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A study of three novel substrates

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For Mum and Dad

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ABSTRACT

The PIM kinase family comprises three constitutively active serine/threonine kinases that affect cell proliferation, survival, and motility, especially when upregulated in hematological malignancies or solid tumors, such as breast cancer. Furthermore, in estrogen receptor α (ERα)-expressing (ER+) breast cancer cells, PIM expression is upregulated in response to estrogen stimulation.

The aim of this PhD project was to explore the physiological roles of PIM kinases (PIMs) and their substrates in ER+ luminal A breast cancer. A variety of pharmacological PIM inhibitors and genetically modified cell lines were used in experiments, where the effects of phosphorylation on the activity of three novel PIM substrates (Notch3, LKB1, and ERα) were investigated. In study I, PIM-catalyzed phosphorylation of Notch3 was shown to inhibit Notch canonical signaling by disrupting interaction with CSL, a crucial component of the Notch transcriptional complex. Despite this, phosphorylated Notch3 supported tumor growth in the chick chorioallantoic membrane (CAM) xenograft model, hinting at an oncogenic CSL-independent Notch3 signaling mechanism. In study II, PIMs were shown to phosphorylate the tumor suppressor LKB1, and in doing so, reduce its catalytic capacity. Moreover, the growth of CAM tumors was slower when the xenografted cells lacked both LKB1 and PIMs, suggesting that PIMs could be a promising therapeutic target for cancer patients with LKB1-deficient tumors. Study III demonstrated that PIMs phosphorylate ERα and play a key role in regulating ERα signaling. While PIMs are known to contribute to resistance against various cancer therapies, we found no evidence that they are involved in endocrine therapy resistance in breast cancer.

Collectively, these study results provide further insight into how PIMs impact breast cancer cell signaling mechanisms.

KEYWORDS: PIM kinases, phosphorylation, inhibitors, breast cancer, Notch3, LKB1, estrogen receptor α

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TIIVISTELMÄ

PIM-kinaasiperheeseen kuuluu kolme konstitutiivisesti aktiivista seriini/treoniinikinaasia, jotka vaikuttavat solujen proliferaatioon, elinkykyyn ja liikkuvuuteen etenkin silloin, kun niitä ilmennetään liiallisesti hematologissa tai kiinteissä kasvaimissa, kuten rintasyövässä. Estrogeenireseptoria α (ERα) ilmentävissä (ER+) rintasyöpäsoluissa PIM-kinaasien ilmentyminen lisääntyy estrogeenistimulaation seurauksena.

Tämän väitöskirjaprojektin tavoitteena oli tutkia PIM-kinaasien ja niiden substraattien fysiologista merkitystä luminaalisissa A-tyypin rintasyöpäsoluissa. Erilaisia farmakologisia PIM-inhibiittoreita sekä geneettisesti muokattuja solulinjoja käytettiin kokeissa, joissa tutkittiin fosforylaation vaikutuksia kolmen uuden PIM-substraatin (Notch3, LKB1 ja ERα) aktiivisuuteen. Tutkimuksessa I osoitettiin, että PIM-kinaasien katalysoima Notch3-fosforylaatio estää Notch-välitteistä kanonista viestintää häiritsemällä Notch3:n vuorovaikutusta CSL:n kanssa, joka on keskeinen komponentti Notch-transkriptiokompleksissa. Tästä huolimatta fosforyloitu Notch3 edisti kasvaimen kasvua kanan korioallantoiskalvon (CAM) siirrännäismallissa, mikä viittaa CSL-riippumattomaan viestintämekanismiin. Tutkimuksessa II PIM-kinaasien osoitettiin fosforyloivan tuumorisuppressiivisen LKB1-kinaasin ja siten vähentävän sen katalyyttistä aktiivisuutta. Lisäksi CAM-kasvainten kasvu oli hitaampaa, kun siirretyistä soluista puuttuivat LKB1:n lisäksi myös PIM-kinaasit. Tämä viittaa siihen, että PIM-kinaasit voisivat olla mahdollinen terapeuttinen kohde syöpäpotilaille, joiden kasvaimista LKB1 puuttuu. Tutkimuksessa III PIM-kinaasien osoitettiin fosforyloivan ERα:n ja olevansa keskeisessä roolissa ERα-signaloinnin säätelyssä. Vaikka PIM-kinaasien tiedetään suojaavan syöpäsoluja erilaisia hoitoja vastaan, emme löytäneet näyttöä siitä, että ne osallistuisivat endokriinisen hoitoresistenssin kehittymiseen rintasyövässä.

Kaiken kaikkiaan tämän tutkimuksen tulokset antavat uutta lisätietoa niistä mekanismeista, joilla PIM-kinaasit voivat vaikuttaa rintasyöpäsolujen viestintään.

ASIASANAT: PIM kinaasit, fosforylaatio, inhibiittorit, rintasyöpä, Notch3, LKB1, estrogeenireseptori α

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Abbreviations

ABCB1	ABC Transporter P-glycoprotein
ABCG2	ATP-Binding Cassette Sub-Family G Member 2
ADAM	A Disintegrin And Metalloproteinase
AF1	Activation Function 1
AF2	Activation Function 2
AI	Aromatase Inhibitor
AKT	Protein Kinase B
AML	Acute Myeloid Leukemia
AMP	Adenosine Monophosphate
AMPK	AMP-Activated Protein Kinase
ATP	Adenosine Triphosphate
AZD1208	(5Z)-[[2-[(3R)-3-amino-1-piperidinyl][1,1'-biphenyl]-3-yl]methylene]-2,4-thiazolidinedione (A Pan-PIM Kinase Inhibitor)
BRSK	Brain Selective Kinase
CAM	Chick Chorioallantoic Membrane
CBP	cAMP Binding Protein
CDK	Cyclin-Dependent Kinase
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
CERAN	Complete Estrogen Receptor Antagonist
CSL	CBF1, Suppressor of Hairless, Lag-1
CTC	Circulating Tumor Cell
DHPCC-9	1,10-dihydropyrrolo[2,3-a]carbazole-3-carbaldehyde (A Pan-PIM Kinase Inhibitor)
DLL4	Delta-Like Ligand 4
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E1	Estrone
E2	Estradiol
E3	Estriol

E4	Estetrol
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
ER-	Estrogen Receptor Negative
ER+	Estrogen Receptor Positive
ERα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
FDA	US Food and Drug Administration
FLIM	Fluorescence Lifetime Imaging Microscopy
GFP	Green Fluorescent Protein
GSK3β	Glycogen Synthase Kinase 3 Beta
HER2/ErbB2	Receptor Tyrosine-Protein Kinase erbB-2
IDC	Invasive Ductal Carcinoma
IFN-α	Interferon Alpha
IFN-γ	Interferon Gamma
IGF-1	Insulin-Like Growth Factor 1
IL-2/3/7/12/15	Interleukin 2/3/7/12/15
IVK	In Vitro Kinase
JAK	Janus Kinase
KO	Knockout
LBD	Ligand-Binding Domain
LCSM	Laser Confocal Scanning Microscopy
LKB1	Liver Kinase B1
MAML	Mastermind-Like Protein
MAPK	Mitogen-Activated Protein Kinase
MARK	Microtubule Affinity-Regulating Kinase
MCF-10A	Michigan Cancer Foundation-10A (A Non-Tumorigenic Epithelial Cell Line)
MCF-7	Michigan Cancer Foundation-7 (A Luminal A Breast Cancer Cell Line)
MDA-MB-231	Monroe Dunaway Anderson-Metastatic Breast-231 (A Triple-Negative Breast Cancer Cell Line)
miRNA	Micro-RNA
MM	Multiple Myeloma
mRNA	Messenger RNA
mTOR	Mammalian Target Of Rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (A reducing tetrazolium dye used to determine cell viability)

