monophosphate-activated protein kinase) (Beharry et al. 2011; Din et al. 2014). Furthermore, both PIM1 and PIM2 activity has been connected to increased glycolysis in cancer (Leung et al. 2015; Zhang et al. 2015a).

Even though PIM kinases differ from other kinases in structure and function, there is another kinase family with somewhat similar downstream targets. The AKT family (v-akt murine thymoma viral oncogene homolog) is also formed by three members called AKT1 a.k.a. protein kinase B, AKT2 and AKT3, respectively (Staal 1987; Masure et al. 1999). The PI3K family proteins catalyze the formation of a phopholipid phosphatidylinositol-3,4,5-triphosphate, thereby leading to a phosphorylation cascade of downstream targets such as AKT (Carnero & Paramio 2014). The consensus sequence for AKT target site (RXRXXS/T, X represent any amino acid) is highly similar to PIM consensus sequence and they also share common substrates (Warfel & Craft 2015). One example of a common substrate is AKT1S1 (AKT1 substrate 1) a.k.a. PRAS40 (proline-rich Akt substrate, 40 kDa), which is phosphorylated and inactivated by PIM and AKT kinases (Kovacina et al. 2003; Zhang et al. 2009b). AKT1S1 on the other hand is an inhibitor of MTOR (mechanistic target of rapamycin) complex 1, which regulates cellular functions supporting cancer progression (Pópulo et al. 2012). Interestingly, MTOR also phosphorylates and activates STAT3, which is an inducer of PIM expression (Yokogami et al. 2000; Narlik-Grassow et al. 2014). While there are similarities in PIM and AKT signaling pathways, there are also differences. For instance, while *PIM* triple knock-out mice are viable, the *AKT* knock-out mice have several abnormalities and most of the knock-out combinations die shortly after birth, only the *AKT2* and *AKT3* double knock-outs being viable and fertile (Mikkers et al. 2004; Peng et al. 2003; Yang et al. 2005; Dummler et al. 2006). Therefore, while both PIM and AKT are important regulators of tumorigenesis, targeting PIM kinases might be less likely to cause serious side effects.

In summary, PIM kinases mediate many important steps in cancer progression and they may also play important roles in the regulation of cancer cell motility and metabolism (Figure 10). Changes in cell metabolism can provide an additional advantage for tumor growth, while PIM kinases also enhance cell survival and proliferation more directly. Furthermore, the influence of PIM kinases on angiogenesis and cell motility can promote the formation of metastases. Hence, PIM kinases are likely to support tumor progression in several different ways.

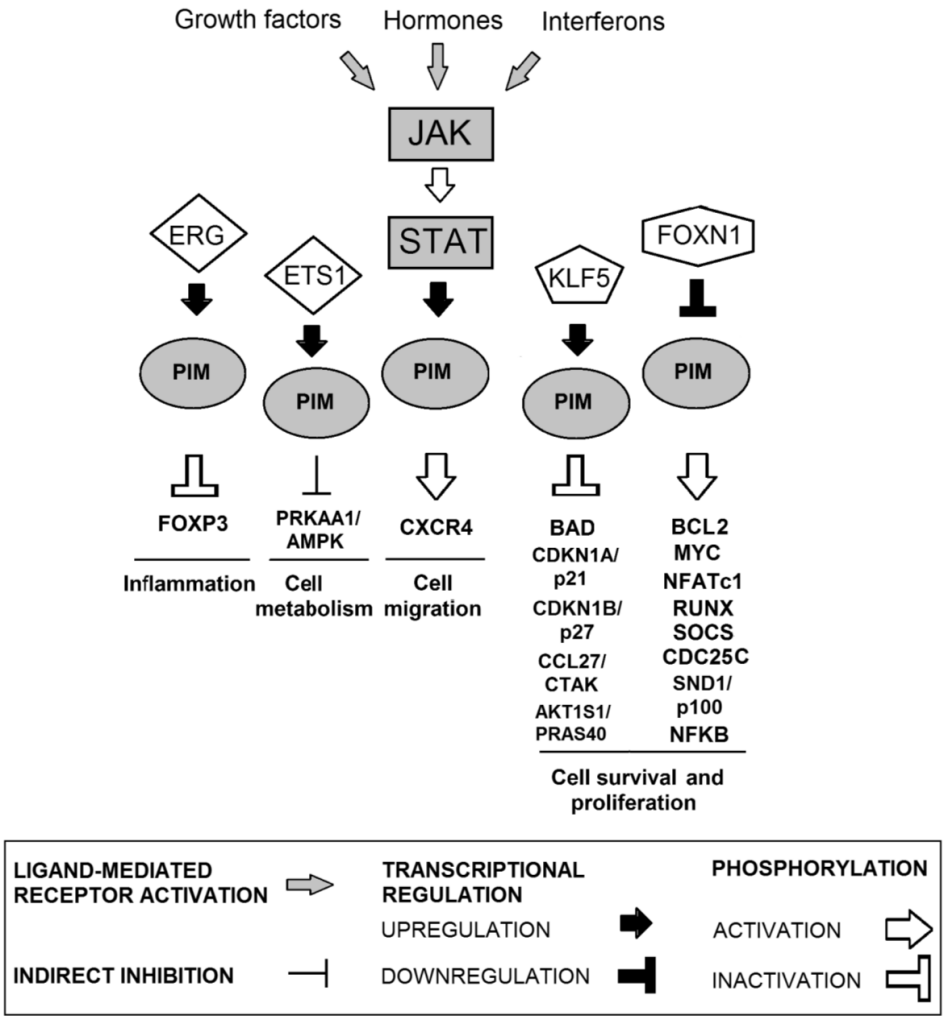

Figure 10 Simplified PIM signaling network
The main activator of *PIM* expression is the JAK/STAT pathway, while several cellular functions are regulated by PIM kinases. Shown are examples of PIM kinase regulators and downstream targets (Bachmann et al. 2004; Nihira et al. 2010; Beharry et al. 2011; Magistroni et al. 2011; Huot et al. 2014; Narlik-Grassow et al. 2014; Li et al. 2014).

2.4.3 PIM kinases in breast and prostate tumorigenesis

While hormones and cytokines play a major role in the regulation of breast and prostate tumorigenesis, they are also important regulators of PIM expression. Therefore, it is not surprising, that PIM kinases have been implicated in breast and prostate tumorigenesis. Breast cancer cell lines such as MCF-7 express higher levels of PIM as compared to non-tumorigenic mammary epithelial cells MCF10A, while in benign epithelial prostate tissues PIM levels have been shown to be weak as compared to cancer tissues (Gapter et al. 2006; Dhanasekaran et al. 2001; Valdman et al. 2004; Xu et al. 2005). Secondly, a connection has been found between PIM kinases and hormonal regulation of tumorigenesis. Prolactin,

progesterone and estradiol can lead to increased PIM expression levels (Buckley et al. 1995; Borg et al. 1999; Gapter et al. 2006; Malinen et al. 2013), while the androgen receptor can be phosphorylated and both stabilized and destabilized by PIM1 (Thompson et al. 2003; van der Poel et al. 2010; Linn et al. 2012; Ha et al. 2013).

PIM1 overexpression can induce polyploidy and tumorigenicity in prostate and mammary epithelial cell lines (Roh et al. 2003; Roh et al. 2008). While PIM upregulation promotes prostate tumor growth (Chen et al. 2005), blocking PIM signaling by RNA interference or antibodies decreases growth of both breast and prostate cancer xenografts (Zhang et al. 2014b; Hu et al. 2009; Zhang et al. 2010). PIM kinases have also been shown to play a role in chemoresistance of prostate cancer cells. While PIM upregulation protects cancer cells from chemotherapy, certain chemotherapeutic agents can also enhance PIM expression (Xie et al. 2006; Zemskova et al. 2008). Furthermore, targeting PIM1 by RNA interference, antibodies or inhibitors sensitizes prostate cancer cells to chemotherapy (Xie et al. 2008; Hu et al. 2009; Mumenthaler et al. 2009).

There is debate about the connection of PIM levels and prostate cancer prognosis. In some cases lower PIM levels have been connected to poor prognosis, while in other cases higher PIM protein levels have been found in more severe states of prostate cancer (Dhanasekaran et al. 2001; Valdman et al. 2004; Dai et al. 2005; van der Poel et al. 2010). In some cases a clear correlation has not been established between PIM levels and prostate cancer progression (Cibull et al. 2006). Despite the discrepancies between studies, PIM expression levels have been suggested to be used as a diagnostic marker for prostate cancer, because of increased PIM levels in more severe cases (He et al. 2007; He et al. 2009). While PSA levels are the common way to detect prostate cancer, higher PSA levels can also be connected to better prognosis, and therefore other diagnostic markers are needed (Stege et al. 2000). After treatment of prostate cancer cells with PSA, PIM1 levels have been shown to be decreased along with xenograft growth (Bindukumar et al. 2005). In addition, phosphorylation of androgen receptor by PIM1 can decrease PSA levels (Ha et al. 2013). Part of the controversy between the results might be explained by the regulation of PIM expression. Whereas increased JAK and STAT activity has been shown to correlate with higher Gleason score and poor prognosis in prostate cancer, the role of other PIM regulators is not as clear (Liu et al. 2012). The transcription factor KLF5 is often deleted or downregulated in prostate cancer (Chen et al. 2003). In addition, the ETS transcription factor ERG mutant forms have been connected to prostate cancer formation, but also to more favorable outcome (Zong et al. 2009; Kumar-Sinha et al. 2008; Saramäki et al. 2008; Kimura et al. 2012).

Even though PIM kinases can support breast and prostate tumor growth, their influence on cell metabolism and the formation of metastases has been studied to a lesser extent. Thereby cell metabolism and motility are important research targets especially for increasing the ability to predict the pros and cons of targeting PIM kinases in cancer.

2.5 PIM kinase inhibitors – targeting PIM in cancer

2.5.1 The benefits of PIM inhibition

Since the PIM kinases clearly play a role in the development and progression of cancer, they also are an intriguing target for anti-cancer drug development. The importance of PIM kinases in cancer is supported by clinical data, which shows PIM overexpression in a wide variety of malignancies (Narlik-Grassow et al. 2014). In addition, there are other properties that make PIM kinases highly attractive therapeutic targets. First, knocking out all three *PIM* genes causes only minor changes in the mouse phenotype, while the average wellbeing and fertility are unaltered (Mikkers et al. 2004). This may refer to a low risk of gaining harmful side-effects after PIM-tageted therapies. Secondly, the unique structure of PIM kinases enables the design of highly selective compounds, which can also reduce the probability of unwanted effects (Qian et al. 2005).

2.5.2 Towards selective PIM inhibition

The first reported kinase inhibitor was an indolocarbazole derivative staurosporine found in the microorganism *Streptomyces staurosporeus* (Omura et al. 1977; Furusaki et al. 1978). Unfortunately, while staurosporine was first identified as a protein kinase C inhibitor, later it was shown to be highly unspecific, targeting several different types of kinases (Tamaoki et al. 1986; Karaman et al. 2008). Co-crystallization of PIM1 with staurosporine has also been determined (Jacobs et al. 2005). Thereafter, there has been an increasing amount of reports of novel more or less PIM-selective kinase inhibitors. Some of them have potential against all PIM kinases and those compounds are thereby called pan-PIM inhibitors, while others inhibit the activity of only one or two PIM family members (Blanco-Aparicio & Carnero 2013; Arunesh et al. 2014).

So far there are no PIM inhibitors in the market to be used for treatment of cancer or other health disorders. The PIM inhibitors that have proceeded into clinical trials have mostly been targeted against hematopoietic malignancies either alone or in combination with other anti-cancer agents. The first PIM inhibitor in clinical trials was an imidazopyridazine called SGI-1776, which showed anti-cancer potential in leukemia and prostate cancer models (Chen et al. 2009; Mumenthaler et al. 2009; Chen et al. 2011). However, this compound caused severe cardiac toxicity and the trials were forced to be discontinued (ClinicalTrials.gov 2015). Thereafter several other compounds have been subjected to clinical trials. Phase I trials have been terminated or completed with an ammonium salt of perfluorooctanoic acid CXR1002 and a thiazolidine-2,4-dione AZD1208, both of which are pan-PIM inhibitors (Barnett et al. 2010; UK Clinical Research Network 2015; Keeton et al. 2014; ClinicalTrials.gov 2015). Phase I (and II) trials are recruiting patients for testing a CK and PIM1 inhibitor triazolobenzonapthyridine CX-4945 and a pan-PIM inhibitor LGH447 (Pierre et al. 2011; ClinicalTrials.gov 2015).

2.5.3 Pyrrolocarbazoles and benzazulenes as PIM inhibitors

While the carbazole-based indolocarbazole staurosporine was highly unselective, pyrrolocarbazoles have shown highly selective inhibitory potential towards all PIM family kinases. Especially DHPCC-9 (dihydropyrrolocarbazole compound 9) has demonstrated great potential and selectivity towards PIM kinases, while a fluorescent derivative PCC-13 (pyrrolocarbazole compound 13) has proven the ability of pyrrolocarbazole-based compounds to enter the cells (Akué-Gédu et al. 2009; Akué-Gédu et al. 2010; Letribot et al. 2012). DHPCC-9 inhibits PIM activity by competing with ATP by binding close to the ATP binding site (Figure 11).

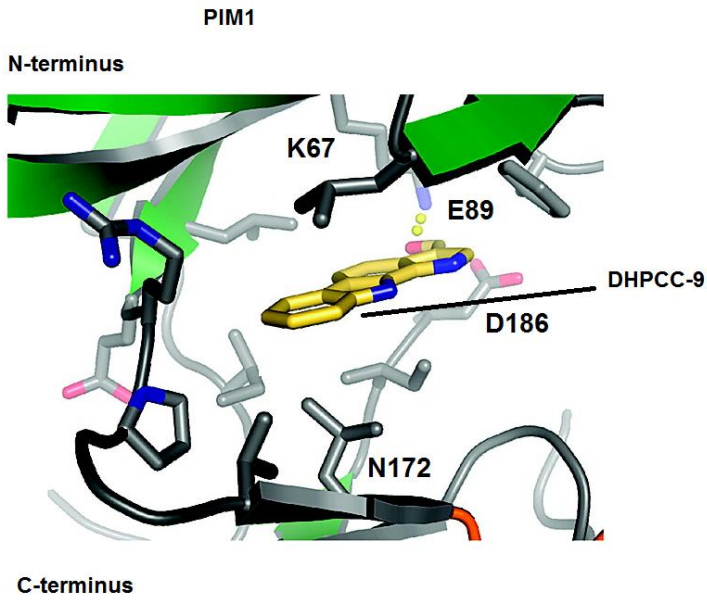

Figure 11 DHPCC-9 binding site in PIM1
The binding site of DHPCC-9 is located close to the ATP binding amino acids (modified from Akué-Gédu et al. 2009).

In nature, the blue-coloured compounds called azulenes can be extracted from various plants such as chamomiles (Lind 2003). The first benzo[*cd*]azulenes a.k.a benzazulenes were synthesized already over 40 years ago, but their potential as selective PIM inhibitors was discovered only recently (Boekelheide & Smith 1966; Kiriazis et al. 2013). Interestingly, the benzazulenes such as BA-1a (benzazulene 1a) have shown potential to inhibit especially PIM1 and PIM3 (Kiriazis et al. 2013).

2.6 Potential players in PIM-mediated cell migration and metabolism

2.6.1 Nuclear factor of activated T-cells 1

The first NFAT transcription factor was identified in activated T-cells as an enhancer of interleukin 2 expression (Durand et al. 1988; Shaw et al. 1988). Nowadays NFAT proteins are known to be involved not only in the function of hematopoietic system but also in epithelial cells. While NFAT activity is important e.g. for inflammation, there is also an increasing number of reports of the role of NFAT family members in cancer (Pan et al. 2013). NFAT pathway is also an attractive target for drug development and inactivation of NFAT signaling might be useful in cancer therapy. Certain drugs are already targeted to this pathway for instance to treat rejection of organ transpants, but long-term immunosuppression can also support formation of cancer, which is problematic (Medyouf & Ghysdael 2008).

The family of NFAT proteins consists of five members NFATC1/NFAT2, NFATC2/NFAT1, NFATC3/ NFAT4, NFATC4/ NFAT3 and NFAT5. The four members NFATC1-4 are more structurally related than the fifth family member NFAT5. Even though all the members have a homological domain called the Rel-homology domain, there are differences in the domain structure and function. While NFATC1-4 regulate transcription in complex with an adaptor protein 1 dimer, NFAT5 does not interact with it (Chen et al. 1998; Lopez-Rodríguez et al. 1999). Furthermore, the N-terminal NFAT-homology region, which is targeted by calcineurin-mediated dephosphorylation and activation, is not present in NFAT5 (Luo et al. 1996; Lopez-Rodríguez et al. 1999). Therefore, only NFATC1-4 activities are upregulated by increases in cellular calcium levels. However, there are also other factors than calcineurin that regulate NFAT activity. For instance, NFATC1 is phosphorylated and inactivated by several kinases such as GSK3 (glycogen synthase kinase 3), while PIM1 can phosphorylate distinct sites and thereby activate NFATC1 (Beals et al. 1997; Rainio et al. 2002).

Interestingly, both PIM kinases and NFATC family members can support cancer progression in both hematopoietic malignancies and solid cancers (Narlik-Grassow et al. 2014; Pan et al. 2013). NFATC1, NFATC2 and NFAT5 can also promote cell motility e.g. in breast cancer and endothelial cells (Jauliac et al. 2002; Seifert et al. 2009; Jang et al. 2010). In addition, NFATC members have also been connected to stimulation of angiogenesis (Graef et al. 2001; Hernández et al. 2001). Thereby NFATC1 may play a role in PIM-induced cell migration. Due to structural similaritites, PIM kinases may even be able to phosphorylate other NFAT family members than only NFATC1.

2.6.2 Chemokine (C-X-C motif) receptor 4

Chemokines are factors that regulate important functions such as leukocyte trafficking, angiogenesis, hematopoiesis and wound healing (Rollins 1997; Raman et al. 2011). Several chemokine ligands and receptors have been identified, while one especially important couple is the CXCR4 receptor and its ligand CXCL12 (Zlotnik & Yoshie 2012). The name CXCR comes from a complex classification of chemokine (C) receptors (R) according to the number and position of conserved cysteine residues in their amino acid sequences (XC) (Murphy et al. 2000; Zlotnik & Yoshie 2000). CXCL12 was originally identified as a stromal cell-derived factor 1 and a regulator of B cell proliferation, while CXCR4 was identified as a regulator of human immunodeficiency virus entry in T cells (Tashiro et al. 1993; Nagasawa et al. 1994; Feng et al. 1996). Later on CXCR4/CXCL12 signaling has been connected to the normal function of the hematopoietic system e.g. in inflammation, hematopoiesis and vascularization, but also to autoimmune and inflammatory disesases as well as cancer (Chatterjee et al. 2014).

CXCR4/CXCL12 pathway is an important regulator of cell migration during hematopoiesis, but it may also support tumorigenesis of hematopoietic malignancies as well as formation of metastases in solid cancers. The cells expressing CXCR4 receptor have been shown to move towards CXCL12 ligand-expressing cells in several cases (Chatterjee et al. 2014). High CXCL12 levels have been detected e.g. in lymph nodes, lungs, liver and bone marrow, while relatively low levels have been shown for prostate, brain and muscle (Müller et al. 2001). Furthermore, increased levels of CXCR4 has been found from malignant breast and prostate cancer cells, while inhibition of the CXCR4/CXCL12 axis has been connected to decreased formation of lung metastases in both cancer types (Müller et al. 2001; Singh et al. 2004; Shanmugam et al. 2011).

Because of its importance, the CXCR4/CXCL12 pathway is also a therapeutic target. Several compounds target them, and there are also attempts to improve the imaging of the pathway (Chatterjee et al. 2014). However, the importance of CXCR4/CXCL12 signaling in normal hematopoietic system may limit the use of the inhibitory compounds. For instance, knocking out either *CXCR4* or *CXCL12* causes perinatal death and severe defects in hematopoiesis in mice (Nagasawa et al. 1996; Ma et al. 1998). Therefore, the potentially harmful effects of complete or long-term inactivation of this pathway should be carefully examined.

PIM1 along with many other factors is also involved in the regulation of the CXCR4/CXCL12 pathway in hematopoietic system. PIM1 can phosphorylate CXCR4, thereby promoting its localization on the cell surface (Grundler et al. 2009). Furthermore, high PIM and cell surface CXCR4 levels have been detected in several hematopoietic malignancies such as acute myeloid leukemia, diffuse large B-cell lymphoma and chronic lymphocytic leukemia (Grundler et al. 2009; Brault et al. 2012; Decker et al. 2014). In addition, CXCR4 is a biomarker for radioresistant cancer stem cells, which implies that the PIM/CXCR4 interaction may partly explain the importance of PIM1 in the radioresistant squamocellular carcinoma cells (Trautmann et al. 2014; Peltola et al. 2009).

2.6.3 Forkhead box P3

Forkhead box proteins form a large family of transcription factors, which influence various targets such as organogenesis, immune and nervous systems, fertility, metabolism and tumorigenesis (Benayoun et al. 2011). The first family member was found in the fruit fly, where mutation of the gene led to replacement of terminal structures of embryos by head-derived elements, leading to a spiked or fork-headed appearance, from which the name originates (Weigel et al. 1989). By now, total of 19 subclasses of forkhead box protein families have been identified and each subclass is described by a letter from A to S (Hannenhalli & Kaestner 2009).

The forkhead box subclass P protein 3, FOXP3, is a marker for regulatory T cells and has been shown to be an important factor regulating immune homeostasis (Fleskens & van Boxtel 2014). FOXP3 also acts as a tumor suppressor e.g. in breast and prostate cancer (Wang et al. 2010b). However, it has shown rather pro-tumorigenic behavior in tumor-infiltrating regulatory T cells expressing high levels of FOXP3. Several studies support the role of intratumoral regulatory T cells in protection of cancer cells from immune defense (Tanchot et al. 2013).

Because FOXP3 is reported to impede cell migration and PIM kinases can inhibit FOXP3 activity, it is reasonable to assume that FOXP3 might also play a role in PIM-induced prostate cancer cell migration (Zhang et al. 2015b; Li et al. 2014; Deng et al. 2015). Furthermore, there are also reports showing inhibition of CXCR4/CXCL12-mediated cell migration and repression of NFAT activity by FOXP3 (Douglass et al. 2014; Bettelli et al. 2005). Therefore, FOXP3 is a highly attractive target for PIM-related research.

2.6.4 Glycogen synthase kinase 3 beta

Glycogen synthase kinases belong to a large protein family consisting of cyclin-dependent kinases, mitogen-activated protein kinases, glycogen synthase kinases and cyclin-dependent kinase-like kinases (Kaidanovich-Beilin & Woodgett 2011). The main function of GSKs is to catalyze the phosphorylation and inactivation of glycogen synthase, which is needed for conversion of glucose into glycogen. The GSK family members are a cyclic-AMP-dependent protein kinase (“GSK1”), a phosphorylase kinase (“GSK2”) and glycogen synthase kinases GSK3A and GSK3B (glycogen synthase kinase 3 alpha and beta). (Huijing & Larner 1966; Embi et al. 1979; Woodgett 1990).

GSK3 serine/ threonine kinases are expressed in several tissues, and in cells they localize to both nucleus and cytoplasm (Kaidanovich-Beilin & Woodgett 2011). GSK3 proteins inhibit glycogen synthase activity, but they have also other substrates such as NFATC1 (Beals et al. 1997). Furthermore, GSK3-mediated signaling pathways have been connected to cellular activities such as regulation of metabolism, development, neuronal functions and cell survival (Kaidanovich-Beilin & Woodgett 2011). GSK3 activity is regulated by various mechanisms such as phosphorylation. Tyrosine phosphorylation leads to GSK3 activation, and hence

GSK3 proteins are usually active in cells (Hughes et al. 1993). However, phosphorylation of GSK3A at Ser^{21} and GSK3B at Ser^{9} leads to kinase inactivation (Sutherland et al. 1993; Sutherland & Cohen 1994).

GSK3 proteins have also been connected to tumorigenesis, but it is not clear, whether they act rather as tumor suppressors or oncogenes (McCubrey et al. 2014). For example in breast and prostate cancer, GSK3B has been reported to have both pro- and anti-tumorigenic effects (Darrington et al. 2012; Kim et al. 2013; Shao et al. 2013; Goc et al. 2014; Zhu et al. 2011). In addition, there are reports showing deregulation of androgen receptor activity by GSK3 (Mazor et al. 2004; Wang et al. 2004). While the role of GSK3 is slightly controveral in cancer, there are still several drugs in market and in clinical trials targeting GSK3 pathway in different diseases such as cancer (McCubrey et al. 2014).

It has been previously suggested in a review article that PIM kinases phosphorylate GSK3B (Narlik-Grassow et al. 2014), but direct evidence for phosphorylation has not been shown. However, the sequence of GSK3B around Ser^{9} (-RPRTTSF-) shares some complementarity with the PIM1 consensus sequence, suggesting that PIM1 may be able to phosphorylate GSK3B. In addition, AKT can phosphorylate and inactivate GSK3B, while inhibition of PI3K/AKT signaling by LY294002 reduces GSK3B phosphorylation e.g. in PC-3 prostate cancer cells (Cross et al. 1995; Liu et al. 2014c). The LY294002 inhibitor is also known to reduce PIM kinase activity, and decreased GSK3B activity has been connected to increased PC-3 cell migration (Jacobs et al. 2005; Liu et al. 2014c). All these data raise the question of whether PIM kinases can directly phosphorylate GSK3B and thereby influence the migratory potential of prostate cancer cells.

2.6.5 Prostaglandin-endoperoxide synthase 2

Prostaglandin endoperoxide synthases are also known as cyclooxygenases (Simmons et al. 2004). They catalyse prostaglandin synthesis by their cyclooxygenase and peroxidase activities, while other enzymes are needed for catalysis of synthesis of the final products such as prostaglandins (Figure 12). Prostaglandins are involved in the regulation of various cellular and physiological functions such as inflammation, vascular smooth muscle cell behavior, platelet aggregation and maintenance of normal tissue functions e.g. in stomach, kidney and lungs (Simmons et al. 2004). Furthermore, several broadly used non-steroidal anti-inflammatory drugs such as acetylsalicylic acid and ibuprofen target cyclooxygenases.

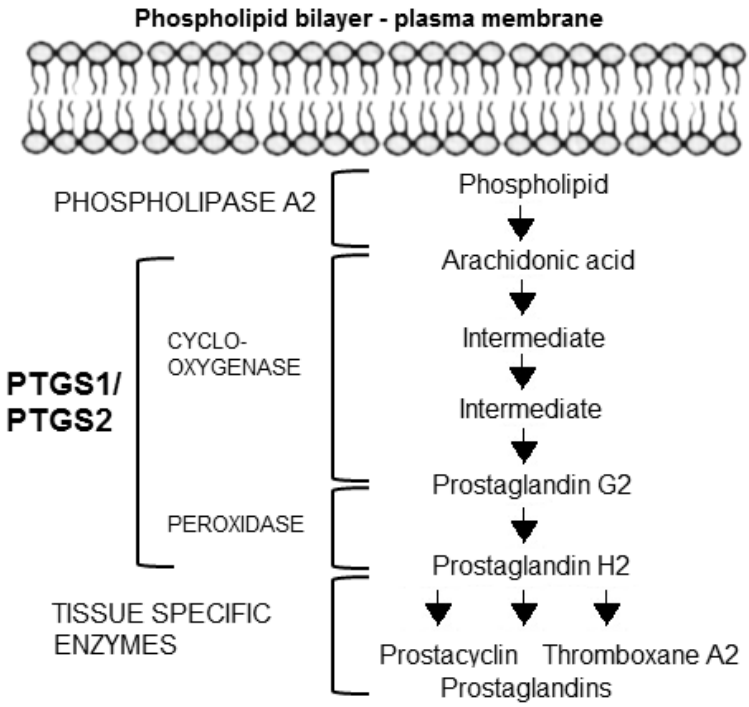

Figure 12 Prostanoid synthesis
After release of phospholipids from the plasma membrane, several enzymes are needed for synthesis of prostanoids (modified from Smith et al. 2000).

Prostaglandin-endoperoxide synthase family is formed by two members PTGS1 and PTGS2 (Hla et al. 1986; Yokoyama & Tanabe 1989; Hla & Neilson 1992). While PTGS1 is generally expected to be expressed more broadly in various tissues as compared to PTGS2, the differences between these two proteins are not entirely clear (Rouzer & Marnett 2009). PTGS2 plays a major role in inflammation, while it can also support tumor formation, angiogenesis and invasiveness in various cancers (Ghosh et al. 2010). For instance, increased PTGS2 activity is likely to promote breast and prostate cancer progression (Howe 2007; Sooriakumaran & Kaba 2005). Furthermore, PTGS2 upregulation has been connected to PC-3 prostate cancer cell migration, which was abrogated by the PI3K inhibitor LY294002, which also inhibits PIM kinase activity (Vo et al. 2013; Jacobs et al. 2005). Thus, PTGS2 can be expected to play a role in PIM-mediated cell migration.

Previously described substrates for PIM kinases, NFATC1, FOXP3 and the potential substrate GSK3B have all been shown to regulate PTGS2 expression e.g. in endothelial or gastric cancer cells (Mena et al. 2014; Hao et al. 2014; Thiel et al. 2006). Due to its importance in cancer and connection to PIM kinase substrates, PTGS2 is a very interesting enzyme for PIM-targeted cancer research.

2.6.6 Integrin receptor proteins

As previously stated, several known PIM kinase substrates have been linked to cell motility. In addition, some of them can regulate the expression of PTGS2, which also affects movement of cells. Both GSK3 activity and PTGS2-mediated signaling have also been connected to integrin-dependent cell adhesion and motility (Roberts et al. 2004; Menter & DuBois 2012). As previously mentioned, the integrins (ITGs) are the main regulators of cell-matrix contact, and thereby they regulate both the cell motility and the cell survival. While the regulation of adhesion is important in normal cellular and physiological conditions, abnormal integrin activity can also promote tumorigenesis (Desgrosellier & Cheresh 2010). Furthermore, the proper regulation of integrins during cytokinesis is highly important, and aberrant integrin signaling may lead to aneuploidy and thereby tumor formation (Högnäs et al. 2012). Hence, integrins may also play a role in tumor progression regulated by the PIM kinases.

Integrin cell surface receptor heterodimers are formed by A (alpha) and B (beta) subunits (Tamkun et al. 1986). Several integrin subunits have been discovered and different heterodimers are known to bind different extracellular matrix components (Table 1) as well as other adhesion molecules (Plow et al. 2000; Takada et al. 2007). Integrin signaling is regulated by complex signaling pathways from outside and inside the cells (Takada et al. 2007; De Franceschi et al. 2015).

Table 1 Integrin binding to extracellular matrix
Here are examples of the ECM (extracellular matrix) components which bind to heterodimers formed by integrin A (alpha) and B (beta) subunits (Plow et al. 2000; Takada et al. 2007).

ECM component	Heterodimers formed by the integrin subunits
Collagens	A1B1, A2B1, A10B1, A11B1, AIbB3, AXB2
Fibronectin	A2B1, A3B1, A4B1, A4B7, A5B1, A8B1, AIIbB3, ADB2, AVB1, AVB3, AVB5, AVB6, AVB8
Laminin	A1B1, A2B1, A3B1, A6B1, A6B4, A7B1, A10B1, AVB3

Due to the importance of integrins in normal and malignant cell function, there are also attempts to target integrins by drugs in various diseases such as cancer (Millard et al. 2011). However, the function of integrins in normal cell behavior can cause problems for the use of these drugs. Though, there are differences between the integrin subunits and their roles in diseases, and thereby selective targeting may reduce or prevent the unwanted effects. Still, the best way might be to target the integrins indirectly through inhibition of other proteins, which regulate integrin-mediated cellular functions and only affect some integrin subunits.

2.6.7 Receptor protein and transcriptional regulator NOTCH1

NOTCH receptors are regulators of development, tissue homeostasis and cancer progression. They are cell surface proteins, which after cleavage enter the cell nucleus and form a transcriptional regulatory complex with other cofactors. PIM kinases support the activity of the transcription factor EBNA2 (Epstein-Barr virus nuclear antigen 2), which forms a complex with a cellular cofactor called RBPJ (recombination signal-binding protein for immunoglobulin kappa J region) a.k.a. CBF1 (C promoter-binding factor 1) prior to regulation of the target genes (Rainio et al. 2005; Henkel et al. 1994). The same cofactor is also a member of the transcriptional regulatory complex formed by NOTCH receptor intracellular domain (NICD) (Tamura et al. 1995). Furthermore, NOTCH signaling supports JAK/STAT activation (Jin et al. 2013). NOTCH can also inhibit expression of PIM kinase substrates such as NFATC1 and CXCR4 as well as promote transactivation of FOXP3 (Tu et al. 2012; Williams et al. 2008; Wang et al. 2009; Xie et al. 2013; Ou-Yang et al. 2009). GSK3 can also inhibit NOTCH1 and NOTCH2 activity (Espinosa et al. 2003; Jin et al. 2009). NOTCH signaling pathway also supports MYC expression (Palomero et al. 2006; Sharma et al. 2006; Weng et al. 2006) In addition, NOTCH signaling can repress androgen receptor activity (Belandia et al. 2005). Taken into account the role of NOTCH receptors in cancer as well as their connection to PIM signaling pathways, NOTCH family members are potential players in the PIM-regulated cellular functions.

NOTCH receptors were originally found when a wing-notching phenotype was characterized in the fruit fly, after which the gene was discovered and named as *notch* (Dexter 1914; Wharton et al. 1985). Thereafter NOTCH receptors have been a target of massive research and four family members have been discovered in mammals, NOTCH1-4 (Kopan & Ilagan 2009). The NOTCH receptor consists of an extracellular domain, a transmembrane domain and an intracellular domain. The activating ligands for the receptors are divided in two groups in mammals, the delta-like and jagged protein families (Kopan & Ilagan 2009).

NOTCH receptor activation is a multi-step process. Prior to cell surface localization the full-length protein undergoes a proteolytic cleavage in the Golgi apparatus (Blaumueller et al. 1997; Watanabe et al. 2009). Thereafter the cleaved receptor protein is transported to the cell membrane. After ligand binding, first a metalloprotease and then a gamma-secretase containing protein complex cleaves the receptor, leading to formation of the active NICD (van Tetering & Vooijs 2011). Then, the NICD can enter the nucleus and regulate transcription in complex with RBPJ a.k.a. CSL (human C promoter binding factor/ fruit fly suppressor of hairless/ nematode LIN-12 and GLP-1) (Henkel et al. 1994; Tamura et al. 1995; Christensen et al. 1996). In addition, the transcriptional complex can be further regulated positively or negatively by other factors (Kopan & Ilagan 2009). Several target genes such as hes family bHLH transcription factors and HES-related family bHLH transcription factors with YRPW motif are regulated by NOTCH intracellular domains (Jennings et al. 1994; Maier & Gessler 2000).