NF-KB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NICD	Notch Intracellular Domain
NLS	Nuclear Localization Signal
NSCLC	Non-Small Cell Lung Cancer
NST	No Special Type
NUAK	Novel AMPK-Related Kinase
P70S6K	70 kDa Ribosomal Protein S6 Kinase
PAM50	Prediction Analysis of Microarray 50
PAX2	Paired Box Gene 2
PC-3	Prostate Cancer-3 (A Prostate Cancer Cell Line)
PCR	Polymerase Chain Reaction
pegRNA	Prime Editing Guide RNA
PI3K	Phosphoinositide 3-Kinase
PIM	Proviral Integration Site for Moloney Murine Leukemia Virus
PIM1S/L	PIM1 Short/Long Isoform
PIMi	Unpublished PIM Kinase Inhibitor
PJS	Peutz-Jeghers Syndrome
PLA	Proximity Ligation Assay
PP2A	Protein Phosphatase 2A
PR	Progesterone Receptor
PROTAC	Proteolysis Targeting Chimera
PTEN	Phosphatase and Tensin Homolog
PTM	Post-Translational Modification
qPCR	Quantitative Polymerase Chain Reaction
RPMI-1640	Roswell Park Memorial Institute 1640 Medium
S2/3/4	Site 2/3/4 (in Notch cleavage)
Ser	Serine
SERCA	Selective Estrogen Receptor Covalent Antagnonist
SERD	Selective Estrogen Receptor Degrader
SERM	Selective Estrogen Receptor Modulator
sgRNA	Single Guide RNA
siRNA	Small Interfering RNA
SRC-1	Steroid Receptor Coactivator 1
STAT	Signal Transducers and Activators of Transcription
STK11	Serine/Threonine Kinase 11
T-47D	No unabbreviated form (A Luminal A Breast Cancer Cell Line)

TFF1	Trefoil Factor 1
TKO	PIM 1/2/3 Triple Knockout
TNBC	Triple-Negative Breast Cancer
TNF-α	Tumor Necrosis Factor Alpha
UK	United Kingdom
USA	United States of America
UTR	Untranslated Region
WHO	World Health Organization
WT	Wild-Type

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Sebastian K J Landor*, Niina M Santio*, William B Eccleshall, Valeriy M Paramonov, Ellen K Gagliani, Daniel Hall, Shao-Bo Jin, Käthe M Dahlström, Tiina A Salminen, Adolfo Rivero-Müller, Urban Lendahl, Rhett A Kovall, Päivi J Koskinen, Cecilia Sahlgren. PIM-induced phosphorylation of Notch3 promotes breast cancer tumorigenicity in a CSL-independent fashion. *J Biol Chem.* 2021; 296:100593.

II Kwan Long Mung, William B Eccleshall, Niina M Santio, Adolfo Rivero-Müller, Päivi J Koskinen. PIM kinases inhibit AMPK activation and promote tumorigenicity by phosphorylating LKB1. *J Cell Commun Signal*. 2021; 19(1):68.

III William B Eccleshall, Kwan Long Mung, Iida Laiho, Cecilia Sahlgren, Päivi J Koskinen. PIM kinases regulate estrogen receptor α signaling in luminal A breast cancer cells. *Manuscript*. Previous version available as preprint at DOI:10.20944/preprints202409.0907.v1

1 Introduction

The proviral integration site for Moloney murine leukemia virus (PIM) kinase family consists of three constitutively active serine/threonine kinases (PIM1, PIM2, PIM3) that influence stem cell differentiation, hematopoietic cell regulation, metabolism, and immune cell function. PIMs, like other kinases, regulate cell signaling by catalyzing the addition of a phosphoryl group (PO_3) to their substrates in a biochemical process known as phosphorylation. Phosphorylation is an ancient mechanism through which nature can alter the function, activity, stability, and/or localization of a biomolecule.

PIM expression levels are low in most adult tissues, however they are upregulated in numerous cancers, where they promote tumorigenesis, cancer progression, and therapy resistance by influencing pathways involved in cell survival and apoptosis, the cell cycle, cell motility, and drug efflux. Several attempts have been made to develop PIM inhibitors and implement them in a clinical setting, however, so far there has been limited success in this area. For this reason, interest has shifted in recent years to the idea of combining PIM inhibition with other therapies, as a means of enhancing therapeutic efficacy. Abemaciclib, the only PIM-targeting drug on the market, epitomizes this premise rather effectively. The primary molecular targets for this compound are cyclin-dependent kinases 4 and 6, however, after its development, it was discovered to have an unintentional co-target, namely the PIM kinase family. It is widely used to treat metastatic breast cancer and is in clinical trials for a wide array of other cancers.

Breast cancer is a challenge to healthcare systems owing to its prevalence and heterogeneity. The most common breast cancer subtype is luminal A, which is characterized by the expression of estrogen receptor α (ERα) and progesterone receptor (PR). Treatment options are generally good for patients with luminal A breast cancer, however a key challenge in this area is therapy resistance. The mechanisms by which tumors develop therapy resistance are varied and complex, making it a particularly challenging hurdle to overcome. In luminal A breast cancer cells, PIM expression is upregulated by ERα signaling, suggesting that the kinases may have an important role in this context, a role that has received little attention.

To better understand the function and importance of PIMs in luminal A breast cancer, this thesis aims to explore the cell and molecular effects that follow PIM-mediated phosphorylation of three cancer-linked signaling proteins.

2 Review of the Literature

2.1 Breast cancer

2.1.1 Epidemiology

Among all cancers, female breast cancer is the most commonly detected and comprised 11.7% of cancer diagnoses in 2020 (Siegel *et al.* 2022). As a proportion of cancers detected in females, this percentage rises substantially to 24.5%, dwarfing colorectal cancer, which comes in at second place at 9.4% (Siegel *et al.* 2022). Treatment strategies are however well established and, in most cases, the outlook for patients is good in comparison to other cancers. This is reflected in the fact that despite comprising a quarter of cancer diagnoses in females, breast cancer deaths account for only 15.5% of cancer deaths in females (Ginsburg *et al.* 2017; Siegel *et al.* 2022). Nevertheless, owing to its prevalence and heterogeneity, breast cancer persists as a significant challenge to healthcare systems worldwide, which on top of treatment, must consider screening, diagnosis, and dissemination of relevant public health information.