NOTCH signaling is important for development and normal tissue homeostasis, while it has been connected to several diseases as well as both hematological malignancies and solid tumor progression (Louvi et al. 2012; Ntziachristos et al. 2014). Interestingly, the role of NOTCH signaling is often highly context-dependent, and there is evidence of a controversial behavior of NOTCH receptors in breast versus prostate cancer. In breast cancer, NOTCH proteins often behave as oncogenes, while in some cases NOTCH2 and NOTCH3 have also shown tumor suppressor functions (Guo et al. 2011; Cui et al. 2013). In prostate cancer, NOTCH1 seems to play the most important role as compared to other family members. NOTCH1 can promote prostate cancer cell survival and motility e.g. in PC-3 cells, while patient and *in vivo* models show a connection between tumor progression and increased NOTCH1 levels (Leong & Gao 2008). However, in some cases NOTCH1 has also been connected to decreased prostate cancer cell proliferation and better patient survival, referring to a tumor suppressive role (Leong & Gao 2008).

NOTCH proteins also regulate cell energy metabolism. For instance in breast cancer, NICD1 enable the use of either glycolysis or oxidative phosphorylation depending on the oxygen levels, while NOTCH1 inactivation leads to defects in mitochondrial function and enhances glycolytic activity (Landor et al. 2011). Thereby NOTCH1 can be considered as the regulator of the "glycolytic switch", which supports efficient energy gain of tumor cells in both high and low oxygen conditions.

Due to its importance in breast and prostate cancer and the connection to PIM signaling, NOTCH family is a very interesting research target. NOTCH1 might be a potential regulator of PIM-mediated cell migration, while NOTCH activity could also connect PIM kinases to the regulation of breast tumorigenesis. In addition, similar to PIM kinases, there are also slightly contradictory data on the influence of NOTCH1 on prostate cancer progression.

3 AIMS OF THE STUDY

Cancer is one of the leading causes of death in the world and cancer therapy needs lots of improvements. The major problems in developing efficient treatments are the harmful side effects due to poor selectivity of chemotherapy. Therefore, it is important to investigate different signaling pathways of cancer cells to discover new druggable pathways to target.

PIM kinases promote cell survival and are often highly expressed in malignancies. Interestingly, *PIM* triple (*PIM1, PIM2* and *PIM3* deleted) knock-out mice have been shown to be born and live normally (Mikkers et al. 2004). They have a slightly altered immune system and decreased body size, but the results still prove that inactivation of PIM kinases is not lethal, as is the case of inactivation of several other cell survival factors. The structure of PIM kinases also differs enough from other kinases to enable the design of selective PIM-targeted compounds (Jacobs et al. 2005; Qian et al. 2005). Thus, it is extremely intriguing to test novel PIM inhibitors to search for a selective and effective compound, which could reduce tumor progression.

In addition to PIM inhibitor development, it is highly important to discover more information about the signaling pathways, which PIM-targeted drugs might affect. The functional roles of PIM kinases have been studied especially in lymphomas and leukemias, but lately also in solid tumors. However, the studies have mostly concentrated on the influence of PIM kinases on cell survival, proliferation and apoptosis (Brault et al. 2010; Narlik-Grassow et al. 2014). The PIM-dependent signaling pathways regulating cancer cell metabolism to support tumor growth, or cancer cell motility to promote formation of metastases have not been properly measured.

The aim of this Doctoral Thesis research was to clarify the importance of PIM kinases in the regulation of cancer cell motility and metabolism as well as to analyse the efficacy of novel PIM kinase inhibitors as anti-cancer agents. The hypothesis was, that PIM kinases may support cancer cell motility and energy gain in certain cancer types. The selective PIM inhibitors were expected to abrogate the PIM-mediated pro-tumorigenic functions. The specific aims to confirm the hypothesis were as follows:

- ✓ Perform cell-based and *in vivo* experiments to study the role of PIM kinases in cell motility, especially in prostate cancer
- ✓ Test efficacy and toxicity of the PIM-selective inhibitors DHPCC-9 and BA-1a
- ✓ Identify novel PIM-mediated signaling pathways in breast and prostate cancer
- ✓ Test, whether PIM kinases regulate glycolytic metabolism in breast cancer

4 MATERIALS AND METHODS

While detailed information is included in the original publications, summaries of materials and methods are presented in the tables 2-12. Methods used in the experiments published in Letribot et al. 2012 and Kiriazis et al. 2013 are not described here, even though part of those results are shown in the Results section.

Table 2 Protein inhibitors and their diluents

Short name	Formal name	Purpose	Used in
BA-1a	8-isopropylidene-4-methoxy-1-methyl-3-trifluoromethyl-8*H*-benzo[*cd*]azulene	PIM inhibitor	II
BA-2c	4-hydroxy-1-methyl-3-trifluoromethyl-8*H*-benzo[*cd*]azulen-8-one	PIM inhibitor	II
Celecoxib	4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]-benzenesulfonamide	PTGS2 inhibitor	III
DAPT	*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester	gamma-secretase inhibitor	IV
DHPCC-9	1,10-dihydropyrrolo[2,3-*a*]carbazole-3-carbaldehyde	PIM inhibitor	I, II, III, IV
DMA	*N,N*-dimethylacetamide	diluent	II
DMSO	Dimethylsulphoxide	diluent	I, II, III, IV
Indomethacin	1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid	PTGS1 inhibitor	III
SGI-1776	*N*-[(1-methylpiperidin-4-yl)methyl]-3-[3-(trifluoromethoxy)phenyl]imidazo[1,2-*b*]pyridazin-6-amine	PIM inhibitor	IV, unpublished (I)

Table 3 Oligonucleotides for RNA interference

Oligonucleotides were ordered from Dharmacon (D) or Sigma-Aldrich (S).

siRNA	Sequence, details and manufacturer	Used
non-targeting (D)	D-001810-01-20 ON-TARGETplus Non-tageting siRNA #1	I, III, IV
non-targeting (S)	MISSION® siRNA Universal Negative Control #1, SIC001	III, IV
human PIM1 (D)	5′- GAU GGG ACC CGA GUG UAU A -3′ J-003923-09-0020 ON-TARGETplus PIM1 siRNA	I, III
human PIM2 (D/S)	5′- GUG GAG UUG UCC AUC GUG ACA UU -3′ 5′- UGU CAC GAU GGA CAA CUC CAC UU -3′	I, III, IV
human PIM3 (S)	5′- GGC GUG CUU CUC UAC GAU AUG UU -3′ 5′- CAU AUC GUA GAG AAG CAC GCC UU -3′	III, IV

Table 4 DNA constructs
Shown are vectors for human (upper case) or mouse (lower case) proteins.

Tag	Backbone	Insert	Origin or cloning	Used in
AVI	MigRI-AVI	FoxP3	A.Rudensky (Howard Hughes Medical Institute)	III
Flag	pBJ5-Flag	NFATC1	S.N. Ho (Stanford)	I
	pFlag-CMV-2	FoxP3	N. Santio	III
	p3xFlag-CMV7.0	Notch3ICD	U. Lendahl (Karolinska)	IV
	pIRES-puromycin	FOXP3	B. Li (Shanghai)	III
GFP	GFP-linker	Notch1ΔE	U. Lendahl (Karolinska)	IV
	pEGFP-C3	GSK3B	N. Santio	III
	pEGFP-N1	FOXP3	R. Mentlein (Kiel)	III
GST	pGEX-2T	CXCR4 (C46)	A. Bullock (Oxford)	II
	pGEX-4T-3	Notch1ICD	U. Lendahl (Karolinska)	IV
	pGEX-6P-1	PIM1	N. Santio	I, II, III, IV
		PIM2	N. Santio	II, IV
		Pim3	R. Vahakoski	II, IV
		FoxP3	S. Eerola	III
	pGEX-6P-3	GSK3B	H. Arola	III
		Notch2ICD	N. Santio; E. Paloniemi	IV
		Notch3ICD	N. Santio; E. Paloniemi	IV
HA	pBJ5-HA	GSK3B	C. Beals (Stanford)	III
RFP	pTag-RFP-N	PIM1	G. Manoharan (Tartu)	III, IV
V5	pcDNA3.1./V5-HisC	PIM1	M. Varjosalo (Helsinki)	I, II, III, IV
		PIM2	M. Varjosalo (Helsinki)	I, II
		PIM3	M. Varjosalo (Helsinki)	I, II, IV
-	pCS2+	Notch1ΔE	U. Lendahl (Karolinska)	IV

Table 5 Mutagenesis
Phosphodeficient (SA), phosphomimicking (SD/SE) and kinase-deficient (KD) human (upper case) or mouse (lower case) proteins were generated by mutagenesis (A=alanine, D=aspartic acid, E= glutamic acid, K=lysine, M=methionine and S=serine).

Protein	Mutation	Mutant name	Origin or mutagenesis	Used in
CXCR4	S339>A	SA	A. Bullock (Oxford)	II
FoxP3	S418>A	S418A	N.Santio	III
FOXP3	S418>A	SA (S418A)	B. Li (Shanghai)	III
	S418>D	SD (S418D)	B. Li (Shanghai)	III
	S422>A	SA (S422A)	B. Li (Shanghai)	III
	S422>D	SD (S422D)	B. Li (Shanghai)	III
GSK3B	S9>A	SA	N. Santio; H. Arola	III
Notch1	S2152>A	SA or S2152A	N. Santio	IV
	S2173>A	S2173A	N. Santio	IV
	S2152>E	SE	N. Santio	IV
PIM1	K67>M	KD	M. Varjosalo	III, IV

Table 6 Bacterial cell culture and experiments
Luria Broth medium was supplemented with kanamycin (50 µg/ml) or ampicillin (50 µg/ml).

***E.Coli* cells**	**Experiments and methods**	**Used in**
DH5a	Culture at 37 °C, plasmid isolation	I, II, III, IV
BL21	Culture at 37 °C, GST-tagged protein production at 30 °C	I, III, IV

Table 7 Nucleic acid transfer to cells

Cells	Nucleic acid	Methods	Used in
Bacterial cells	DNA	Heat shock (42 °C, 2 min)	I, II, III, IV
		Electroporation (capacitance 25 µF, voltage 1.8 kV)	III
Eukaryotic cells	DNA	Electroporation (capacitance 975 µF, voltage 230 kV)	IV
		Lipofection (Fugene 6/HD, Promega, 3:1 to DNA or JetPEI, Polyplus Transfection™)	I, II, III, IV
	siRNA	Lipofection (Oligofectamine™, Invitrogen, 100-200 nM siRNA)	I, III, IV

Table 8 Eukaryotic cell culture

Roswell Park Memorial Institute (RPMI-1640) medium or Dulbecco's Modified Eagles Medium (DMEM) were supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM L-glutamine.

Cell line	Cell type	Medium details	Used in
A549	human, lung, carcinoma	DMEM	unpublished (I)
FDC-P1 (Stable cell lines FD/Neo, FD/Pim44)	murine, bone marrow, lymphoblast	RPMI-1640 + conditioned medium from WEHI3B cells	I
HeLa	human, cervix, adenocarcinoma	DMEM	unpublished (I)
MCF-7 (Stable cell line MCF-7/Notch1ΔE)	human, mammary gland/breast, adenocarcinoma	DMEM + non-essential amino acids, growth induction by estradiol (Stable Notch1ΔE induction by 1 ug/ml of Doxycycline)	IV
MDA-MB-231	human, mammary gland/breast, adenocarcinoma	DMEM	unpublished (I, IV)
MG-63	human, bone, osteosarcoma	DMEM (conditioned medium: 0,5 µg/ml of ascorbic acid (10 days) and 0.1% BSA + ascorbic acid (two days)	I
PC-3 (Stable cell lines PC-3/Mock/Tomato, PC-3/PIM1/Tomato, PC-3/PIM3/Tomato)	human, prostate, adenocarcinoma	RPMI-1640 (Stable cell line selection by 200 µg/ml of G418)	I, II, III, IV (stable lines in II, III, IV)
Saos-2	human, bone, osteosarcoma	DMEM	unpublished (I)
UT-SCC-12A	human, head and neck, squamous cell carcinoma	DMEM	I
WEHI3B	murine, peripheral blood, leukemia	RPMI-1640	I

Table 9 ***In vitro*, cellular and animal experiments**
Animal experiments were licenced by the Provincial State Office of Western Finland with the licence IDs ESAVI/4068/04.10.07/2013, ESAVI/2008-05531 and ESAVI/3937/04.10.03/2011.

Experiment	Short protocol	Used in
Cell adhesion	Follow up by xCelligence (Roche) on collagen I/ fibronectin or poly-L-lysine -coated plates	III
Cell confluency/ proliferation	Automatic imaging (IncuCyte™, Essen Instruments)	IV
Cell invasion	Invasion in an invasion insert, fixation, staining, mounting, light microscopy	I
Cell migration	Automatic scratching, imaging (WoundMaker™ and IncuCyte™, Essen Instruments)	I
	Manual scratching, light microscopy	I, II, III, IV
Cell viability	MTT assay, spectrophotometry	I, II, IV
	Tryban blue staining, light microscopy	I, III
Cellular protein phosphorylation	Whole cell lysis, (protein immunoprecipitation), Western blotting	I, II, III, IV
Glucose uptake	Fluorescent glucose probe uptake, flow cytometry	IV
In vitro kinase assay	Reaction with radioactive or non-radioactive ATP, SDS-PAGE, autoradiography or Western blotting, protein staining by PageBlue™ or Ponceau S	I, II, III, IV
Lactate production	Convertion of lactate to pyruvate from conditioned medium, production of NADH, spectrophotometry	IV
Mass spectrometry	*In vitro* kinase assay, SDS-PAGE, in-gel trypsin-digestion, phosphopeptide enrichment, liquid chromatography-tandem mass spectrometry	IV
NOTCH activation	Cell culture on jagged 1-coated or control wells	IV
NOTCH activity	Luciferase reporter and control beta-galactosidase transfection, luminometry	IV
Orthotopic xenografts in adult nude mice	Cell inoculation into the prostate, daily treatments and monitoring, sacrifice after 25 days, tissue collection and staining, light microscopy	II, IV
Protein localization and interaction	Localization: fixed cover glass samples, (antibody staining), confocal microscopy Colocalization: sequential scanning Interaction: fluorescence-lifetime imaging or proximity ligation assay	II, III, IV
Subcutaneous xenografts in adult nude mice	Cell inoculation into the back side, daily monitoring, fluorescence imaging, sacrifice after 24 days, tissue collection and staining, light microscopy	II
Toxicity in adult mice	Daily treatments and monitoring for 6-17 days, sacrifice, tissue collection and staining, light microscopy	II
Toxicity in zebrafish embryos	After spawning, collection of embryos, treatments from 6-50 hours post fertilization, dechorionation, light microscopy	II
Tumor growth in chicken eggs	Cell inoculation on the chorioallantoic membrane of a nine-day-old embryo, treatments for 5 days, tumor collection and weighing	IV

Table 10 Primary antibodies

Incubations were performed at +4 over/night, with few exceptions (1 h*, 2 h** or 2,5 h*** at room temperature). Shortenings: Western blotting (WB), immunoprecipitation (IP), immunofluorescence (IF), immunohistochemistry (IHC), proximity ligation assay (PLA), phospho- (p), serine (Ser) and threonine (Thr). Antibody targets are either human (upper case) or mouse (lower case) proteins or tags (tag).

Antibody target	Company and product code	Dilution	Used in
ACTB	Sigma-Aldrich, D13K4803	1:20000 (WB)	II, III, IV
	Cell Signaling Technology, 13E5, #4970S	1:1000 (WB)	IV
Bad	Cell Signaling Technology, #9292	1:1000 (WB)	I
Bad $pSer^{112}$	Cell Signaling Technology, #9291S	1:1000 (WB)	I
Cd34 (blood vessels)	Santa Cruz,ME 14.7, sc-18917	1:50 (IHC)	II
CXCR4	Abcam, ab2074	1:1000 (WB) 1:1000 (IF) 1:200 (IHC)	II
CXCR4 $pSer^{339}$	Abcam, ab74012	1:1000 (WB) 1:1000 (IF) 1:500 (IHC)	II
Flag (tag)	Sigma-Aldrich, F1804	1:500 (WB) 1:500 (IF)	I, III, IV
FOXP3/ FoxP3	Santa Cruz, H-190, sc-28705	1:1000 (WB)	III
GAPDH/ Gapdh	Sigma-Aldrich, G8795	1:20000 (WB)	I, II
GFP (tag)	Cell Signaling Technology, #2956S	1:1000	III, IV
GSK3B	Cell Signaling Technology, #9315	1:3000 (WB)	III
GSK3A $pSer^{21}$/ GSK3B $pSer^{9}$	Cell Signaling Technology, #9331S	1:3000 (WB)	III
HA (tag)	Sigma-Aldrich, H3663	1:5000 (WB)	III
HSPA8 a.k.a. HSC70	Enzo Life Sciences, 1B5	1:1000 (WB)	IV
Lyve1 (lymphatic vessels)	Dr. Jackson, WIMM, Oxford, UK	1:200 (IHC)***	II
MT-CO2 a.k.a. COXII	Santa Cruz, 12C4, sc-65239	1:25 (PLA)*	IV
activated NOTCH1/ Notch1	Abcam, ab8925	1:1000 (WB)	IV
cleaved NOTCH1/ Notch1	Cell Signaling Technology, Val1744, #D3B8	1:1000 (WB) 1:500 (IHC)*	IV
	Sigma-Aldrich, SAB4502019	1:1000 (IP)	IV
NOTCH1	Santa Cruz, C20, sc-6014	1:33 (IF)* 1:33 (PLA)*	IV
NOTCH3	Cell Signaling Technology, #C2211	1:1000 (WB)	IV
pH3F3 (mitosis)	Cell Signaling Technology, #9701	1:200 (IHC)	II

pSer/Thr	Cell Signaling Technology, #9614	1:1000 (WB)	IV
PIM1/Pim1	Santa Cruz, 12H8, sc-13513	1:500 (WB)	I, II, III, IV
	Abcam, ab117525	1:25 (IF)* 1:25 (PLA)*	IV
	Cell Signaling Technology, #2907	1:1000 (WB)	IV
PIM2/Pim2	Atlas Antibodies, HPA000285	1:1000 (WB)	I
	Cell Signaling Technology, D1D2, #4730	1:1000 (WB)	I, II, III, IV
PIM3/Pim3	Cell Signaling Technology, D17C9, #4165	1:1000 (WB)	I, II, III, IV
PTGS2	Cayman Chemicals, 160116	1:2000 (WB)	III
V5 (tag)	Life technologies (Invitrogen), #46-0705	1:500 (WB)	I
	Abcam, ab95038	1:500 (IHC)**	II

Table 11 Secondary antibodies
Incubations were performed at room temperature for 30 min for Western blotting (WB), 1 h for immunofluorescence (IF) and 2 h for immunohistochemistry (IHC), with few exceptions (30 min at 37 °C and 30 min at room temperature* or 30 min at room temperature**). Proximity ligation assays (PLA) were performed according to manufacturer's protocol.

Antibody details	**Company and product code**	**Dilution**	**Used in**
chicken anti-rabbit (Alexa Fluor® 488)	Life Technologies (Invitrogen), A21441	1:1000 (IF)*	II
horse anti-mouse	Cell Signaling Technology, #7076	1:5000 (WB)	I, II, III, IV
goat anti-rabbit	Cell Signaling Technology, #7074	1:5000 (WB)	I, II, III, IV
donkey anti-mouse (Alexa Fluor® 488)	Life Technologies (Invitrogen), A21202	1:200 (IF)	IV
donkey anti-rabbit (Alexa Fluor® 546)	Life Technologies (Invitrogen), A10040	1:200 (IF)	IV
goat anti-rabbit	Vector, BA-1000	1:200 (IHC)	II
horse anti-goat	Vector, BA-9500	1:200 (IHC)	II
goat anti-rabbit	Immunologic, DPVR55HRP	1:500 (IHC)**	IV
rabbit anti-rat	DAKO, 097(101)E0468	1:200 (IHC)	II
Duolink® In Situ Orange Starter Kit	Sigma-Aldrich, DUO92102	(PLA)	IV

Table 12 Statistical analyses and preparation of diagrams
$P<0.05$ was considered as a significant difference (marked in diagrams by *). Error bars represent standard deviation.

Test	**Software**	**Used in**
ANOVA variance analyses	IBM SPSS Statistics 22	II
Non-linear regression fitting	GraphPad Prism v.5.0	I
Student´s t-test	Microsoft Excel	II, III, IV
Z-test	Microsoft Excel	I

5 RESULTS

5.1 Selective Pim inhibition by DHPCC-9 in cells (I)

5.1.1 DHPCC-9 decreases Pim1-dependent myeloid cell survival

Survival of FDC-P1 (factor-dependent cell progenitor 1) murine myeloid cells is highly dependent on interleukin 3, but stable overexpression of Pim1 prolongs cell survival in the absence of interleukin 3 (Aho et al. 2004). Thereby this cell-based model is well suitable for studying of Pim-selective inhibition of cell proliferation. Previously, DHPCC-9 had been identified as a PIM-selective inhibitor *in vitro* (Akué-Gédu et al. 2009). In this study, FD/Neo (control) and FD/Pim44 (stable overexpression of Pim1 44 kDa isoform) cells were cultured with or without interleukin 3. After DHPCC-9 treatment, survival of only FD/Pim44 cells was decreased in both cases (I: Figure 1A-B). Furthermore, the IC_{50} value for DHPCC-9 was lower in FD/Pim44 cell line as compared to the control FD/Neo cell line, when cells were cultured in the absence of interleukin 3 (I: Figure 1C). Similar results were gained by measuring the metabolic activity as a sign of cell well-being (MTT assay) and direct staining of dead cells (Trypan blue staining) (I: Figure 1B, D). To sum up, Pim inhibition by DHPCC-9 only affected the survival of Pim1-overexpressing cells and not control cells, referring to a selective Pim inhibition.

5.1.2 DHPCC-9 inhibits Pim activity in myeloid cells

Pim1 has previously been shown to phosphorylate and inactivate the pro-apoptotic protein Bad at Ser^{112} (Aho et al. 2004). Therefore, Bad phosphorylation was analysed after Pim inhibition by DHPCC-9 in FDC-P1 cells. After 8 hours of interleukin 3 withdrawal, Pim expression was decreased in control cells, along with Bad phosphorylation, while stable Pim1 expression maintained Bad phosphorylation also in the absence of interleukin 3 (I: Figure 2A-B). As expected, DHPCC-9 treatment led to a decrease in Bad phosphorylation in the Pim1-overexpressing cells, while Pim44 protein levels were not influenced (I: Figure 2A-B). Pim levels were also measured in the presence of interleukin 3 as a control and no major changes were detected after DHPCC-9 treatment (I: Figure 2C). Thus DHPCC-9 efficiently blocks cellular Pim kinase activity without affecting Pim protein levels.

5.1.3 DHPCC-9 is not cytotoxic like SGI-1776

The behavior of DHPCC-9 was first studied in the stable FDC-P1 cells, where it did not show signs of cytotoxicity (I). The IC_{50} value of DHPCC-9 had also been shown e.g. in human prostate cancer PC-3 cells (Akué-Gédu et al. 2009). As an additional control to exclude the possibility of severe cytotoxicity, DHPCC-9 was also compared to a well-known cytotoxic PIM kinase inhibitor SGI-1776 (Chen et al. 2011; Clinical Trials.gov 2015). Again, DHPCC-9 only decreased Pim-dependent survival in FD/Pim44 cells, while SGI-1776 decreased the survival of both control and Pim-overexpressing FDC-P1 cells as well as naïve PC-3 cells (Figure 13A-B). This further confirmed the Pim-selectivity of DHPCC-9.

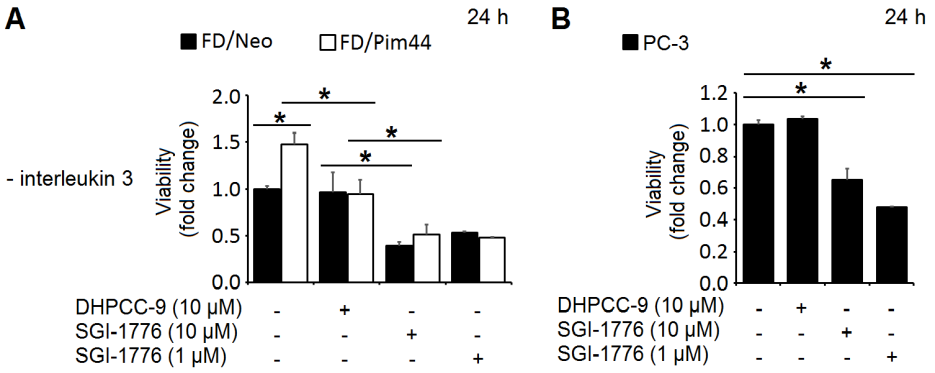

Figure 13 Comparison of PIM inhibitors DHPCC-9 and SGI-1776
A, DHPCC-9 selectively decreased the viability of PIM-overexpressing cells (FD/Pim44) in the abscence of interleukin 3, while SGI-1776 affected the control cells (FD/Neo), too. **B,** In PC-3 cells, DHPCC-9 did not alter cell survival, while SGI-1776 led to a massive cell death. DMSO concentration was kept at 0.1% in each sample. (N. Santio & L. Vahtera, unpublished data)

5.1.4 Fluorescent pyrrolocarbazole derivative gains access into cells

To confirm the ability of pyrrolocarbazoles to enter cells, a fluorescent derivative was developed. A fluorescent dansyl group was added to the pyrrolo[2,3-*a*]carbazole backbone to create a 5-(Dimethylamino)-*N*-({1-[4-(3-formylpyrrolo[2,3-*a*]carbazol-10(1*H*)-yl)butyl]-1*H*-1,2,3-triazol-4-yl}methyl) naphthalene-1-sulfonamide a.k.a. PCC-13 (Letribot et al. 2012). In PC-3 cells, PCC-13 was observed in the cytoplasm, but not in the nucleus (Letribot et al. 2012). However, the fluoresencent group influenced the size and function of the compound and higher residual PIM activities were detected in *in vitro* kinase assays after addition of PCC-13 as compared to DHPCC-9 a.k.a. compound A or other derivatives (Letribot et al. 2012). Nonetheless, these results demonstrated the ability of pyrrolocarbazoles to enter PC-3 cells (Figure 14A-B).

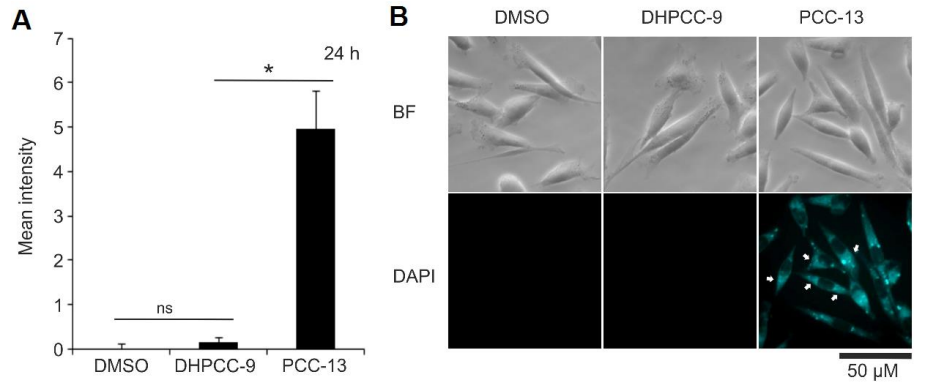

Figure 14 Fluorescent pyrrolocarbazole is visualized in PC-3 cells
PC-3 cells were treated with 10 µM Pim inhibitor DHPCC-9, its fluorescent derivative PCC-13 or 0,1% DMSO as a control. **A,** Mean intensity values were measured according to the fluorescent signal. **B**, Cells were imaged by fluorescence microscopy (DAPI filter) and light microscopy (Bright field = BF). (Modified from Letribot et al. 2012, experiment performed by N. Santio)

5.2 PIM kinases promote cancer cell motility (I)

5.2.1 PIM inhibition decreases cell migration and invasion

Increased cell motility is essential for cancer progression. Since the role of PIM kinases is slightly contradictory in prostate cancer, it was of interest to study their effects on prostate cancer cell motility. Thus, invasive human prostate cancer PC-3 cells were treated with the PIM inhibitor DHPCC-9 or DMSO as a control, after which cell migration was studied. Interestingly, DHPCC-9 reduced cell migration, without influencing PIM protein levels or cell viability (I: Figure 3A-E). After analysis of different concentrations, 10 μM DHPCC-9 was shown most efficient. Since the concentration was wanted to keep as low as possible, 10 μM concentration was also used in the following experiments. The most dramatic change in cell motility was gained after treatment of PC-3 cells with DHPCC-9 during serum removal, when the movement of cells was almost completely abolished (I: Additional Files 1-2). Since PIM kinases had been connected to the response of human head and neck squamocellular carcinoma cells to radiation therapy (Peltola et al. 2009), the migration of those cells was also analysed. In UT-SCC-12A (University of Turku - squamous cell carcinoma) cells, DHPCC-9 behaved similarly as in PC-3 cells (I: Figure 4A-D) further supporting the importance of PIM kinases in the regulation of cancer cell migration.

To confirm the PIM-dependency of the results gained with PIM inhibitors, PIM-targeted RNA-interference and PIM upregulation were also measured. Silencing of PIM kinases decreased PC-3 cell migration in somewhat similar way to PIM inactivation (I: Figure 5A-B). To study cell motility on a broader scale, cell invasion was also analysed. PC-3 cells were allowed to invade into a Matrigel invasion insert for 72 hours towards MG-63 cell-conditioned medium, which contains CXC chemokines such as CXCL12 as chemoattractants (Proost et al. 1993; Perissinotto et al. 2005). Silencing of PIM kinases by RNA interference or inhibition of PIM activity by DHPCC-9 led to a significant decrease in the invasion rate (I: Figure 5C-D). In addition, overexpression of either PIM1, PIM2 or PIM3 increased cell migration in wound healing assays, while PIM inhibition by DHPCC-9 decreased migration in each case (I: Figure 6A-B). Again, no major differences were detected in PIM protein levels after DHPCC-9 treatment (I: Figure 6C). These results demonstrated the importance of PIM activity in prostate and squamocellular carcinoma cell migration and prostate cancer cell invasion.

Another PIM inhibitor, BA-1a was also used to treat both PC-3 and UT-SCC-12A cells (Kiriazis et al. 2013). After analysis of wound healing, cell viability and Pim protein levels, highly similar results were obtained with BA-1a as compared to DHPCC-9 (Figure 15A-F). This further confirmed that PIM kinases are needed to promote cell migration at least in two different types of cancers.

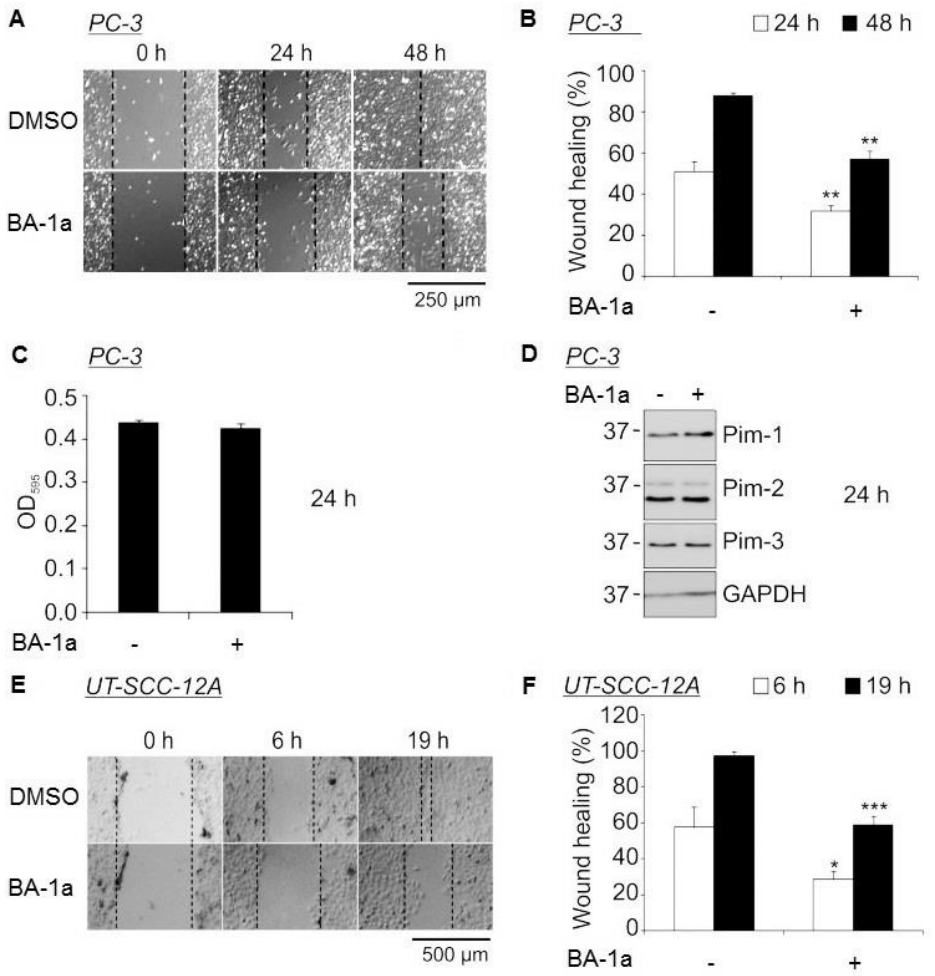

Figure 15 PIM inhibitor BA-1a decreases cancer cell migration
A-B, PC-3 cell migration was measured by wound healing assays. **C**, Cell viability was measured by the MTT assay, which detects the mitochondrial activity of the cells. **D,** PIM expression levels were detected by Western Blotting. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control. **E-F,** UT-SCC-12A cell migration was similarly measured by wound healing assays. PC-3 and UT-SCC-12A cells were treated either with 10 µM BA-1a or 0,1% DMSO as a control. (Modified from Kiriazis et al. 2013, experiments performed by N. Santio)

5.2.2 PIM substrate NFATC1 increases cell migration

NFATC1 transcription factor has previously been shown to promote cell migration (Seifert et al. 2009; Jang et al. 2010). In addition, NFATC1 activity is increased by PIM1 (Rainio et al. 2002). Therefore, we wanted to test whether NFATC1 activation by PIM1 plays a role in PC-3 cell migration. Overexpression of NFATC1 promoted PC-3 cell migration and PIM inhibition by DHPCC-9 efficiently blocked the migration even during NFATC1 upregulation (I: Figure 7A-C). Thus, enhanced activation of NFATC1 may support the pro-migratory effects of PIM kinases.

5.2.3 PIM kinases regulate cell migration in a cell type –dependent manner

Our studies identified PIM kinases as mediators of cell motility in prostate cancer cells as well as in head and neck squamocellular carcinoma cells *in vitro*. To clarify whether this is a general phenomenon or cell type-dependent, other cancer cell lines were also tested. PIM expression has been observed in various common adherent epithelial cell lines such as cervical adenocarcinoma HeLa cells and lung carcinoma A549 cells (Yan et al. 2006; Nasser et al. 2008). In addition, PIM expression was detected in MDA-MB-231 breast carcinoma and Saos-2 (sarcoma osteogenic 2) osteosarcoma cell lines (Figure 14A). Interestingly, PIM-targeted RNA-interference has been associated with decreased breast tumor growth, while the PIM kinase substrate NFATC1 supports breast cancer cell motility (Seifert et al. 2009; Zhang et al. 2014b). Therefore, these cell lines were chosen for wound healing analyses. Surprisingly, PIM inhibition only decreased the migration of the HeLa cells, while the other cell lines were not significantly influenced (Figure 16B-E). Thus, the effects of PIM kinases on cell motility appear to depend on the cell type.