Breast cancer is more frequently diagnosed in high-income countries as compared to low-income countries (Ginsburg *et al.* 2017; Siegel *et al.* 2022). It is likely that this is underpinned in part by the different risk factors associated with high-/low-income regions such as diet and alcohol consumption (Danaei *et al.* 2005), the age of the mother when her first child is born and the number of children she bears (Althuis *et al.* 2005; Colditz *et al.* 2006), and medical interventions such as birth-control and other hormonal medications (Mørch *et al.* 2017; Busund *et al.* 2018; Del Pup *et al.* 2019). There are, however, other factors at play. Breast cancer diagnosis also depends on the regional availability of detection methods, and, while in high-income regions, breast cancer is usually detected earlier, and multiple treatment options are available, in low-income regions breast cancer is usually detected at a later stage, and there is often reduced patient access to suitable treatments. This is reflected in the higher breast cancer mortality rates in these

regions (Allemani *et al.* 2015). An individual's ethnicity also predisposes them to different types of tumors. It is known that women with African ancestry are more likely than others to be diagnosed with triple negative breast cancer (TNBC), and more likely to display metastatic disease and undifferentiated or poorly differentiated tumors (Kohler *et al.* 2015). Thus, it is evident that the likelihood of developing, detecting, and dying from breast cancer depends on a multifarious chain of events including, genetics, environment, lifestyle choices, income, access to screening and other diagnostic platforms, treatment quality, and treatment availability.

2.1.2 Future directions and challenges in breast cancer research and treatment

Last year was tamoxifen's 50th birthday. The hormonal treatment was first approved for use in the UK as a treatment for breast cancer in 1973 and it revolutionized our understanding of and ability to treat the disease (Jordan 2003). Half-a-century on, where do the main challenges in this field lie? Firstly, breast cancer is heterogenous, and there is no silver bullet to cure all patients. Certain breast cancer subtypes are easier to treat than others, and across all subtypes, patients can display or acquire resistance to therapies that are working perfectly well in other patients. Secondly, more work is needed to decode the risk factors in this disease. For example, women of African descent are more likely to die of breast cancer than any other population group (Giaquinto *et al.* 2022); what governs this and is there anything we can do to change this? Finally, a prominent ethical and logistical challenge is how we, as a society, minimize regional differences in the accessibility of high-quality care across the globe.

2.1.3 Histological subtypes

Cellular carcinogenesis is driven by the transformation of normal cells into cancerous ones, which occurs through the accumulation of genetic mutations and epigenetic alterations (Hanahan and Weinberg, 2011). In over 99% of cases, breast cancer originates in the epithelia of the milk ducts or lobules of the breast (Feng et al., 2018).

Breast cancer can be divided into subclasses based on histological, genetic, and molecular patterns. The World Health Organization (WHO) described 19 major histotypes in their 2014 breast tumor classification edition (Harbeck *et al.* 2019). Accounting for 70% – 80% of breast cancer cases is invasive breast carcinoma of no special type (NST), also referred to as invasive ductal carcinoma (IDC) (Weigelt *et al.* 2008; Weigelt *et al.* 2010). IDC is further broken down into two; the 2019 WHO guidelines define tumors that display 10% or less of a specific histological type as

non-mixed IDC, while if between 10% and 90% of the tumor displays a more defined histological pattern, they are designated mixed IDC-special (Tan *et al.* 2020). The remaining 20% - 30% of breast cancers are specific histological types (Weigelt *et al.* 2010).

Histological distinctions are important for characterizing tissue architecture and offer some prognostic relevance (Ellis *et al.* 1992; Fitzgibbons PL, Page DL 1999). Histology can inform a physician as to the likelihood that a breast tumor is primary or metastatic, a distinction that is essential when deciding on a rational treatment route. Likewise, morphologically similar tumors may have a better or worse prognosis depending on the site where they have formed. Arguably more important than histological subtype is the molecular subtype of a breast tumor (see section 2.1.4), which directly informs the choice of pharmacological intervention and prognosis (Giaquinto *et al.* 2022). Above these factors, however, is the stage of the cancer upon detection. Simply put, the earlier the cancer is caught, the better a patient's chances of survival, which is consistent, regardless of histology, grade, or molecular subtype (Bonotto *et al.* 2014; Giaquinto *et al.* 2022).

2.1.4 Molecular subtypes

The molecular subtype of a breast cancer tumor is classified according to specific molecular signatures. The prediction analysis of microarray 50 (PAM50) "intrinsic subtype" system considers the expression signatures of 50 genes within a tumor when assigning a tumor to a specific group (Perou *et al.* 2000; Shiovitz and Korde 2015; Bray *et al.* 2018). Clinically, the most commonly used subtypes are part of the "surrogate intrinsic subtype" labelling system (Table 1). This system separates breast cancers into five groups by assessing both the histology of the tumor and the expression levels of estrogen receptor α (ERα), progesterone receptor (PR), receptor tyrosine-protein kinase erbB-2 (HER2 or ErbB2) and Ki67, which serves as an index of proliferation. Luminal A breast cancers express ERα (ER+) and PR (PR+), lack HER2 expression (HER2-), and typically have a low proliferation index and a good prognosis (Harbeck *et al.* 2019). Luminal B breast cancers are ER+ and PR+, however ERα and PR levels are lower as compared to luminal A cancers. Tumors are further divided into HER2-expressing (HER2+) and HER2- types. Both subtypes are higher grade and more aggressive than the luminal A subtype, with a worse prognosis (Harbeck *et al.* 2019). The remaining subtypes lack ERα (ER-) and PR (PR-) expression. Of these, HER-enriched, so called because the tumors are HER2+, are higher grade and more aggressive than the luminal A and B subtypes, however, generally have an intermediate prognosis if patients have access to targeted therapies, which are usually effective at slowing disease (Harbeck *et al.* 2019). TNBC is ER-, PR- and HER2-, and generally these tumors will display the highest

grade and highest proliferation rate (Harbeck *et al.* 2019). TNBC has the worst prognosis of all subtypes, a reality that is compounded by the fact that there is a lack of targeted therapies for these tumors. The molecular subtypes of breast cancer have different rates of mortality; the lowest 5-year relative survival rate is for TNBC, HER2-enriched is next, followed by luminal B then luminal A (National Cancer Institute 2020). This correlates with the proliferation rates and grades that are associated with each tumor type and reflects the ease with which each subtype can be treated.

2.1.5 Breast cancer treatments

The molecular subtype of breast cancer greatly influences the strategy used to treat the cancer and likelihood of success. The mainstay of breast cancer treatment is surgery, where the primary goal is to remove the tumor. Generally, surgeons will also try to preserve as much of the healthy breast tissue as possible, in what is known as "breast-conservation" (Harbeck *et al.* 2019). Following surgery, patients are likely to be treated with post-operative radiation or chemotherapy, along with hormonal or HER2-targeting treatments, depending on the characteristics of the tumor. In cases where an individual has a high risk of developing breast cancer, owing to genetic predisposition, it is becoming increasingly common to pre-empt tumorigenesis by undergoing a prophylactic mastectomy. A systematic review of clinical studies has suggested that removal of both breasts prior to cancer diagnosis does indeed reduce disease-associated mortality in high-risk patients, however after cancer has been diagnosed, pre-emptive removal of the contralateral breast does not improve chances of survival (Carbine *et al.* 2018).

Metastasis, the spread of cancer cells from the original tumor to distant organs or tissues, significantly complicates treatment efforts. ER+/PR+ breast tumors usually metastasize to the bone and/or lymph nodes, HER2+ breast tumors have tropism for the brain, while TNBC tumors commonly spread to the lungs and/or the brain (Harbeck *et al.* 2019). As the disease becomes more systemic rather than localized, it becomes increasingly difficult to cure. Metastasis often involves multiple organs or regions that cannot be surgically removed or treated in isolation. In such cases, surgical intervention will not cure disease, however there are indications that it may extend survival duration (Alghamdi & Mahmood 2023). As advanced metastatic disease is considered incurable, treatment goals are to increase the length and quality of a patient's life.