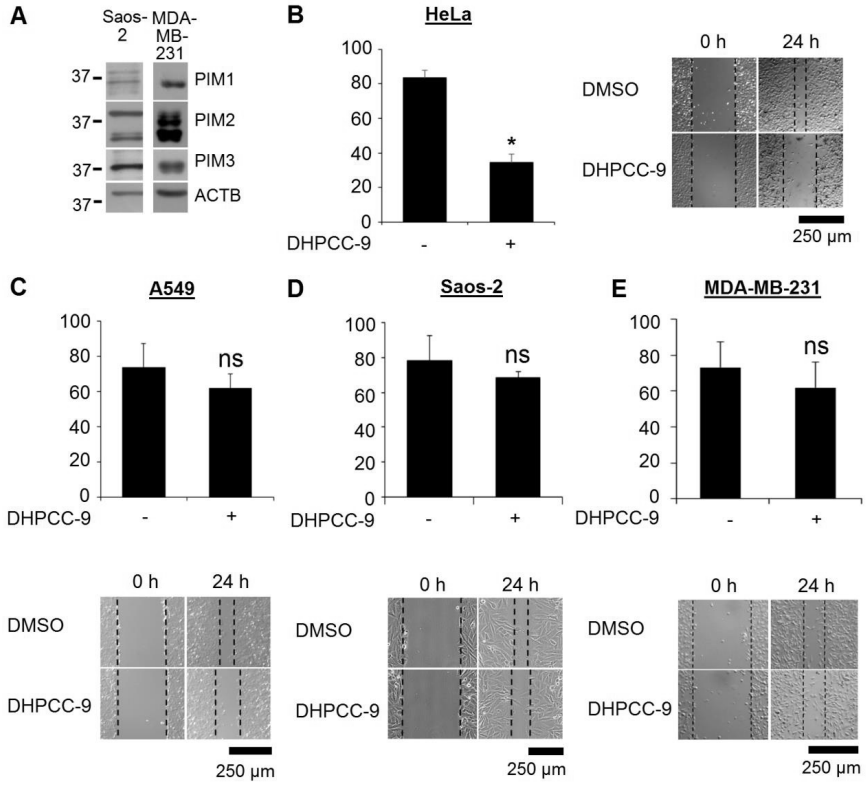

Figure 16 PIM-regulated cell migration is cell type-dependent
A, PIM kinase expression was detected in Saos-2 osteosarcoma and MDA-MB-231 breast cancer cell lines. ACTB (actin beta) was used as a control to demonstrate equal loading of the wells. **B-E,** Migration of cervical cancer HeLa, lung cancer A549, osteosarcoma Saos-2 and breast cancer MDA-MB-231 cells was analysed by wound healing assays. PIM kinase activity was inhibited by 10 µM DHPCC-9, while 0,1% DMSO was used as a control. (N. Santio, unpublished data)

5.3 PIM kinases and PIM inhibitors DHPCC-9 and BA-1a in prostate cancer progression (II)

5.3.1 PIM3 upregulation promotes subcutaneous tumor growth

While PIM1 and PIM2 are known to enhance subcutanous PC-3 tumor growth in mice (Chen et al. 2005), data for PIM3 has been lacking. Therefore, PC-3 cells stably overexpressing the Tomato^{TM} fluorescent marker and either PIM3 or control vector were subcutaneously inoculated into the back sides of nude mice. Tumors were allowed to grow for approximately three weeks, during which tumor growth was followed by fluorescence-based imaging and manual palpation. Based on these measurements, we discovered that stable overexpression of PIM3 results in increased tumor size (II: Figure 1A-B). The results from the fluorescence-based imaging also correlated well with the manually performed measurements (Supplementary Figure S1A). Immunohistochemical staining of paraffin-embedded tissue samples revealed an increase in the number of mitotic cells in the PIM3- overexpressing tumors, as compared with controls (II: Figure 1C, Supplementary Figure S1B). Whole tumor scanning also clearly demonstrated the differences in tumor sizes formed by the different cell lines (II: Supplementary Figure S1C). The stable overexpression of PIM3 in PC-3 cells was also analysed during a three-week-period, which was the duration of the animal experiments. Western blotting confirmed overexpression of PIM3 after three weeks of antibiotic-free cell culture (II: Supplementary Figure S1D). In summary, PIM3 upregulation supports prostate tumor growth.

5.3.2 Cell migration is enhanced by stable PIM upregulation

To test the influence of stable PIM overexpression on cell migration, wound healing assays were performed with stable PIM-overexpressing PC-3 cells. In addition to the control and PIM3-overexpressing cells, a stable cell line overexpressing PIM1 and the Tomato^{TM} fluorescent marker were also established. Furthermore, two small molecule PIM inhibitors were studied. Both DHPCC-9 as well as BA-1a were previously shown to inhibit PIM1 and PIM3 activity (Akué-Gédu et al. 2009; Kiriazis et al. 2013). While stable PIM overexpression significantly promoted PC-3 cell migration in wound healing assays, both inhibitors efficiently reduced it (II: Supplementary Figure S2A-B).

5.3.3 Cell viability is reduced by DHPCC-9 and BA-1a

We had previously followed PC-3 cell viability during the 24 hour wound healing experiments to confirm that differences in migration were not due to changes in cell viability after PIM inhibition (I, Kiriazis et al. 2013). Therefore, we also analysed cell viability in our stable cell lines. PIM overexpression did not significantly affect cell viability (II: Supplementary Figure S2C). After 24 hours of inhibitor treatments, no major changes were detected in the control cell viability, while an obvious decrease was detected in the stable PIM-overexpressing cells. However, cell viability was dramatically decreased after 72 hours, confirming the importance of PIM activity for PC-3 cell survival (II: Figure S2C).

5.3.4 PIM kinases induce prostate xenograft cell invasion to lungs

Since the subcutaneous tumors did not form metastases, a more metastatic prostate cancer model was chosen. According to previous results, a more natural microenvironment might support the formation of metastases after an orthotopic inoculation of the cells (Stephenson et al. 1992). Thus control or PIM3-overexpressing PC-3 cells were orthotopically inoculated into the prostates of athymic nude male mice as a preliminary test (orthotopic set 1). After three weeks, tumor size was measured and tissue samples were subjected to immunohistochemical analysis. While no major differences were seen in tumor growth, increased number of mitotic cells was again detected in the PIM-overexpressing tumors as compared to the control tumor (II: Figure 1D). Previously, parental PC-3 cells have been shown to invade to the prostate-draining lymph nodes in the orthotopic model, while other metastases are rather rare (Tuomela et al. 2008; Tuomela et al. 2009). Despite these previous findings, we searched for metastases also in other tissues. These results revealed that PIM3-overexpressing cells had invaded to both lymph nodes and lungs (II: Figure 1E). Thus, PIM upregulation promotes cell migration and invasion *in vivo*.

5.3.5 DHPCC-9 and BA-1a are well tolerated *in vivo*

Since the novel PIM-selective inhibitors DHPCC-9 and BA-1a decreased PC-3 cell viability and migration, it was of interest to test their effects in the orthotopically induced metastatic PC-3 prostate cancer model. Prior to the efficacy experiments, the general toxicity of the compounds was studied. Toxicity was first measured in zebrafish embryos, which were treated with the inhibitors from 6 to maximum of 50 hours post fertilization. Neither DHPCC-9 nor BA-1a showed major decrease in embryo survival unlike the cytotoxic control BA-2c (II: Supplementary Table S1, Supplementary Figure S3A). After analysis of body size and structure, no major changes were detected either (II: Supplementary Table S1, Supplementary Figure S3B). However, DHPCC-9 did cause slight changes in the body curvature and lead to an increase in the size of the pericardiac sac (II: Supplementary Figure S3C-D), referring to a potential role of PIM activity during zebrafish embryo development. Thereafter, the inhibitors were intraperitoneally injected to white female or male mice. DHPCC-9 was diluted to DMSO, while BA-1a was dissolved in DMA, since it could not be dissolved in DMSO at high enough concentration. DHPCC-9 did not show any signs of cytotoxicity, while DMA caused irritation at the site of injection as well as restless behavior after injection and decrease in weight (II: Supplementary Figure S4A-B). Luckily, after reducing the amount of DMA to less than half, the signs of cytotoxicity were diminished (II: Supplementary Figure S4C). According to these results, it was reasonable to proceed to test the efficacy of DHPCC-9 and BA-1a in the orthotopic model.

5.3.6 DHPCC-9 counteracts PIM-induced prostate tumor growth and the formation of metastases

While PIM3 upregulation supported tumor progression in the orthotopic model, also PIM1 was included in the next set of experiments. This was done to find out, whether PIM1 and PIM3 similarly support prostate cancer cell invasion to lymph nodes and lungs. Stable PC-3/PIM1, PIM3 or control cells were orthotopically inoculated into the prostates of athymic nude male mice (orthotopic set 2). According to previous observations, increased tumorigenic behavior was expected in PIM3-overexpressing cells as compared to controls. Therefore, mice bearing PIM3-overexpressing cells were also daily injected intraperitoneally with the PIM inhibitors DHPCC-9 or BA-1a. Control treatments (DMSO or DMA) were given to mice bearing either PC-3/control or PIM3 cells. Mouse behavior and weight gain were carefully followed during the three-week experiment, but no major inhibitor-related changes were detected (II: Supplementary Figure S5). After sacrifice, tumors and organs were collected and fluorescent imaging was performed to detect the PC-3 cell-derived Tomato^{TM} signal in tumors and other tissues (II: Supplementary Figure S6A). All animals had not developed tumors and thereby only tumor-bearing mice were taken into account in the final analyses (Supplementary Table S2). Interestingly, overexpression of PIM kinases led to increased tumor sizes, while suppression of PIM activity by DHPCC-9 had efficiently decreased tumor growth (II: Figure 2A-D). Unfortunately, BA-1a treatment did not lead to similar results as DHPCC-9 treatment (II: Figure 2C-D). After immunohistochemical staining of tumor tissues, a decrease was also detected in the number of mitotic cells after DHPCC-9 treatment (II: Figure 2E). As a conclusion, PIM upregulation supports tumor growth, which is decreased by PIM inhibition by DHPCC-9 *in vivo*.

For detection of metastases, several organs were collected from mice bearing the orthotopic prostate tumors. Due to the weak fluorescent signal in the organs, immunohistochemical staining was needed for visualization of metastases. After careful analysis, metastases were found in lymph nodes as well as in lungs. While all cancer cell types were able to form lymph node metastases, again only PIM-overexpressing cells had metastasized to the lungs (II: Figure 3A). In addition, PIM inhibition by DHPCC-9, but not by BA-1a, decreased the amount of metastases in both cases (II: Figure 3B-C, Supplementary Table S3). However, no major differences were detected in the size or proliferation potential of the metastases (II: Supplementary Figure S6B). An additional staining of the V5-tag was performed to confirm stable PIM expression in tumor tissues and metastases (II: Supplementary Figure S7). In summary, PIM kinases promote formation of metastases, while DHPCC-9 treatment efficiently prevents it.

5.3.7 PIM activity promotes prostate xenograft vascularization

To further analyse the differences in tumor growth and formation of metastases, vasculature was also stained from the orthotopic prostate xenografts. There PIM overexpression had led to an increase in the blood vessel area, while lymphatic vessels were less influenced (II: Figure 4A-B). Furthermore, PIM inhibition by DHPCC-9 decreased the area of blood and lymphatic vessels (II: Figure 4A-D). The increase in angiogenesis mediated by PIM kinases may partially explain the dependency of tumor growth and the number of metastases on PIM activity.

5.3.8 CXCR4 may support PIM-mediated formation of metastases

To identify PIM-dependent signaling pathways involved, the CXCR4/CXCL12 pathway known to promote the formation of metastases was studied. PIM1 has been previously shown to phosphorylate CXCR4 at Ser^{339}, leading to an increased CXCR4 cell surface expression (Grundler et al. 2009). In addition, DHPCC-9 was earlier shown to decrease PC-3 cell invasion towards the MG-63 conditioned medium, which contains e.g. CXCL12 as a chemoattractant (I). Therefore, the effects of PIM kinases and their inhibitors on CXCR4 pathway was studied in PC-3 cells and the orthotopic tumors.

First, the phosphorylation of CXCR4 was analysed in the stable or transiently transfected as well as parental PC-3 cells. PIM1 and PIM3 overexpression increased CXCR4 phosphorylation, while PIM inhibition by DHPCC-9 decreased it (II: Figure 5A-B, Supplementary Figure S8). *In vitro* kinase assays clarified direct phosphorylation of CXCR4 by human PIM1 and murine Pim3 but not human PIM2 (II: Figure 5C). Thereafter, localization and signal intensity of phosphorylated CXCR4 was measured in the stable PC-3 cells. While the stable control and PIM-overexpressing cells showed membrane positive phospho-CXCR4 staining, DHPCC-9 treatment led to a decreased, weaker and more distributed signal (II: Figure 5D-E). Furthermore, a slightly higher nuclear CXCR4 signal was detected in DHPCC-9-treated as compared to DMSO-treated cells (II: Figure 5E). For visualization of CXCR4 pathway in the xenografts, both phospho-CXCR4 and CXCR4 were stained. Interestingly, the stable PIM-overexpressing tumors showed higher phospho-CXCR4 signal as compared to the control tumors, while PIM inhibition by DHPCC-9 significantly decreased phospho-CXCR4 signal (II: Figure 5F). These results imply the potential role of CXCR4/CXCL12 pathway as a mediator of PIM-induced metastatic progression of prostate tumors.

5.4 FOXP3, GSK3B, PTGS2 and the integrins – connection to the PIM-promoted prostate cancer cell migration (III)

5.4.1 PIM substrates FOXP3 and GSK3B inhibit cell migration

FOXP3 and GSK3B have been shown to decrease cell migration rate e.g. by downregulating PTGS2 expression (Hao et al. 2014; Thiel et al. 2006). GSK3B has been suggested to be a substrate for PIM1, but direct phosphorylation has not been shown (Narlik-Grassow et al. 2014). GSK3B is known to be inactivated by phosphorylation at Ser^{9}, while the sequence aroung Ser^{9} nicely resembles the PIM1 consensus sequence (Sutherland et al. 1993; Friedmann et al.1992). Therefore, GSK3B phosphorylation was analysed by *in vitro* kinase assays, which confirmed direct GSK3B phosphorylation at Ser^{9} by PIM1 (III: Figure 1A-B). Furthermore, cellular GSK3B phosphorylation was decreased in PC-3 cells after PIM inhibition by DHPCC-9, further confirming that GSK3B is a true substrate for Pim1 (III: Figure 1C). Overexpression of GSK3B wild-type or a phospho-deficient S9A mutant were used as controls. As an additional control, AKT phosphorylation at Ser^{473} was measured as a sign of AKT activity, which remained unaffected by DHPCC-9 (III: Figure 1D) as expected according to previous results (Akué-Gédu et al. 2009). To investigate the role of GSK3B phosphorylation in PC-3 cell migration, wound healing assays were performed. After overexpression of GSK3B wild-type or the phospho-deficient mutant, PC-3 cell migration was decreased (III: Figure 1E-F). Thereby, it is possible that PIM kinases increase prostate cancer cell migration by inhibition of GSK3B activity by Ser^{9} phosphorylation.

FOXP3 has been reported to reduce cell migration, while Ser^{418} phosphorylation is known to be necessary for full activity of FOXP3 (Douglass et al. 2014; Nie et al. 2013). Still, the kinase phosphorylating Ser^{418} has not been identified. Due to the resemblance of the amino acid sequence around murine FoxP3 Ser^{418} to PIM1 consensus site, FoxP3 phosphorylation was studied by *in vitro* kinase assays. Interestingly, PIM1 phosphorylated FoxP3 at Ser^{418} but also other phosphorylation sites appeared to be present, since only slightly decreased phosphorylation was observed after mutagenesis of Ser^{418} to alanine (III: Figure 2A-B). FoxP3 overexpression also significantly reduced prostate cancer cell migration, while the phosphodeficient mutant S418A was completely inactive and did not cause any changes in cell motility (III: Figure 2C-D). However, it was more important to determine the function of human FOXP3 than murine FoxP3, in order to gain further inside into the function of FOXP3 in human prostate cancer. Human FOXP3 activity has been shown to be directly upregulated by phosphorylation at Ser^{422} by PIM1 (Douglass et al. 2014; Li et al. 2014; Zhang et al. 2015b). Endogenous FOXP3 expression was found in the PC-3 cells, while overexpression of FOXP3 wild-type or phosphodeficient (S418A or S422A) or phosphomimicking (S418D or S422D) mutants confirmed the importance of Ser^{418} and Ser^{422} phosphorylation for FOXP3 activity and FOXP3-mediated inhibition of cell migration (III: Figure 3A-C). The phosphomimicking mutant S422D even slightly rescued the effect of PIM inhibition by DHPCC-9, further supporting the role of PIM-mediated phosphorylation of FOXP3 at Ser^{422} and inactivation of FOXP3 in prostate cancer cells.

FOXP3 and GSK3B wild-types and phosphomutants were also overexpressed in the stable mock or PIM1-overexpressing PC-3 cells to clarify their potential phosphorylation-dependent role in PIM-mediated cell migration (III: Supplementary Figure S1A-C). PIM overexpression significantly promoted cell migration during wild-type FOXP3 or GSKB overexpression, while the phospho-deficient S422A and S9A mutants were able to keep the migration rate low (III: Figure 4A-D). Interestingly, cell viability was not significantly altered by transient overexpression of the human FOXP3 or GSK3B proteins in the stable cell lines (III: Figure 4E-F). Hence, PIM kinases are likely to enhance cell migration by inhibition of FOXP3 and GSK3B activity by phosphorylation of FOXP3 at Ser^{422} and GSK3B at Ser^{9}.