2.1.6 Endocrine therapies

Luminal A and B cancers are ER+ and usually depend upon the canonical estrogen signaling pathway for cancer progression. They are therefore generally treated with endocrine therapies (also referred to as hormonal therapies) that target estrogen signaling. Estrogen signaling will be described in this thesis in more detail in section 2.5. In breast cancer, endocrine therapies inhibit the estrogen signaling cascade via numerous mechanisms. Aromatase inhibitors (AIs), for example, suppress the production of estrogens by inhibiting aromatase (also known as estrogen synthetase), which is responsible for converting androgens to estrogens (Chumsri *et al.* 2011). Examples include aminoglutethimide, testolactone, anastrozole, letrozole, and exemestane. Selective estrogen receptor modulators (SERMs) compete with estradiol at ERα by binding the activation function domain 2 (AF2) of the receptor (Patel *et al.* 2023). In breast tissue, when SERMs bind AF2, ERα is more likely to interact with co-repressors of target gene expression, and in this way the receptor's transcriptional activity is hindered, although some agonist-induced signaling persists as SERMs do not inhibit activation function domain 1 (AF1) in any way. Examples include tamoxifen, toremifene, raloxifene, and broparestrol. Selective estrogen receptor degraders (SERDs) bind ERα and prevent it from translocating to the nucleus, thus rendering ERα-driven transcription impossible. The drug-bound receptor is then targeted for proteasomal degradation, thus amplifying its anti-estrogenic effects further (Patel *et al.* 2023). Examples of SERDs include fulvestrant, and elacestrant.

There are additional ERα-targeting drug classes that are still in their infancy, and, as of yet, no drugs of these classes have been approved for use in a clinical setting. However, there is considerable optimism that they might represent the next generation of drug compounds for ER+ breast cancer, offering improved safety and efficacy profiles. These include proteolysis targeting chimeras (PROTACs), a general class of drug molecule that hijacks the proteolytic machinery of a cell. PROTACs are engineered small molecules containing a domain that binds a target protein, covalently attached to a domain that binds E3 ubiquitin ligase with a linker region in between. When a PROTAC is simultaneously bound to its target protein and E3 ligase, the components are brought into close proximity and the target protein is ubiquitinated. Consequently, the ubiquitinated protein is targeted for degradation via the proteasomal pathway (Békés *et al.* 2022). Vepdegestrant (ARV-471) is an ERα-targeting PROTAC that is currently being compared with fulvestrant in phase 3 clinical trials (ClinicalTrials.gov Identifier: NCT05654623). Complete estrogen receptor antagonists (CERANs) block ERα-mediated gene expression by recruiting co-repressors to AF1 and directly block AF2 (Shastry & Hamilton 2023). OP-1250 is an orally bioavailable CERAN that is currently in phase 2 clinical trials (ClinicalTrials.gov Identifier: NCT04505826). Selective estrogen receptor covalent

antagonists (SERCAs) inhibit ERα by covalently binding a distinctive cysteine residue that is not found in other hormone receptors (Shastry & Hamilton 2023). H3B-6545 is an example of a SERCA that is currently in phase 2 clinical trials (ClinicalTrials.gov Identifier: NCT03250676).

2.1.7 Endocrine therapy resistance

The molecular mechanisms that facilitate endocrine therapy resistance are complex. There are numerous papers highlighting the relevance of genetics (Jeselsohn *et al.* 2018; Lei *et al.* 2018; Pejerrey *et al.* 2018), epigenetics (Zhou *et al.* 2019; Achinger-Kawecka *et al.* 2020), the cistrome (Hurtado *et al.* 2011; Ross-Innes *et al.* 2012; Fu *et al.* 2016; Fu *et al.* 2019; Nagarajan *et al.* 2020; Xu *et al.* 2020) and DNA damage repair (Haricharan *et al.* 2017) in therapy-resistant tumors, while other studies have pointed to mechanisms involving signal rewiring (Kirkegaard *et al.* 2005; Staka *et al.* 2005; Frogne *et al.* 2009; McClaine *et al.* 2010; Miller *et al.* 2010; Turner *et al.* 2010; Fox *et al.* 2011) and post-translational modifications (PTMs) (Likhite *et al.* 2006; Yamnik *et al.* 2009; Chen *et al.* 2013; Zheng *et al.* 2014; Guo *et al.* 2016). It therefore appears that the mechanisms that underpin the therapy-resistant phenotype are context-dependent and should be considered on a case-by-case basis. The mechanisms described in the literature are numerous, and in the following section some of the most prominent findings are presented.

Mutations in the ligand-binding domain (LBD) are an established, albeit uncommon means by which patients may develop therapy resistance, owing to the receptor being expressed as a form that can be activated independently of its natural ligand (Jeselsohn *et al.* 2018; Pejerrey *et al.* 2018). Furthermore, fusions in the ERα gene (ESR1) can lead to functionally altered ERα protein with similar consequences (Lei *et al.* 2018). Another study explored the role of the MutL mismatch repair complex in therapy resistance. In healthy individuals, the MutL complex, consisting of MLH1/3 and PMS1/2, plays an important role in mismatch recognition, strand discrimination, and strand removal (Guarné 2012). Haricharan *et al.* (2017) showed that MutL-deficient tumors are more likely to display intrinsic endocrine therapy resistance owing to their impaired ability to repair single-strand DNA breaks. With respect to epigenetics, two studies have suggested that three-dimensional reorganization of chromatin may be characteristic of endocrine resistance in breast cancer (Zhou *et al.* 2019; Achinger-Kawecka *et al.* 2020). Other studies, considering the ESR1 cistrome have suggested that FOXA1, a forkhead DNA-binding protein that acts as a pioneer factor for ERα in ER+ breast cancer, is associated with poor patient outcomes and development of endocrine therapy resistance. In patients and cell lines, FOXA1 has been linked to reprogramming of the ERα cistrome, such that ERα occupies enhancers that are associated with oncogenic transcriptional pathways

(Hurtado *et al.* 2011; Ross-Innes *et al.* 2012), and in the context of therapy resistance FOXA1 may interact with other transcription factors to negate the reduction in ERα availability (Fu *et al.* 2016; Fu *et al.* 2019). Another cistrome-linked mechanism involves loss of ARID1A, a transcriptional regulator (Nagarajan *et al.* 2020; Xu *et al.* 2020). These findings were published in the same issue of Nature Genetics, and while Xu and colleagues reported that ARID1A facilitates ERα chromatin binding as well as regulating components in the ESR1 cistrome, Nagajaran *et al.* reported that ARID1A is a part of the ERα transcriptional complex where it acts as a repressor.