5.4.2 FOXP3 and GSK3B interact with PIM1 in PC-3 cells

To support the hypothesis of FOXP3 and GSK3B interactions with PIM1 in PC-3 cells, their colocalization and interaction were measured using fluorescently-tagged fusion proteins. After preparation of the fluorescent constructs, PC-3 cells were transfected and the correct protein sizes were confirmed by Western blotting (III: Figure 5A). Thereafter, confocal microscopy revealed colocalization of both FOXP3 and GSK3B with PIM1 in the nuclei of PC-3 cells (III: Figure 5B). Furthermore, decreased fluorescent lifetime of the FOXP3 and GSK3B fusion proteins in the presence of the PIM1 fusion protein indicated interactions of FOXP3 and GSK3B with PIM1 (III: Figure 5C-D).

5.4.3 PIM kinases promote PTGS2 expression

To study the role of PTGS2 in PIM-dependent cell migration, PIM1 was overexpressed in PC-3 cells simultaneously with PTGS2 inhibition by Celecoxib. While PIM overexpression led to an increase in both migration and PTGS2 levels, Celecoxib efficiently blocked migration even during PIM upregulation (III: Figure 6A-B, Supplementary Figure S2A). As an additional control, PIM kinases were also silenced by gene-specific siRNAs, after which PTGS2 levels were analysed. Both PIM-targeted RNA interference and inactivation by DHPCC-9 led to a decrease in PTGS2 expression levels (III: Figure 6C). Thereafter the connection of GSK3B Ser^{9} and FOXP3 Ser^{422} phosphorylation to the regulation of PTGS2 expression levels were determined. Overexpression of the wild type or the phosphodeficient S9A mutant of GSK3B downregulated PTGS2 levels, but overexpression of FOXP3 did not have a similar effect (III: Figure 6 D-E, Supplementary Figure S2B-C). As an additional control for PTGS activity, Indomethacin was used to inhibit PTGS1, and it did not significally alter PC-3 cell migration (III: Supplementary Figure S3). Thereby, PIM-mediated phosphorylation and inactivation of GSK3B are likely to prevent the ability of GSK3B to inhibit PTGS2 expression.

5.4.4 PIM kinases regulate integrin-mediated cell adhesion

Integrins are important mediators of cell adhesion and thereby also affect cancer cell invasiveness (Desgrosellier & Cheresh 2010). PTGS2 can also increase the expression of integrin subunits such as ITGA2 and ITGA5 (Liu et al. 2010; Han et al. 2005). Therefore, the connection of PIM-dependent cell motility and integrin activity was studied. Integrin-dependent cell adhesion was followed on collagen and fibronectin –coated wells. Poly-L-lysine coating was used as an integrin-independent adhesion control. Interestingly, PIM inhibition decreased integrin-mediated adhesion (III: Figure 7A-B). Furthermore, adhesion to both collagen and fibronectin was decreased, referring to a connection of PIM kinases to several integrins. However, this pathway was not dependent on the PTGS2 activity, because the influence was seen already after 1 h treatment by DHPCC-9, while PTGS2 inhibition by Celecoxib did not cause clear integrin-related changes in cell adhesion (III: Supplementary Figure S4A-B). ITGA2 is known to form an active receptor heterodimer with ITGB1 subunit. When both ITGA2 expression and ITGB1 activity were analyzed, no changes were detected in cell surface ITGA2 expression or ITGB1 activity after PIM inhibition (III: Supplementary Figures S5 and S6A-C). Thus, the exact signaling pathways through which PIM kinases regulate integrin-mediated cell adhesion remain to be clarified.

5.5 PIM-NOTCH interplay in prostate and breast cancer (IV)

5.5.1 PIM kinases increase NOTCH transcriptional activity

Taking into account the potential connection between PIM and NOTCH signaling discussed above, it was reasonable to test, whether these pathways actually interact. Due to the importance of NOTCH in both breast and prostate cancer, MCF-7 breast cancer and PC-3 prostate cancer cells were used in the study. First, both cell lines were shown to express PIM kinases as well as NOTCH1 receptor, while NOTCH3 was only present in MCF-7 cells (IV: Figure 1A). Thereafter, endogenous NOTCH activity was measured in MCF-7 cells, where PIM-targeted RNA interference decreased and PIM overexpression increased the low basal NOTCH activity (IV: Figure 1B-C). Also, inhibition of PIM activity by DHPCC-9 or SGI-1776 decreased endogenous NOTCH activity in both cell lines, as shown by the 12xCSL-dependent luciferase assays, while the constitutively active CMV control promoter activity was not decreased (IV: Figure 1D-G). After *in silico* analysis of patient data, correlation was also found in breast carcinomas between *NOTCH1* or *NOTCH3* and *PIM1* and in prostate cancer between *NOTCH1* and *PIM1* mRNA levels (IV: Supplementary Figure S1A-B). These results implied the potential of PIM kinases to promote NOTCH activity in cancer.

5.5.2 PIM kinases phosphorylate NICD1 and NICD3

To measure the regulation of NOTCH activity by PIM-mediated phosphorylation, Notch intracellular domains (NICD) were subjected to *in vitro* kinase assays. Murine NICD1 and NICD3 were phosphorylated by human PIM kinases, while NICD2 was not (IV: Figure 2A, Supplementary Figure S2A). To study the interaction of PIM and NOTCH in human cells, PIM activity was inhibited by DHPCC-9 in MCF-7 cells, after which NICD1 was immunoprecipitated. PIM inhibition also led to a decrease in cellular NICD1 phosphorylation (IV: Figure 2B). To reveal the phosphorylation sites in NICD1, both mass spectrometry and *in silico* analysis were used. Two potential phosphorylation sites were detected in NICD1, while Ser^{2152} phophorylation was detected by mass spectometry (IV: Supplementary Figure S2B-C). Thereafter serine to alanine (SA) mutations were introduced to generate phosphodeficient mutans of NICD1. *In vitro* kinase assays revealed phosphorylation of NICD1 at Ser^{2152} by all PIM family members (IV: Figure 2C-D). Interestingly, both phosphorylation sites were conserved in mouse and human, while sequence analysis of NICD4 did not reveal any similarities to the PIM1 consensus site (IV: Supplementary Table S1). For further experiments both phosphodeficient (SA) and phosphomimicking (SE) mutants of N1CD1 Ser^{2152} were generated.

5.5.3 PIM1 interacts with NICD1 and increases NICD1 activity

To further investigate the regulation of NOTCH activity by PIM kinases, their localization, colocalization and interaction were measured. First, NICD1 localization was imaged in PC-3 cells. There the phosphodeficient mutant showed increased cytoplasmic signal as compared to the wild-type and the phosphomimicking mutant (IV: Figure 3A-B). Similarly, Pim inhibition decreased the amount of nuclear NICD1 (IV: Figure 3A-B). The Notch1ΔE construct produces a membrane-tethered form, which needs to be cleaved by the gamma-secretase to form an active NICD. Thereby, the gamma-secretase inhibitor DAPT prevented NICD1 nuclear localization (IV: Figure 3 A-B). The correct protein sizes and expression levels were confirmed by Western blotting (IV: Figure 3C). The effect of phosphorylation on NICD1 activity was measured by luciferase assays in both PC-3 and MCF-7 cells. The phosphodeficient mutant of NICD1 showed significantly decreased transcriptional activity as compared to the wild-type or the phosphomimicking mutant in both cell lines (IV: Figure 3D-E). As an additional control, NICD1 was overexpressed in PC-3 cells in the presence of DHPCC-9 or DAPT, after which NOTCH activity was decreased (IV: Figure 4F). Similarly, DHPCC-9 decreased the activity of wild-type NICD1 in MCF-7 cells (Figure 17). On the other hand, DAPT was previously shown to decrease NOTCH activity in MCF-7 cell line (Landor et al. 2011). These results show that phosphorylation of Ser^{2152} supports NICD1 nuclear localization and activity.

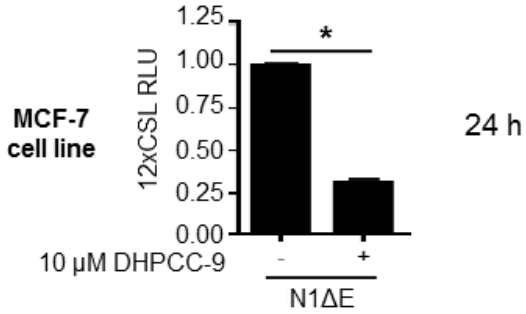

Figure 17 PIM inhibition decreases NICD1 activity in MCF-7 cells
MCF-7 cells were transiently transfected with 12xCSL luciferase reporter construct, beta-galactosidase and either a control plasmid or a Notch1ΔE construct. At the next day, cells were treated with 10 µM Pim inhibitor DHPCC-9 or 0,1% DMSO for 24 hours. Then, cells were lysed and luciferase activities were measured by luminometry. Beta-galactosidase-derived signal was used as a control. (S. Landor, unpublished data)

The colocalization and interaction of ectopically overexpressed NICD1 with PIM1 was first studied in prostate cancer cells. Confocal microscopy and fluorescence-lifetime imaging method confirmed colocalization and interaction of PIM1 with NICD1 wild-type and the phosphomutants (IV: Figure 4A-C). DHPCC-9 and DAPT treatments were used as controls. As expected, after analysis of PIM1 interaction with the phosphomutants, DHPCC-9 did not influence the interaction between NICD1 and PIM1 (IV: Figure 4B). Again, DAPT prevented nuclear entry of the Notch1ΔE and thereby no interaction was seen between NICD1 and PIM1 after DAPT treatment (IV: Figure 4B). The interaction of endogenous PIM and NICD1 was studied in MCF-7 cells. While the ectopically overexpressed NICD1 and PIM1 interacted mainly in the nuclei in PC-3 cells, the colocalization and interaction of endogenous proteins were detected in the cytoplasm of MCF-7 cells (IV: Figure 5A-B). MT-CO2 a.k.a. COXII (mitochondrially encoded cytochrome c oxidase II) was used as a negative control. In summary, these results show interaction of endogenous as well as ectopically overexpressed PIM1 and NICD1, while mutagenesis or PIM inhibition does not prevent the interaction.

5.5.4 NICD1 activity supports PIM expression

The JAK/STAT signaling pathway can be activated by NOTCH in breast cancer (Jin et al. 2013). However, it is dependent on TP53 inactivation. In MDA-MB-231 cells TP53 is mutated, while MCF-7 cells express the wild type TP53 (Jin et al. 2013). In PC-3 cells, TP53 is inactivated (Rubin et al. 1991). Thereby a positive feedback loop was search for between NOTCH activation and PIM expression in both breast and prostate cancer cells. First, MDA-MB-231 cells were treated with the gamma secretase inhibitor DAPT to inhibit the formation of NICD1. This led to a decrease in the expression levels of all three PIM family members (Figure 18).

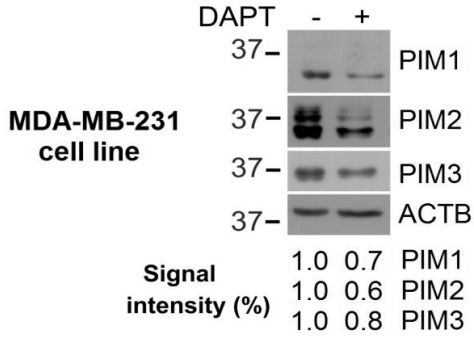

Figure 18 Inhibition of NOTCH cleavage reduces PIM expression in MDA-MB-231 cells
MDA-MB-231 cell line was treated with the gamma secretase inhibitor DAPT to prevent the formation of Notch1 intra cellular domain. DMSO was used as a control treatment. Thereafter, PIM protein levels were analysed from the cell lysates by Western blotting. ACTB was used as a loading control. (N. Santio, unpublished data).

Next MCF-7 and PC-3 cells were analysed. Interestingly, overexpression of NICD1 increased PIM protein levels in both MCF-7 and PC-3 cells (IV: Figure 6A-B). However, higher upregulation was detected in PC-3 cells, as expected according to their TP53 status. After transient overexpression of NICD1 phosphomutants in PC-3 cells, the behavior of the phosphomimicking mutant was quite similar to the wild-type, while the phosphodeficient mutant rather decreased PIM levels (IV: Figure 6B). Similar to MDA-MB-231 cells, DAPT treatment also led to a decrease in PIM levels in PC-3 cells (IV: Figure 6C). However, inhibition of PIM activity did not have any major effect on the levels of cleaved NOTCH1 (IV: Figure 6D). To conclude, increased NICD1 activity leads to an upregulation of PIM protein levels and might partially be dependent on cellular TP53 status and NOTCH-mediated activation of the JAK/STAT pathway.

5.5.5 Phosphorylation of NICD1 promotes prostate cancer cell migration

To clarify the possible role of NOTCH signaling in PIM-dependent cell migration, various wound healing assays were performed with PC-3 cells. First, PIM1 was overexpressed simultaneously with inhibition of NOTCH cleavage by DAPT. Interestingly, DAPT treatment decreased PC-3 cell migration even during PIM upregulation (IV: Figure 7A). On the other hand, jagged 1-mediated activation of NOTCH signaling induced PC-3 cells migration, while PIM inhibition by DHPCC-9 prevented the migration during NOTCH activation (IV: Figure 7B). Furthermore, phosphorylation of NICD1 clearly promoted its pro-migratory effects and the phospho-mimicking mutant even slightly rescued the decrease caused by Pim inhibition after DHPCC-9 treatment (IV: Figure 7C). As an additional control, PC-3 cell proliferation was measured. Both PIM1 and the NICD1 wild-type slightly decreased PC-3 cell proliferation, which confirmed that the faster wound healing was not caused by higher cell number in those samples (IV: Supplementary Figure S3A-B). These results show that PIM-mediated phosphorylation and activation of NICD1 support prostate cancer cell motility.

5.5.6 Phosphorylation of NICD1 regulates breast cancer cell metabolism

Both PIM and NOTCH signaling have been previously shown to regulate the ability of a cell to produce energy by aerobic or anaerobic means (Beharry et al. 2011; Din et al. 2014; Landor et al. 2011; Leung et al. 2015; Zhang et al. 2015a). Therefore, different metabolic experiments were performed in MCF-7 cells. First, PIM inhibition showed clear increase in glucose uptake as well as mitochondrial membrane potential (IV: Figure 8A-B). Similarly, also overexpression of the phosphodeficient NICD1 led to an increase in glucose consumption (IV: Figure 8C). To further clarify the influence of NOTCH phosphorylation on the glycolytic metabolism, lactate production was measured. The overexpression of the phosphodeficient NICD1 increased lactate production in a similar way as PIM inhibition, while PIM1 overexpression led to a decrease in it (IV: Figure 8D-E). Overexpression of NICD1 phosphomimicking mutant also prevented the increase in lactate production after PIM inhibition (IV: Figure 8D). Additional proliferation and viability experiments with NICD1 or PIM1 overexpression or inhibition confirmed that the results of the lactate assays were independent of changes in cell number (IV: Supplementary Figure S3B-F). These data support the role of PIM kinases in cell metabolism and refer to the connection of PIM and NOTCH signaling in the regulation of glycolytic metabolism and mitochondrial function in breast cancer cells.

5.5.7 PIM-NOTCH interaction affects tumor progression *in vivo*

The chorioallantoic membrane supports tumor growth well, and the CAM model is a relatively easy and cheap model to study tumor growth *in vivo* during a short period of time (Deryugina & Quigley 2008). Thus the NICD1 wild-type and the phosphomutants were overexpressed in MCF-7 cells cultured on top of the CAM. Tumor growth was followed in the presence and absence of estradiol due to the hormone-dependency of MCF-7 cell proliferation *in vivo* (Levenson & Jordan 1997). The pro-tumorigenic effect of NICD1 was dramatically enhanced by estradiol, but decreased by PIM inhibition (IV: Figure 9A-B). Overexpression of the NICD1 phosphomimicking mutant also led to an increase in tumor size as compared to the phosphodeficient counterpart (IV: Figure 9A-B). Both PIM1 and PIM3 protein levels were increased after estradiol treatment (IV: Figure 9C).

Since NOTCH1 was shown to upregulate PC-3 cell migration, the effect of NOTCH activity on PC-3 cell tumor growth was also measured. First, PC-3 cells were cultured on the CAM and treated with the PIM inhibitor DHPCC-9 or the gamma-secretase inhibitor DAPT. In both cases tumor growth was decreased, while the most efficient results were gained by combinatorial treatment, referring to both interaction and co-operation of PIM and NOTCH proteins to support prostate tumorigenesis (IV: Figure 9D). As an additional control, the stable mock or PIM1-overexpressing PC-3 cells were cultured on the CAM in the presence of DAPT. While PIM upregulation promoted tumor growth, DAPT treatment was able to decrease tumor growth of both mock and PIM1-overexpressing cells (IV: Supplementary Figure S4). In addition to the CAM experiments, the connection of NOTCH1 activity to PIM-mediated prostate tumor growth was analysed in tumors

from the second orthotopic set, which was previously described (II). Therefore, cleaved NOTCH1 was stained from paraffin-embedded tumor samples, after which the number of cells with nuclear positive NOTCH1 were counted in each tumor. An increased nuclear localization of cleaved NOTCH1 was detected in the tumors with stable PIM overexpression, while DHPCC-9 treatment of mice decreased the amount of nuclear NOTCH1 in the tumors (IV: Figure 9E-F). These results connected the NOTCH1 nuclear localization to the PIM-mediated prostate tumor progression.

In summary, NOTCH activity mediates the effects of PIM kinases on promoting hormone-dependent breast tumor growth as well as prostate cancer progression. This might be due to the ability of PIM kinases to support energy gain in breast cancer cells and the motility of prostate cancer cells through phosphorylation of NICD1 at Ser^{2152} (IV: Figure 10).