Signal rewiring is another important mechanism to consider. There are myriad examples in the literature, across multiple cancer types, of signal rewiring facilitating resistance to various therapies. In the case of HER2-expressing tumors (HER2+) there are a wealth of studies indicating that targeted inhibition of either HER2 or ERα may enrich signaling via the other (Gutierrez *et al.* 2005; Lipton *et al.* 2005; Munzone *et al.* 2005; Massarweh *et al.* 2006; Creighton *et al.* 2008), thereby attenuating the efficacy of the therapy. Pre-clinical studies have also highlighted a potential role for several receptor tyrosine kinase pathways in endocrine resistance development (Frogne *et al.* 2009; McClaine *et al.* 2010; Turner *et al.* 2010; Fox *et al.* 2011). Moreover, immunohistochemical stains of breast tumor samples often indicate that the PI3K/AKT/mTOR pathway is associated with endocrine therapy resistance (Kirkegaard *et al.* 2005). In these cases, enrichment of the PI3K-AKT pathway may enable ER+ breast cancer cells to retain their proliferative phenotype in the presence of antiestrogens (Miller *et al.* 2010). Signal rewiring can present cancer cells with mechanisms to negate or nullify the intended effect of cancer therapies, however it is important to remember that signal rewiring also plays a role in cancer more broadly. The sustained proliferative and survival signaling that we associate with a cancer cell is often underpinned by abnormalities in a cell's phosphorylome, brought on by dysregulation of kinases and phosphatases. Over the years, kinase inhibitors have proved to be one of the most popular classes of drug molecules, with 37 having been approved by the FDA for use in patients with cancer of various types (Bhullar *et al.* 2018).

Table 1: Surrogate intrinsic breast cancer subtypes and their features (Harbeck *et al.* 2019; National Cancer Institute 2020; Sakach *et al.* 2022). ERα, estrogen receptor α; HER2, receptor tyrosine-protein kinase erbB-2; NST, no special type; PARP, poly ADP-ribose polymerase; PR, progesterone receptor.

	Luminal A-like	Luminal B-like HER2–	Luminal B-like HER2+	HER2-enriched (non-luminal)	Triple-negative
ERα expression	+++	++	+	-	-
PR expression	+++	++	+	-	-
HER2 expression	-	-	+	++	-
Commonly associated histotypes	NST, tubular cribriform, classic lobular	NST, micropapillary, lobular pleiomorphic	NST and pleiomorphic	NST	NST, metaplastic, adenoid cystic, medullary-like, secretory
Five-year survival rates in USA (2013-2016)	95		91	86	77
Chemotherapy	Depending on risk of recurrence		Neoadjuvant		Neoadjuvant or adjuvant
Preferred treatment alongside surgery and chemotherapy	Adjuvant endocrine therapy If metastatic, combine with CDK4/6 inhibition		HER2-blocking antibodies		Non-responders may receive 1) platinum chemotherapy drugs 2) PARP inhibitors 3) immunotherapies 4) Trop-2-directed antibody and topoisomerase inhibitor
Population prevalence (%)	60 – 70	10 – 20	13 – 15		10 – 15
Highest risk ethnic group and frequency within that group	Woman of European descent (~75%)		Woman of Asian descent (~16%)	Women of Hispanic descent (~7%)	Women of African descent (~28%)
Grade	+	++	+++	++++	+++++

2.2 PIM kinases

2.2.1 History

The proviral integration site for Moloney murine leukemia virus (PIM) kinase family consists of three serine/threonine kinases (PIM1, PIM, PIM3). The first PIM family gene (PIM1) was discovered in mouse lymphoma DNA as an integration site gene for the mink cell focus-forming leukemia virus by Cuypers *et al.* (1984). Subsequent studies in mouse lymphomas showed that the 5' and 3' untranslated regions (UTR) of PIM1 often contained proviral insertions and that this usually occurred in conjunction with elevated levels of PIM1 mRNA (Selten *et al.* 1985; Selten *et al.* 1986). PIM2 was discovered in 1989 as another proviral integration site in mouse lymphoma (Breuer *et al.* 1989), followed by PIM3 in 1998 from pheochromocytoma cells as a gene encoding a kinase that is induced by depolarization or treatment with forskolin (Feldman *et al.* 1998). Since their discovery, these kinases have been studied by researchers across the world. Numerous substrates have been identified (Figure 1) and a PubMed search for "PIM kinase" yields 1414 results as of February 2024.

2.2.2 Genetics and biochemistry

The PIM1 protein is expressed as one of two isoforms, PIM1-Long (PIM1L) or PIM1-Short (PIM1S), which are differentially translated from the PIM1 gene depending on the translation initiation site (Saris *et al.* 1991). PIM2 mRNA contains 3 different translation initiation sites, which enable PIM2 to be expressed as one of three isoforms (Van Der Lugt *et al.* 1995), while PIM3 has only been characterized as a single isoform (Qian *et al.* 2005). Considering the shortest isoforms of PIM1 and PIM2, the amino acid sequences of PIM1 and PIM2 are 53.7% homologous, PIM1 and PIM3 are 66.6% homologous, PIM2 and PIM3 are 54.9% homologous.

PIM kinases (PIMs) are constitutively active serine/threonine kinases and their influence in a cell is directly correlated with their expression level (Qian *et al.* 2005). X-ray crystallography has shown that PIM1 possesses a conserved catalytic domain but does not contain a regulatory domain (Qian *et al.* 2005), a distinctive feature that grants PIMs their unusual characteristics. Recombinant PIM1 generated in *Escherichia coli* has been shown to autophosphorylate at Ser-8 and Ser-261 (Bullock *et al.* 2005; Jacobs *et al.* 2005), however it is unclear what role, if any, this plays in regulating PIM activity. Indeed, there is little evidence of PTMs on PIMs themselves in the literature, which partly explains the prevailing dogma that PIMs are only regulated at the level of transcription and expression. Cytokines and growth hormones, including IFNα, IFNγ, IL-2, IL-3, IL-7, IL-12, IL-15, erythropoietin,

prolactin, leukemia inhibitory factor, and granulocyte-macrophage colony-stimulating factor, are regulators of PIM1 expression (Dautry *et al.* 1988; Sato *et al.* 1993; Miura *et al.* 1994; Buckley *et al.* 1995; Yip-Schneider, Horie & Broxmeyer, 1995; Matikainen *et al.* 1999; Malinen *et al.* 2013; Mary Photini *et al.* 2017; James *et al.* 2021). The JAK/STAT pathway mediates this process, and its inhibition causes a notable reduction in the levels of all PIM kinase family members (Szydłowski *et al.* 2017).

Due to the limited evidence of PTMs on PIMs, our understanding of their impact is poor. There are no examples of PTMs regulating PIM subcellular localization, and while protein phosphatase 2A (PP2A) has been shown to regulate PIM stability, and to dephosphorylate PIM3, it is unclear in the study how direct the link between dephosphorylation and degradation is (Losman *et al.* 2003). A pair of more recent papers have challenged the notion that PIM activity cannot be regulated post-transcriptionally. In 2017, Iyer *et al.* performed some elegant *in vitro* experiments in which they demonstrated that SUMOylation both increases PIM1 catalytic activity and increases the rate of protein turnover. Shortly after this, Takami *et al.* (2018) reported that protein kinase C enhances the catalytic activity and stability of PIM1L by phosphorylating Ser-65. Therefore, although PIMs are unusual in their constitutive activity, it is likely that PTMs regulate their structure and function in a manner that is consistent with our understanding of molecular biology.

Functional redundancy among PIM isoforms has been observed in various *in vitro* and *in vivo* settings (Mikkers *et al.* 2004; Bullock *et al.* 2005; Mukaida *et al.* 2011; Narlik-Grassow *et al.* 2012; Saurabh *et al.* 2014). This is something that we have encountered in our own research with most substrates; *in vitro* kinase (IVK) assays with recombinant PIM proteins and putative substrates usually show that the different PIMs do not differ in terms of substrate specificity. However, there are exceptions, such as the substrate CXCR4, which is phosphorylated by PIM1 and PIM3 but not PIM2 (Santio *et al.* 2015). Furthermore, differences in chromosomal location and tissue expression distribution hint at discrete roles for these enzymes. Chromosome 6, the X chromosome, and chromosome 22 are the respective locations of the PIM1, PIM2, and PIM3 genes in the human genome. Taken together, these reports suggest that a potential blind spot in this research area is a tendency to consider the PIMs as a singular entity. The field may benefit from thinking about these kinases with more nuance.

The PIM1 consensus sequence as defined by Friedmann *et al.* (1992) is the following: (K/R)3-X-S/T*-X', where X' is neither a basic nor a large hydrophobic residue. This initial definition was then updated; to (K/R)2-R-K/R-L-S/T*-X, where X represents a small chain residue (Palaty *et al.* 1997), and it was later determined that PIM1 phosphorylates the following consensus sequence with 20-times more efficiency than the previously defined consensus sequence: R-X-R-H-X-S*, where

X represents any amino acid (Peng *et al.* 2007). PIM2 was shown to share this consensus sequence (Peng *et al.* 2007), but a consensus sequence for PIM3 is yet to be published. Owing to the overlaps in substrate specificity between the PIM kinases it seems likely that the PIM3 consensus target sequence is highly similar, if not identical, to the consensus shared by PIM1 and PIM2. Interestingly, the PIM consensus sequence is strikingly similar to the AKT consensus sequence, R-X-R-X-X-S*/T* (Obata *et al.* 2000), which in part explains why the AKT and PIM families co-regulate a number of signaling pathways, particularly in the context of cancer (Warfel and Kraft 2015). Another intriguing aspect of this topic is that PIM1's autophosphorylation site, Ser-261, is in fact a non-consensus site within the sequence R-Q-R-V-S-S*-E (Jacobs *et al.* 2005). This highlights the intrinsic flexibility of kinase-substrate targeting.

2.2.3 Healthy tissues

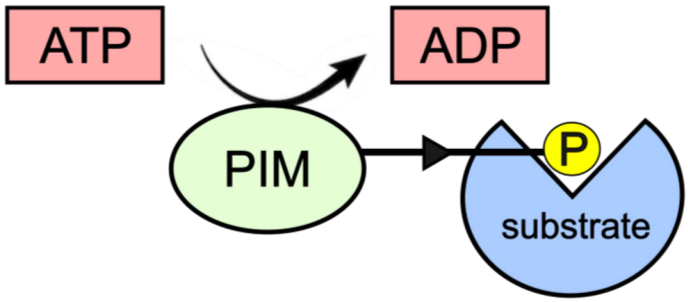
RNAseq data from The Human Protein Atlas [proteinatlas.org] shows that PIM family members are not highly expressed in any adult human tissues. However, moderate expression is observed in several tissues, with transcript levels ranging from 100 to 1000 transcripts per million (TPM).

- PIM1 is moderately expressed in adipose tissue (148 TPM), bone marrow (216 TPM), esophagus (196 TPM), skin (106 TPM), and vagina (110 TPM).
- PIM2 is moderately expressed in bone marrow (101 TPM), lymph nodes (111 TPM), spleen (160 TPM), and tonsils (102 TPM).
- PIM3 is moderately expressed in bone marrow (113 TPM), kidney (110 TPM), lung (104 TPM), skeletal muscles (108 TPM), skin (113 TPM), and stomach (115 TPM).

Despite our general understanding of PIM distribution across tissues, the roles of PIMs in healthy adult tissues are either physiologically limited, mostly inconsequential, or poorly understood, which is reflected in the complete lack of scientific review articles that are explore this area. In development, PIM function has been studied in humans, mice, and quails. The first point to note is that complete knockout of all PIM family members (PIM triple knockout or TKO) results in viable mice with a modest reduction in body size at birth and throughout life (Mikkers *et al.* 2004). Therefore, in spite of being needed to avoid developmental abnormalities, PIMs are not indispensable in mice. The same study noted abnormalities in B cell differentiation and T cell survival, which is consistent with numerous earlier studies that linked PIMs to various hematological processes. PIM1 expression exhibits notable enrichment in hematopoietic tissues and testes in mice and humans, and in hematopoietic cells various cytokines have been shown to stimulate PIM1

expression (Meeker *et al.* 1987; Dautry *et al.* 1988; Sorrentino *et al.* 1988; Amson *et al.* 1989; van Lohuizen *et al.* 1989; Wingett *et al.* 1992; Matikainen *et al.* 1999). Immunohistochemical analysis during human fetal hematopoiesis indicates that PIM1 is robustly expressed in the liver and spleen, whereas in adults, it is far more difficult to detect (Amson *et al.* 1989). In adult mice, PIM2 has been reported to be coexpressed with PIM1 in the spleen, thymus and bone marrow (Allen *et al.* 1997; Eichmann *et al.* 2000), while in mice embryos the expression pattern of PIM2 aligns more closely with PIM3; the two can be detected in the epithelia of all tissues (Eichmann *et al.* 2000). In a developing quail embryo, the quail PIM3 homologue displays a ubiquitous pattern of expression that is not limited to few tissues (Eichmann *et al.* 2000).

Alongside its reported roles in regulating B cell differentiation and T cell survival, two studies have reported that PIM regulates erythrocyte size *in vivo* (Larid *et al.* 1993; Mikkers *et al.* 2004). Additionally, PIMs may not just have a role in regulating T cell survival, but also in regulating T cell activation, owing to the fact that PIM1 is reportedly upregulated during T cell activation (Wingett *et al.* 1996). PIM1-overexpressing mice have enlarged spleens and are more susceptible to hematologic malignancies (a facet that will be covered in more detail in the next section), while bone marrow-derived mast cells from PIM1-deficient mice display an impaired ability to grow in response to interleukin-3 (Domen *et al.* 1993). A later study provided a detailed characterization of hematopoietic abnormalities in adult TKO mice, providing compelling evidence that PIM activity has roles beyond development, for example as an important factor in governing elements such as stem cell renewal and expansion (An *et al.* 2013). Thus, our understanding of the roles of PIMs in a healthy organism are largely pieced together based on observational findings in PIM-deficient or -overexpressing embryos and postnates. The difference between PIM kinase levels in embryos and in postnates strongly suggests that PIM expression is important in developmental biology, particularly given that PIM activity is regulated at the level of expression.