6 DISCUSSION

6.1 PIM kinases support prostate cancer progression (I, II, IV)

PIM kinases promote tumor progression in various *in vivo* models, while increased PIM levels have been found in several human malignancies (Narlik-Grassow et al. 2014). PIM activity has been mostly studied in the regulation of cell survival, proliferation and apoptosis, while a major part of this thesis concentrated on their role in prostate cancer cell motility. At the same time, other research groups have connected PIM kinases to the regulation of cell migration in malignant and nonmalignant cell lines. However, our observations of changes in prostate cancer, cervival cancer and squamocellular carcinoma cell motility after PIM inhibition, silencing or upregulation were first to connect all PIM family members directly to the promotion of cancer cell migration and invasion (I; Kiriazis et al. 2013 and unpublished data). Simultaneously others also reported the involvement of PIM kinases in squamocellular carcinoma cell motility (Tanaka et al. 2009). On the other hand, PIM3 was shown to promote endothelial cell migration and tube formation, suggesting a role in angiogenesis (Zhang et al. 2009). PIM1 was also shown to regulate lymphocyte homing through CXCR4/CXCL12 chemokine pathway (Grundler et al. 2009). Later on, PIM kinases were connected to increased bone invasion of sarcoma cells as well as vasculogenesis in pancreatic cancer (Narlik-Grassow et al. 2012; Liu et al. 2014b). Recently, PIM3 was also shown to promote ovarian cancer cell migration (Zhuang et al. 2015). Taken together, all these results nicely support each other and confirm the important role of PIM kinases in promoting cell motility especially in cancer.

Previous experiments with subcutaneous PC-3 xenograft mouse models show how upregulation of PIM1 or PIM2 supports prostate tumor growth (Chen et al. 2005; Zhang et al. 2010). However, similar experiments were not performed with PIM3. In addition, in both cases the xenografts were subcutaneously injected and thereby the models were not suitable for analysis of metastases (Chen et al. 2005; Zhang et al. 2010). In our experiments, PIM3 upregulation also led to increased tumor growth in the subcutanous model (II). Thereafter a better model was needed for analysis of the role of PIM activity in the formation of metastases. Previous results showed metastases in prostate-draining lymph nodes in an orthotopic mouse model for prostate cancer (Stephenson et al. 1992; Tuomela et al. 2008; Tuomela et al. 2009). The orthotopic model was tested and, both PIM1 and PIM3 upregulation led to larger tumors, enhanced angiogenesis and increased numbers of lymph node as well as lung metastases (II). The formation of lung metastases may even be a PIM-dependent phenomenon, since parental PC-3 cells rarely invade as far as into lungs in this model (Tuomela et al. 2008; Tuomela et al. 2009). This is further supported by the data from an orthotopic hepatocellular carcinoma model, where silencing of PIM1 decreases the amount of lung metastases (Leung et al. 2015). The chicken embryo chorioallantoic membrane model is useful for measuring tumor growth and invasion. The advantage of this model is that it also serves as an alternative to basic animal models (Deryugina & Quigley 2008). Therefore, the growth of PC-3 cell-derived tumors was also measured on the chorioallantoic

membrane. In these studies, PIM inhibition led to a decrease in tumor growth, further supporting the role of PIM kinases in prostate tumorigenesis (IV).

To summarize, both prostate tumor growth as well as formation of metastases are positively regulated by increased PIM expression. This is likely to be dependent on the ability of PIM kinases to enhance tumor angiogenesis as well as invasive potential of prostate cancer cells. These results were the first to confirm that PIM kinases are not only able to increase prostate cancer cell survival and proliferation but also motility *in vivo*.

6.2 DHPCC-9 – a novel, potent and selective PIM inhibitor (I, II, III, IV)

In cancer therapy, the common problems are the side effects caused by unselective drug compounds. This emphasises PIM kinases as very interesting drug targets. The structure of PIM kinases is different enough as compared to other kinases to be able to design highly selective drugs (Qian et al. 2005). Furthermore, several oncoproteins may play so important roles in normal cell function, that they cannot be fully inactivated during cancer therapy. PIM kinases have been shown to play a role in normal organ function and thereby PIM inhibition might cause some unwanted side-effects (Eichmann et al. 2000). However, an *in vivo* mouse model shows that inactivation of all three PIM family members is not lethal, and even though it leads to changes in mouse size, the animals are still viable and fertile (Mikkers et al. 2004). Besides, a developing embryo or a young individual is often more sensitive to drugs than an adult (Niebyl & Simpson 2008, Mohammed 2013, Stephenson 2005). Thereby, these minor changes, such as the defects in growth in the PIM triple knock-out mice, might suggest that selective PIM inhibition may not lead to severe side-effects at least in adult human patients. Hence, PIM kinases have become an important target for drug research and development. Still, it should not be forgotten that replacement of inactivated PIM kinases by other kinases such as AKT in tumor cells might cause drug resistance. Therefore, combination of different therapy methods is still likely to be most efficient. This is further supported by our CAM results, where combined inhibition of both PIM and NOTCH was the most efficient way to inhibit tumor growth (IV).

While several multitargeted drugs influence PIM signaling pathways, increasing numbers of selective drugs are being developed. While our group was testing the selectivity and efficacy of pyrrolocarbazole and benzazulene derivatives, other groups identified simultaneously several potential PIM inhibitors (I, II, Kiriazis et al. 2013; Blanco-Aparicio & Carnero 2013; Arunesh et al 2014). Especially intriguing is the development of pan-PIM inhibitors targeting the whole family, since there is evidence of overlapping expression as well as functional redundancy between the PIM proteins (van der Lugt 1995; Eichmann et al. 2000; Mikkers et al. 2002; Saurabh et al. 2014). Some of the novel inhibitors have also been subjected to clinical trials, even though the first test with the compound SGI-1776 was discontinued due to the cardiac toxicity (Narlik-Grassow et al. 2014, ClinicalTrials.gov 2015). However, SGI-1776 also efficiently inhibits another kinase called FLT3 (fms-related tyrosine kinase 3/ FMS-like tyrosine kinase 3) which functions upstream from PIM kinases (Chen et al. 2009; Kim et al. 2005).

Although the actual reason for the cardiac toxicity is difficult to determine, the influence on FLT3 might be one of them due to its importance in cardiomyocytes (Pfister et al. 2014). Interstingly, the PIM inhibitor DHPCC-9 did not show general cytotoxicity in the FDC-P1 myeloid control cell line or in the PC-3 prostate cancer cell line, while SGI-1776 behaved differently leading to cytotoxic effects in both cell lines. Similar cytotoxicity has also been seen in primary acute myelogenous leukemia cells (Chen et al. 2011). This refers to possible PIM-independent cytotoxic effects of SGI-1776. Anyway, the clinical trials with the other PIM-targeted compounds are likely to clarify the PIM-dependent effects more precisely.

The dihydropyrrolocarbazole DHPCC-9 selectively inhibits all three PIM kinases *in vitro* (Akué-Gédu et al. 2009). Our results also confirm obvious Pim-selective effects on murine myeloid cell survival by DHPCC-9 (I). Other experiments further support this by showing similar results with PIM inhibition by DHPCC-9 and by PIM-targeted RNA interference (I, III, IV). On the other hand, PIM overexpression causes opposite changes in cancer cell behavior as compared to DHPCC-9 treatment (I, II, III, IV). The use of the pyrrolocarbazole derivative with the fluorescent tag also confirms that the pyrrolocarbazoles are able to enter PC-3 cells (Letribot et al. 2012). These results suggest that DHPCC-9 can selectively and efficiently inhibit PIM activity *in vitro* and in cell-based models.

Prior to the *in vivo* efficacy experiments, the general toxicity of DHPCC-9 was needed to be tested. Zebrafish embryos were chosen as the research model, since they are a highly cost-effective and a rapid animal model for analysis of drug safety. The experiments performed under 120 hours post fertilization are not legally considered as animal experiments (Strähle et al. 2012). Furthermore, experiments with zebrafish emryos can easily contain a big sample number, which is harder to achieve in experiments with adult animals or mammals such as mice. Both DHPCC-9 and the benzazulene BA-1a were given to zebrafish embryos, but no major changes were detected in body structure or viability (II). However, PIM inhibition caused slightly curved tailes and a pericardiac oedema, which might refer to some sensitivity of embryo development on PIM inactivation (II). Previously, PIM expression has been detected in the myocardium of quail embryos, but not in mouse embryos (Eichmann et al. 2000). Thereby, PIM kinases may also play a more important role in the cardiac development in some vertebrates such as fish or bird as compared to mammals such as mouse or human. On the other hand, this effect might also be associated with the above mentioned cardiotoxicity of SGI-1776 and might be related to the function of PIM kinases. After zebrafish embryonal experiments, the toxicity of the inhibitors in mammals still needed to be clarified. Treatment of adult mice with DHPCC-9 did not cause any detectable changes in animal healt (II). Unfortunatelly, the results with the benzazulene BA-1a were not as promising. While BA-1a itself is not likely to be toxic, its insolubility caused problems, because it needed to be diluted to DMA (II). Thus, of the two tested inhibitors, DHPCC-9 was more suitable for *in vivo* experiments.

There is a wide variety of known common side-effects of chemotherapy such as nausea, constipation, diarrhea, fatigue, pain, blood disorders, nerve damage,

appetite loss, hair loss, infertility and problems in memory (Cancer.net 2016b). These can be studied more or less successfully by animal experiments. However, monitoring of all these effects is very important, if the drug is about to proceed into clinical trials. In our experiments, DHPCC-9-treated mice did not show changes in their eating habbits or basic behavior, which might suggest that the mice did not suffer from pain, tiredness or digestive disorders (II). The orthotopic experiments were performed in nude mice, in which hair loss was impossible to detect. However, the toxicity experiments were performed in white mice, but no hair loss was detected (II). However, the test-period might have been too short for detecting hair loss. Since the PIM triple knock-out mice are fertile, it is not expected that any PIM inhibitors would cause infertility (Mikkers et al. 2004). Nerve damage, which can cause various problems such as memory loss, is perhaps one the most difficult things to detect in the animal experiments. Blood disorders might be possible to detect from the blood samples with proper tools. The PIM inhibitor DHPCC-9 was proven to be safe and effective under *in vivo* conditions, but it is not likely to be suitable for actual patient treatment due to its solubility only to DMSO. Even though in certain cases humans can be treated with DMSO, it is not accepted as a diluent for chemotherapeutic agents (Santos et al. 2003; Capriotti & Capriotti 2012). However, development of DHPCC-9 derivatives might lead to the discovery of more soluble and usable compounds.

Even though DHPCC-9 is not proceeding into clinical trials, the results from the experiments were promising. They showed that both the cellular and the animal models were well-chosen for analyzing PIM inhibitor efficacy, selectivity and safety. While our cell-based models, especially the stable PIM-overexpressing FDC-P1 and PC-3 cells, were proven to be very useful in studying PIM-dependent cell survival and migration, the orthotopic model was also well-suited for analysis of PIM-mediated tumor progression. The FDC-P1 cell lines were optimized for PIM research previously (Aho et al. 2004). This thesis showed that the stable PC-3 cell lines and the orthotopic model can also be used later on for studying the efficacy of novel PIM inhibitors in cells and whole organisms (II-IV). The chorioallantoic membrane model is also a potential option to be optimized for preliminary evaluation of the ability of PIM inhibitors to reduce tumor growth and formation of metastases (IV; Kobayashi et al. 1998). The experiments performed with DHPCC-9 obey the classical pattern of preclinical testing of a novel druggable compound, starting from *in vitro* assays, followed by cell culture-based experiments and finally *in vivo* models (Table 13). These results gained with DHPCC-9 can be used as a control in the future. DHPCC-9 treatment led to a decrease in prostate tumor growth, angiogenesis, lymphangiogenesis and the number of metastases in the orthotopic model and decreased prostate tumor growth on the CAM (II, IV). Similar effects can be expected with all other efficient PIM-selective drug compounds in these research models. Thus, these results show that DHPCC-9 can reach tumor tissue and inhibit PIM activity *in vivo*. Thereby DHPCC-9 serves as a suitable control for analysis of other PIM inhibitors and it can also be used as a tool to study the influence of PIM kinase inactivation in other models.

Table 13 DHPCC-9 preclinical testing
PIM inhibition by DHPCC-9 was measured by several different assays (I; II, III, IV; Akué-Gédu et al. 2009; Letribot et al. 2012).

Experiment	Method	Concentration	Result
In vitro efficacy	Kinase assays	10 µM	Residual activities (%): PIM1 - 2 PIM2 - 7 PIM3 - 1 Decreased activity of Pim kinase substrates
In vitro selectivity	Kinase assays	10 µM	Lowest residual activities of other kinases (%): ERK - 20 BRSK2 - 22 HIPK2 - 25 ROCK2 - 25
Cellular selectivity and toxicity	Stable FD/Neo and FD/Pim44 cell lines	10 µM	Decrease in PIM-mediated cell survival
	IC_{50} value in different cell lines	variable	PC-3 - 9.5 µM FD/Neo - 6.0 µM FD/Pim44 - 4.7 µM
Cellular efficacy	PIM substrate phosphorylation	10 µM	Decreased phosphorylation (and activity) of Pim kinase substrates
	Cell motility	10 µM	Decreased cell migration and invasion
	Cell metabolism	10 µM	Increased glucose uptake and lactate production
	Visualization of a fluorescent derivative	10 µM	Detection of a derivative in PC-3 cells by confocal microscopy
In vivo toxicity	Zebrafish embryos	1-10 µM	No major changes in survival (curved tail and pericardiac oedema by 10 µM DHPCC-9)
	Adult mice	50 -100 mg/kg	No detectable changes
In vivo efficacy	Intraperitoneal injections to mice with stable PIM3-overexpressing orthotopic prostate tumor	50 mg/kg	Decrease in tumor growth, angiogenesis, lymphangiognesis, formation of metastases, phosphorylation and localization of Pim kinase substrates
	Treatment of PC-3 cell-derived tumors grown on the chicken embryo chorioallantoic membrane	100-200 µM	Decrease in tumor growth alone and in combination with other druggable combounds

6.3 PIM signaling in prostate cancer cell motility (I, II, III, IV)

PIM kinases are constitutively active, which means that PIM activity is mainly dependent on transcriptional regulation (Qian et al. 2005). The most well-known regulators of PIM expression are cytokines, while direct induction of PIM expression is mediated by activation of the JAK/STAT pathway (Narlik-Grassow et al. 2014). There are also several known substrates for PIM kinases such as NFATC1, CXCR4 and FOXP3 (Rainio et al. 2002; Grundler et al. 2009; Li et al. 2014). While the the interaction of these substrates with PIM kinases has been previously studied in hematopoietic systems, individually these substrates have been connected to the motility of breast or prostate cancer cells (Singh et al. 2004; Seifert et al. 2009; Douglass et al. 2014). After PIM kinases were shown to promote prostate cancer cell migration and the formation of metastases, the potential signaling pathways needed to be examined.

First the influence of known PIM kinase substrates was studied in PC-3 cells by wound healing assays. Previously, NFAT family members had been reported to play a role in cell migration and invasion (Jauliac et al. 2002; Seifert et al. 2009; Jang et al. 2010). In line with the previous findings, in PC-3 cells, overexpression of NFATC1 also promoted cell migration (I). In addition, the pro-migratory behavior of NFATC1 was efficiently blocked by PIM inhibition, suggesting a role for NFATC1 in PIM-mediated cell motility (I).

CXCR4 is known to be phosphorylated by PIM1, which activates the CXCR4/CXCL12 pathway (Grundler et al. 2009). On the other hand, CXCR4-positive cell migration towards CXCL12-expressing cells has been connected to tumor progression (Chatterjee et al. 2014). Tissues such as lymph nodes and lungs also express CXCL12, while PC-3 cell invasion depends on the CXCR4/CXCL12 pathway (Müller et al. 2001; Singh et al. 2004; Kukreja et al. 2005). In our experiments, PIM inhibition decreased PC-3 cell invasion towards a chemoattractant containing CXCL12 (I). Therefore, the phosphorylation of CXCR4 was analysed in cultured and xenografted PC-3 cells. Interestingly, PIM1 and PIM3 increased CXCR4 phosphorylation *in vitro*, in cell culture and in the orthotopic xenografts (II). Since PIM upregulation supported tumor growth, angiogenesis, formation of metastases and CXCR4 phosphorylation, CXCR4/CXCL12 pathway is likely to support PIM-induced formation of metastases (II).

PIM kinases have been reported to phosphorylate and inactivate the tumor suppressor protein FOXP3, which like NFATC1 also is a transcription factor (Li et al. 2014; Deng et al. 2015). Furthermore, GSK3B phosphorylation by PIM1 has been suggested previously, but it has not been directly studied (Narlik-Grassow et al. 2014). Both FOXP3 and GSK3B have been previously shown to inhibit breast or prostate cancer cell migration (Douglass et al. 2014; Liu et al. 2014c). The results from the wound healing assays confirmed that the phosphorylation of FOXP3 at Ser^{422} and GSK3B at Ser^{9} plays an important role in PC-3 cell migration (III). Phosphorylation by PIM1 is likely to inactivate both FOXP3 and GSK3B, which prevents their negative influence on cell migration (III). There might also

be a negative regulatory loop affecting FOXP3 activity after phosphorylation by PIM1. This depends on the importance of phosphorylation of Ser^{422} as compared to Ser^{418}, which both are phosphorylated by PIM1 with completely opposite effects on the activity of FOXP3. However, Ser^{418} phosphorylation might have more severe effects on FoxP3 function in mice, due to the absence of Ser^{422} in mouse FoxP3 (UniProt Consortium 2015).

PTGS2 is a key player in inflammation, but changes in PTGS2 activity have also been connected to tumor progression and increased cell motility e.g. in the PC-3 cells (Ghosh et al. 2010; Vo et al. 2013). NFATC1, FOXP3 and GSK3B have been shown to up- or downregulate PTGS2 expression levels (Hernández et al. 2001; Corral et al. 2007; Hao et al. 2014; Thiel et al. 2006). Integrins are known to be the main regulators of cell adhesion and PTGS2-mediated signaling pathway has been connected to the integrin-mediated cell motility and upregulation of integrin expression (Desgrosellier & Cheresh 2010; Menter & Dubois 2012; Liu et al. 2010; Han et al. 2005). PIM kinase activity was shown to increase PTGS2 expression (III). This was at least partly regulated by the ability of PIM kinases to phosphorylate and inactivate GSK3B, thereby preventing GSK3B-mediated inhibition of PTGS2 expression (III). A connection between phosphorylation of NFATC1 or FOXP3 and PTGS2 levels was not found (III and unpublished data). In addition, PIM kinase activity also regulated integrin-mediated cell adhesion of PC-3 cells, while integrin cell surface expression or activity were not altered (III). The effect of PIM inhibition on integrin-mediated cell adhesion was quite fast as compared to the effect of PTGS2 inhibition (III). This refers to another more direct PIM-dependent pathway not including PTGS2. To sum up, FOXP3 and GSK3B are potential mediators of PIM signaling leading to changes in prostate cancer cell migration. However, another yet unspecified pathway is likely to regulate integrin-mediated cell migration downstream from the PIM kinases.

NOTCH family members have not been connected to PIM kinases previously, even though there are reports showing connection of NOTCH to PIM substrates such as NFATC1, FOXP3, CXCR4 and GSK3 (Tu et al. 2012; Ou-Yang et al. 2009; Williams et al. 2008; Wang et al. 2009; Xie et al. 2013; Espinosa et al. 2003; Jin et al. 2009). Furthermore, PIM kinases activate the EBNA2 transcription factor, which forms a transcription regulatory complex with the same cofactor as the NOTCH intracellular domain (NICD) (Rainio et al. 2005; Henkel et al. 1994; Tamura et al. 1995). Changes in NOTCH1 activity have also been connected to prostate cancer, where NOTCH1 may play an oncogenic role (Leong & Gao 2008). Therefore, the influence of NICD1 activity on PIM-mediated cell migration and tumor growth was measured. NICD1 was endogenously expressed in PC-3 cells, while NICD3 was not present (IV). *In vitro* kinase assays revealed phosphorylation of NICD1 and NICD3, but not NICD2. Phosphorylation of NICD1 Ser^{2152} by PIM kinases increased NICD1 nuclear localization and transcriptional activity (IV). NICD1 phosphorylation also promoted PIM expression levels in PC-3 cells, leading to a positive regulatory loop between NICD1 activity and PIM1 expression (IV). PC-3 cell migration was also enhanced by PIM-mediated phosphorylation of NICD1, while inhibition of PIM and NOTCH activity significantly decreased

prostate tumor growth in the chorioallantoic membrane model (IV). Upregulation of PIM activity also lead to increased amount of nuclear NOTCH1 in the orthotopic prostate xenografts, where PIM kinases supported tumor growth, angiogenesis and the formation of metastases (II; IV). Thereby, NOTCH1 is also expected to mediate the effects of the PIM kinases on prostate cancer cell migration and invasion. Furthermore, prostate tumor growth can be decreased by targeting either PIM1 or NOTCH signaling pathways, but the most prominent results might be gained by combinatorial therapy targeting them both (IV).

In summary, PIM kinases can enhance prostate cancer cell motility and tumor progression by phosphorylation of several substrates. These results also support the importance of CXCR4/CXCL12 pathway in the formation of metastasis as well as tumor suppressor activity of FOXP3 and GSK3B and oncogenic role of NFATC1 and NOTCH1 in prostate cancer. PIM kinases can either activate or inactivate these substrates leading to changes in various downstream targets. This creates a complex signaling cascade supporting cell motility not only in cell culture but also in *in vivo* models and probably also in human prostate cancer (Figure 19).

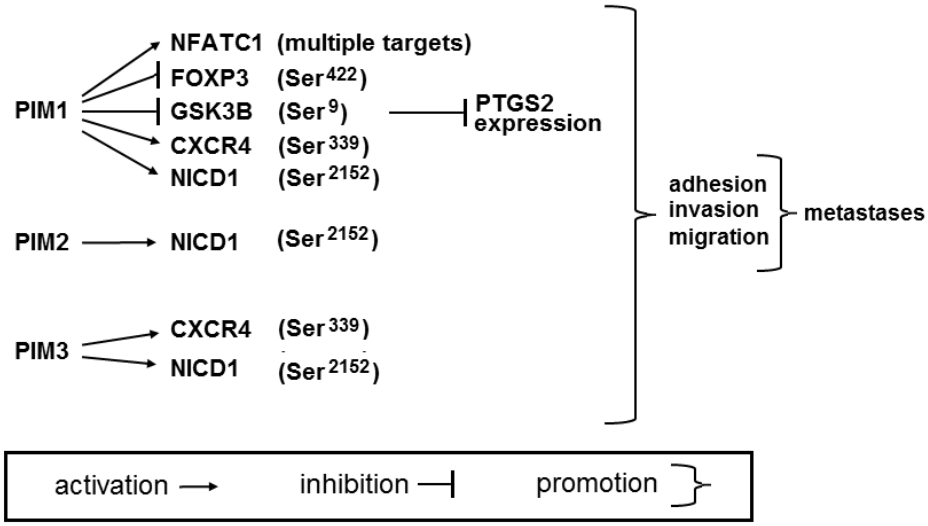

Figure 19 PIM kinases in prostate cancer cell motility
PIM kinases phosphorylate several substrates, thereby regulating multiple downstream targets and the adhesive, migratory and invasive properties of cancer cells, supporting prostate tumor growth and the formation of metastases (I, II, III, IV, unpublished data).

6.4 PIM-mediated regulation of NOTCH receptors in breast cancer (IV)

In breast cancer, NOTCH1 has been described to mostly function as an oncogene, (Guo et al. 2011). As previously mentioned, NOTCH receptors have connections to the substrates of PIM kinases as well as the JAK/STAT pathway, which upregulates PIM expression. Furthermore, there are also reports on the connections between NOTCH or PIM signaling and cell energy metabolism (Landor et al 2011; Beharry et al. 2011; Din et al 2014; Zhang et al. 2015a). Due to the previously reported importance of NOTCH activity in breast cancer progression, and the

hormone-dependency of PIM kinase axpression (Landor et al. 2011; Malinen et al. 2013), the effect of PIM-mediated NOTCH activity on cell metabolism and tumor growth was studied in the hormone-dependent MCF-7 breast cancer cells (IV).

NOTCH1 has been previously connected to the regulation of glycolytic breast cancer cell metabolism, supporting cell viability in low oxygen conditions (Landor et al. 2011). NICD1 has been shown to give tumor cells the advantage to efficiently switch from oxidative phosphorylation to glycolysis when needed. On the other hand, NOTCH1 inactivation is expected to cause problems in mitochondria, thereby leading to increase in glycolysis and decrease in oxidative phosphorylation. PIM kinases can also regulate mitochondrial function and glycolysis (Beharry et al. 2011; Din et al. 2013; Yu et al. 2013). Therefore, the interaction of endogenous PIM1 and NICD1 was measured in MCF-7 cells and they did interact in the cytoplasm. NICD activity was similarly dependent on PIM-mediated phosphorylation in MCF-7 cells and in PC-3 cells. Glycolytic metabolism was measured by detection of glucose uptake, mitochondrial membrane potential and lactate production. Prevention of NICD1 phosphorylation by targeted mutagenesis or inhibition of PIM activity similarly led to increased glycolysis in MCF-7 cells (IV). This was likely to depend on defects in mitochondrial function as shown by increased mitochondrial membrane potential after PIM inhibition (IV). Previous results also show similar results by inhibition of NOTCH cleavage by DAPT treatment (Landor et al. 2011). However, this phenomenon is likely to be cell-type dependent, since in colorectal and hepatocellular carcinoma cells PIM inhibition leads to a decrease in glycolytic metabolism (Leung et al. 2015; Zhang et al. 2015a).

Previously estradiol treatment has been shown to upregulate expression of PIM1, which plays a role in the proliferation of the hormone-dependent MCF-7 cells (Malinen et al. 2013). The connection of PIM activity and estrogen receptor activation or anti-estrogen resistance have not been reported. However, estradiol is known to activate JAK/STAT signaling pathway, which is the main route inducing PIM expression, and this might explain the increase in PIM expression after estradiol treatment (Coughlan et al. 2013; Narlik-Grassow et al. 2014). Therefore, the influence of NICD1 phosphorylation on the growth of MCF-7 tumors was studied in the chorioallantoic membrane model in the presence of estradiol. Interestingly, the overexpression of the NICD1 phosphomimicking mutant supported tumor growth as compared to the phosphodeficient mutant (IV). There was also a clear dependency on hormone levels. Estradiol treatment promoted the NICD1-mediated tumor growth, while both PIM1 and PIM3 were upregulated following estradiol treatment (IV). This refers to increased phosphorylation and activation of NICD1 by PIM kinases after estradiol treatment due to increased PIM epression levels. The changes in breast tumor growth might have also been partly dependent on the ability of NICD1-PIM1 interaction to support breast cancer cell energy gain.

Inhibition of NICD1 formation decreased PIM expression levels in MDA-MB-231 cells, while opposite effect was seen after NICD1 activation in MCF-7 cells, supporting the role of NOTCH proteins in upregulation of PIM expression levels

(IV). Previously, NOTCH receptors have been shown to promote activation of the JAK/STAT signaling pathway in a TP53-dependent manner in MDA-MB-231 cells but not in MCF-7 cells (Jin et al. 2013). Upregulation of PIM expression was also detected in PC-3 prostate cancer cells (IV). Thus NOTCH activation is likely to upregulate PIM expression through increased interleukin production and JAK/STAT activation especially in the cells with inactivated TP53 such as MDA-MB-231 and PC-3 cells. However, other mechanisms may exist as shown by the slightly increased PIM expression levels after NOTCH upregulation in MCF-7 cells expressing the wild type TP53.

These results show the interaction and co-operation of PIM kinases and NOTCH receptors during breast tumor progession (Figure 20). While PIM kinases phosphorylate and activate NICD1, it should be kept in mind that there might also be other kinases able to phosphorylate NICD1 at the same target site as the PIM kinases. Furthermore, the effects of PIM-mediated phosphorylation and activation of NOTCH1 receptor might lead to different results depending on the cancer type.

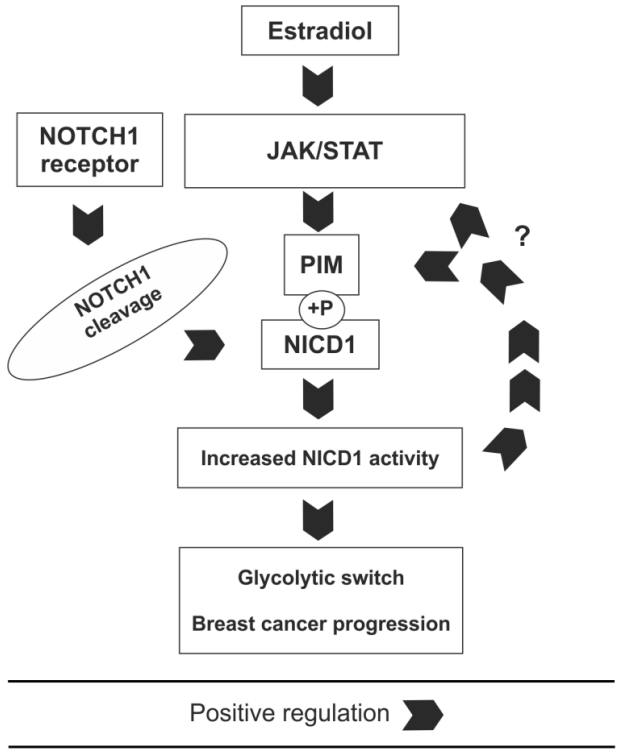

Figure 20 PIM-mediated activation of NOTCH1 in breast cancer
In hormone-dependent breast cancer, estradiol is likely to upregulate PIM expression (Coughlan et al. 2013; Narlik-Grassow et al. 2014; Malinen et al. 2013). The cleaved NOTCH1 intracellular domain (NICD1) can be phosphorylated by PIM kinases leading to enhanced activation of NICD1 (IV). Thereby PIM-mediated phosphorylation of NICD1 can regulate various cellular functions such as energy metabolism leading to hormone-dependent increase in tumor growth (IV). NOTCH activation can support JAK/STAT-mediated transcription of PIM kinases in certain cell lines such as the hormone-independent MDA-MB-231 cells (Jin et al. 2013), but the NOTCH-mediated pathway upregulating PIM expression in MCF-7 cells is not known.

7 CONCLUSIONS

The oncogenic PIM kinases have previously been shown to promote tumor progression by supporting cell proliferation and preventing apoptosis. They have been connected to both hematopoietic malignancies as well as formation of solid tumors. This research concentrated on novel functions of PIM kinases in the regulation of prostate cancer cell migration and breast cancer cell metabolism. Simultaneously to these observations, other investigators found somewhat similar changes in different research models. In this dissertation, PIM kinase upregulation is shown to enhance prostate cancer cell migration, adhesion and invasion in cell culture-based assays as well as increased angiogenesis and formation of metastases in an orthotopic prostate cancer model. Furthermore, PIM activity regulated breast tumor growth on the chorioallantoic membranes of chicken eggs. This is likely to depend partly on the PIM-mediated activation of NOTCH1 leading to improved energy gain and thereby increased cell survival and proliferation.

This thesis also summarizes the preclinical testing pattern of a novel drug compound, DHPCC-9. DHPCC-9 decreased tumor growth, angiogenesis and formation of metastases in a mouse model for prostate cancer. DHPCC-9 was also shown to be selective and effective PIM inhibitor and it serves as a valuable tool to study PIM-dependent signaling pathways later on. Several *in vivo* models were also used in this study such as zebrafish embryos, the chicken embryo chorioallantoic membrane model as well as subcutaneous and orthotopic mouse models for prostate cancer. These models were proven to be functional and suitable for PIM kinase research and they can be used also later on in testing other PIM-targeted drug compounds.

Another aim of this study was to clarify the PIM-dependent signaling pathways promoting cell motility. Several previously known PIM kinase substrates were associated with prostate cancer cell motility, while also novel substrates were found. According to the results, PIM kinases are likely to enhance cell migration through phosphorylation of NFATC1, FOXP3, GSK3B and NOTCH1. A wider signaling cascade was also discovered, connecting PIM kinases to prostaglandin and integrin-mediated cell motility. The orthotopic prostate cancer model also implied the potential role of the CXCR4 chemokine receptor to support the migration of PIM-expressing cancer cells towards a CXCL12 gradient to form metastases to distant organs such as lungs.

Experiments with Notch1 intracellular domain (NICD1) connected PIM kinases to the regulation of breast cancer cell metabolism and hormone-dependent breast tumor growth. PIM kinases activated NICD1 by phosphorylation similarly in breast cancer cells as in prostate cancer cells. Furthermore, phosphorylation of the NICD1 promoted breast tumor growth in the chorioallantoic membrane model. The influence of NOTCH1-PIM1 interaction on breast tumor growth is expected to be hormone-dependent, and it is likely to be at least partly mediated by changes in cell metabolism. Notch3 ICD (NICD3) was also shown to be phosphorylated by PIM1, while the PIM-mediated regulation of NICD3 remained outside the scope

of this thesis. However, further studies with PIM kinases and NICD3 are likely to give more information on the differences between NICD1 and NICD3 activity in cancer and the role of PIM kinases in the regulation of NOTCH receptors.

While many of the PIM kinase substrates regulate cell motility, they can also affect cell survival and thereby tumor progression in some cancer types. In addition, there are other kinases such as AKT that may be able to target these same substrates simultaneously as the PIM kinases or replace the function of PIM kinases, if PIM activity was decreased. Some of these substrates can also interact or co-operate with each other or form various regulatory loops reaching even as far back as to the regulation PIM expression levels. To sum up, these results suggest that there are several different pathways through which PIM kinases are able to promote tumor progression. Furthermore, this thesis showed the connection between PIM kinases and integrin-mediated cell adhesion, while the exact mechanisms remain to be discovered. Thus the integrin receptors and integrin-dependent changes in cell adhesion and cell cytoskeleton form an interesting research target for PIM-related research in the future.

The ability of tumor cells to invade to form metastases is highly dependent on the adhesive properties of cells, but also on tumor angiogenesis, which was enhanced by PIM upregulation. However, the exact PIM-mediated mechanisms regulating angiogenesis were not a part of this thesis. On the other hand, the growth of the primary tumor is highly dependent on the availability of oxygen and nutrients. While angiogenesis supports the oxygen and nutrient gain, the PIM-mediated changes in NOTCH1 activity may regulate the metabolic changes thereby promoting tumor growth. The metabolism can also vary a lot between different tumor types e.g. in prostate and breast cancer. Therefore, the role of PIM kinases in the regulation of tumor angiogenesis and metabolism should be clarified in more detailed later on.

In summary, the main aim of this thesis was to study the role of PIM kinases in cancer cell motility and metabolism, because of lacking information from this area. These results show part of the complex signaling cascade mediated by the PIM kinases in the regulation of cell motility, metabolism and tumor progression. Thereby, these results also reveal new interesting directions for PIM kinase research. Especially since there are several compounds already in clinical trials targeting PIM kinases, it is extremely important to predict as precisely as possible the PIM-mediated cellular functions to be able to estimate the possible effects of PIM inhibition. Finally, while this research clarified the PIM-dependent signaling pathways in breast and prostate cancer, it also showed promising results with the PIM inhibitor DHPCC-9 and supported the importance of developing more selective and well-tolerated inhibitory compounds against PIM kinases to treat cancer.

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