Activity/stability

Physiological process	Promoted	Compromised	Altered
Apoptosis	MDM2	MAP3K5, BAD, BCL2L11	
Cell cycle	CHEK1, CDC25A/C	MARK3, CDKN1A/B	
Chemokine receptors	CXCR4		
Efflux proteins	ABCB1, ABCG2		
Hormone receptors			AR
Lymphoid signaling	SOCS1/3, FLT3		
Metabolism	LDHA	GSK3B, AKT1S1	
Mitosis	CBX3, NUMA1		
Motility		CAPZA1, CAPZB	
Transcriptional regulators	MYBL1, MYB, MYC, H3-3A, NFATC1, NOTCH1, NFKB2, RELA, RUNX1, XBP1	FOXO1, FOXO3, FOXP3, NOTCH3, RUNX3	
Translation	EIF4B, SND1	TSC2, EIF4EBP1	
Other	GBP1, NKX3-1, MAP3K8, NOS3	MAP3K7	YWHAZ

Figure 1: PIM kinases exert their influence in a cell by phosphorylating target substrates. Numerous substrates have been reported, with phosphorylation affecting substrate output or stability in various ways. Substrates are listed above using gene nomenclature. Note: “promoted”, “compromised”, and “altered” refer to substrate activity/stability, not to the associated physiological process. (Information compiled from the following sources: Santio *et al.* 2017; Santio *et al.* 2020; Bellon and Nicot 2023; Fisch *et al.* 2023; Ma *et al.* 2023; Mung *et al.* 2023)

2.2.4 PIMs in cancer

The relationship between PIM overexpression and cancer is unsurprising given that PIMs are constitutively active kinases with close ties to cell proliferation and survival. PIMs are well-known drivers of hematological malignancies, which is consistent with what is known about their tissue distribution and function in healthy organisms (Bellon and Nicot 2023). PIMs are also known to support some solid

cancers, in which context they have been linked to neoplasms in tissues of the breast, brain, gastrointestinal tract, lung, ovary, and prostate, amongst others (Nawijn *et al.* 2011; Santio and Koskinen 2017).

Numerous studies indicate that, on their own, PIMs may not be enough to trigger or support cancer, however in cooperation with other oncogenes PIMs can have a synergistic effect. Examples of PIMs working in synergy with other factors include with: c-myc, N-myc, bcl2, bcl6, runx2, E2a-Pbx1, and Frat 1 in T cell lymphomas (van Lohuizen *et al.* 1989; Berns 1991; Shinto *et al.* 1995; Allen *et al.* 1997; Feldman *et al.* 1997; Jonkers *et al.* 1997; Blyth *et al.* 2001; Baron *et al.* 2012); c-myc, bcl2, and bcl6 in B cell lymphomas (Acton *et al.* 1992; Shinto *et al.* 1995; Allen *et al.* 1997; Baron *et al.* 2012); c-myc in pre-B cell leukemia (Verbeek *et al.* 1991); c-myc in prostate cancer (Kim *et al.* 2010; Wang *et al.* 2010; Wang *et al.* 2012); and c-myc in triple negative breast cancer (Brasó-Maristany *et al.* 2016; Horiuchi *et al.* 2016).

2.2.5 PIMs in cancer therapy resistance

PIMs have been linked to cancer therapy resistance in various settings, where they confer resistance by at least three routes: regulating drug efflux pumps, promoting survival pathways, and inhibiting apoptosis (Malone *et al.* 2020). The different treatment modalities against which PIMs can facilitate resistance are numerous and include chemo-/radiotherapy and targeted inhibition of the PI3K/AKT/mTOR pathway, angiogenesis, the HER2/epidermal growth factor receptor (EGFR) pathway, and hepatocyte growth factor receptor. In this section of the thesis, I will provide a brief overview of PIMs in therapy resistance with no particular focus on the class of therapy or type of cancer.

ATP-binding cassette super-family G member 2 (ABCG2) is a drug efflux pump that actively transports drug molecules out of a cell against a concentration gradient (Taylor *et al.* 2017). In their 2008 study, Xie *et al.* demonstrated that PIM-mediated phosphorylation of ABCG2 is essential for the protein to form a functional multimer that can localize to the plasma membrane. The authors also showed that in mitoxantrone and docetaxel-resistant prostate cancer cell lines the levels of PIM1L and ABCG2 are elevated and demonstrated that by knocking down PIM1L they could reduce cell surface expression of functional ABCG2, thereby restoring the sensitivity of these cells to chemotherapeutic agents. Subsequently, a team of researchers partially comprised of scientists involved in the previous study, showed that another efflux pump, ABC transporter P-glycoprotein (ABCB1), is also a PIM substrate and that phosphorylation protects ABCB1 from degradation (Xie *et al.* 2010). The study in question went on to show that the small molecule PIM inhibitor SGI-1776 sensitizes ovarian cancer cells to doxorubicin. Similar results have since been reported in leukemia cells, where SGI-1776 treatment causes a decrease in the

cell surface expression of ABCB1 and ABCG2 and thereby increases sensitivity to various chemotherapeutic agents (Natarajan *et al.* 2013), and in a panel of drug-resistant cancer cell lines in a study that utilized the second-generation PIM inhibitor molecule TP-3654 (Wu *et al.* 2021). Together these studies provide concise evidence of a direct mechanism via which PIMs influence drug molecule sensitivity.

In terms of survival, PIM1-mediated activation of nuclear factor κB (NF-κB) has been shown to initiate pro-survival pathways that rescue prostate cancer cells from docetaxel treatment (Zemskova *et al.* 2008). This occurs as a counterproductive consequence of docetaxel treatment, which promotes phosphorylation and activation of STAT3 leading to enhanced PIM1 expression. Shortly after this, early evidence for PIM involvement in radioresistance emerged in the context of head and neck squamocellular carcinoma (Peltola *et al.* 2009). The study reported that in radioresistant cells, PIM1 nuclear translocation was increased, and that this promoted cell survival via a mechanism that was not explored further. Subsequently, Kim *et al.* drew a direct line between PIMs and inhibition of apoptosis in their 2012 study on radioresistance of non-small cell lung cancer cells. The mechanism they describe is initiated by a dose of radiation that causes aberrant upregulation of PIM1 expression. This leads to increased phosphorylation of the PIM substrate PRAM40, which then sequesters the transcription factor FOXO3a in the cytoplasm, thereby preventing FOXO3a from initiating a pro-apoptotic transcriptional program. In a subsequent study they showed that sensitivity to radiation therapy could be restored by pharmacologically inhibiting PIMs (Kim *et al.* 2013). More recently, there has been interest in cancer stem cells, which are established mediators of therapy escape in many contexts (Zhou *et al.* 2021). PIM inhibition has, for example, been linked to a reduction in stemness of breast cancer cells of various subtypes (Liu *et al.* 2020), highlighting the fact that we are only just beginning to chart the complex territory of PIMs in cancer therapy resistance.

2.2.6 PIMs in breast cancer

Research into the roles of PIMs in breast cancer stretches back about twenty years, and in recent years there has been an explosion of interest in the topic. There are a wealth of papers looking into PIM's roles in the different molecular subtypes, its promise as a therapeutic target, and its overlap with various oncogenic processes and pathways. An early study showed that PIM1 is expressed in mouse mammary glands during development and that expression levels increase upon exposure to progesterone (Gapter *et al.* 2006). The same study also noted that in commercially available breast cancer cell lines, the level of PIM1 expression was higher as compared to normal epithelial breast cancer cells, hinting at a potential role for PIM in oncogenesis. As well as progesterone, PIM kinase expression is induced in breast

cancer cells following treatment with estradiol (Malinen *et al.* 2013; Santio *et al.* 2016a). In MCF-7 cells, PIM1 depletion by siRNA has been shown to hamper proliferation (Malinen *et al.* 2013). The same study looked at benign and malignant breast tissue samples and noted higher expression in malignant samples, and furthermore reported a correlation between PIM1 expression and tumor grade. PIM is also linked to enhanced tumorigenicity of MCF-7 cells via a mechanism in which PIM phosphorylates and thereby increases the activity of the context-dependent oncoprotein Notch1 (Santio *et al.* 2016a).

Other studies have branched out and explored PIM in ER- contexts. In TNBC cells, the micro-RNA (miRNA) miR-486-5p has been shown to suppress proliferation and promote cell cycle arrest and apoptosis by targeting PIM1 mRNA (Zhang *et al.* 2014). In HER2+ breast cancer PIM1 inhibition has been shown to reduce cell viability and decrease metastatic capacity by a mechanism that, at least in part, is due to a downregulation in HER2 expression (Wang *et al.* 2021). Likewise, in TNBC cell lines, PIM1 has been shown to increase growth and migration, but only in cooperation with MYC (Maristany *et al.* 2013), a finding which was corroborated in a later study that showed that PIM inhibition reduced the growth of MYC-driven TNBC xenografts (Horiuchi *et al.* 2016). PIMs have been linked to chemoresistance in various contexts, making it unsurprising that PIM inhibition has been shown to sensitize TNBC cell lines and xenografts to chemotherapeutic agents (Brasó-Maristany *et al.* 2016). Other studies have reported that inhibition of PIM2 alone with PIM2-specific inhibitors, HJ-PI01 or HS140, is enough to promote apoptosis of TNBC cells and inhibit tumor xenograft growth in mice (Y.-Q. *et al.* 2016; Cobb *et al.* 2017). In spite of several lab-based experiments identifying a role for PIMs in TNBC, it may be that in patients, PIMs are rarely overexpressed. One study examined 141 TNBC patient samples and found that only 15 expressed PIM1 (Ntzifa *et al.* 2021), suggesting that PIM1-expressing TNBC tumors may be an uncommon occurrence.

PIMs are becoming increasingly associated with cell motility (Santio and Koskinen 2017), which may in part explain the reports that have surfaced linking PIMs to the metastatic cascade in breast cancer. One study reported that PIM2 expression promotes epithelial-mesenchymal transition (EMT) in TNBC cells by initiating a positive feedback loop with STAT3, which ultimately results in activation of ZEB1 (Uddin *et al.* 2015). Another study looking at luminal A breast cancer cell lines showed that interleukin-6 (IL-6) promotes EMT and stemness features via a mechanism that depends on PIM1 and c-MYC (Gao *et al.* 2019). PIM inhibition has also been recommended as a means of enhancing the effectiveness of immunotherapies in breast cancer patients. Jiménez-García *et al.* (2017) reported that overexpression of PIM1 or PIM2 in the breast tissue of transgenic mice results in an increased occurrence of breast tumors, a large inflammatory response, and an

increased presence of cancer stem cells as compared to tumors with normal levels of PIMs. Furthermore, PIM inhibition in combination with immune checkpoint blockade has recently been shown to have antitumor activity in models of TNBC. The same study also showed that PIM inhibition leads to a decrease in the level of the immunosuppressive cytokine S100A8/A9, which has recently been associated with breast cancer progression (Begg *et al.* 2023; Zhang *et al.* 2023). Moreover, PIM inhibition has been shown to work synergistically with the inflammatory cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) to combat growth in a murine breast cancer model (Anwar *et al.* 2024). These studies point to, as yet untapped possibilities for PIM inhibitors in the field of immuno-oncology and metastasis therapy.

2.2.7 PIM inhibitors: is a combinatorial approach inevitable?

Combinatorial approaches towards cancer treatment can lead to favorable patient outcomes. By targeting a single entity or pathway, there is the risk that the patient will derive no therapeutic benefit owing to the ability of overlapping or distinct molecular pathways to compensate. By targeting two or more pathways simultaneously, one can inhibit the original and compensatory pathway(s), thereby dramatically improving the effectiveness of a treatment in a phenomenon that is referred to as synthetic lethality (Chen *et al.* 2023b). Combinatorial treatments involving PIM inhibitors are under investigation for this very reason. Moreover, given that PIM is usually a weak oncogene that must work cooperatively with other oncogenes, combined inhibition along with another oncogenic pathway is considered a rational approach.

A triple-action inhibitor, that targets PIM, PI3K, and mTOR (IBL302 or AUM302) was developed owing to the fact that PIM kinase levels are often elevated in breast tumors treated with PI3K inhibitors. Kennedy *et al.* (2020) showed that the compound is efficacious in multiple breast cancer cell lines of different subtypes both *in vitro* and in *in vivo* xenografts. Combined PIM/PI3K/mTOR inhibitor molecules have since garnered further interest from academia and industry (Martínez-González *et al.* 2021). Other examples include the cyclin-dependent kinase (CDK) 4/6 inhibitor abemaciclib, which has been shown to target PIM kinases as well [CDK4 K_i (nM) = 0.07; CDK6 K_i (nM) = 0.52; PIM1 K_i (nM) = 7.7; PIM2 K_i (nM) = <100; PIM3 K_i (nM) = 8.5 (Chen *et al.* 2016a)]. A study using ER+ cell lines showed that abemaciclib works synergistically with a PI3K inhibitor to suppress proliferation (Gelbert *et al.* 2014; Litchfield *et al.* 2020). Another study reported efficacy of a combined PIM and CK2 inhibitor in luminal A and HER2-overexpressing cell lines (Koronkiewicz *et al.* 2022). Converse findings have been reported about the potential of using PIM inhibitors and proteasomal inhibitors in

combination. While one study reported synergy in TNBC cells (Kunder *et al.* 2022), another reported that PIM2 inhibition alone was enough to induce apoptosis, and that its efficacy was in fact reversed if the proteasome was inhibited at the same time (Katsuta *et al.* 2022). The latter study utilized the pan-PIM inhibitor JP11646 and found that treatment with this inhibitor induced proteasomal-dependent degradation of PIM2 but not PIM1 or PIM3. By inhibiting the proteasome in this context, the authors could protect PIM2, the responsible oncogene, from degradation. Together these reports show that combinatorial approaches with PIM are indeed a promising avenue, and that suitable combinations can be planned rationally, but are occasionally stumbled upon serendipitously.

2.2.8 PIM inhibitors in the clinic

Given that complete knockout of all PIM family members results in viable mice (Mikkers et al. 2004), researchers have generally expected pan-PIM inhibitors to be relatively safe. However, it is important to note that this line of thinking overlooks the contrast between the absence of PIMs during development and their abrupt inhibition in a PIM-expressing adult; the two phenomena are not directly comparable. Nonetheless, several pan-PIM inhibitors have been, or are currently under clinical investigation, however as of February 2024 no intentional PIM-targeting therapies have reached the market. This is an important distinction to make, as it is known that, for example, the FDA-approved CDK 4/6 inhibitor abemaciclib targets PIM kinases (Gelbert *et al.* 2014; Litchfield *et al.* 2020) – an unintended but potentially useful off-target effect with this molecule. Indeed, abemaciclib is currently in clinical trials again for use in combination with sunitinib in metastatic renal cell carcinoma. In the trial description PIM1 is acknowledged as an intended target (NCT03905889). Given the data surrounding abemaciclib and the knowledge that kinase inhibitors are seldom entirely specific, it seems probable that PIM kinases also fall under the list of off-targets for other clinically available drugs.

A majority of the clinical trials involving PIM inhibitors have tested their safety and efficacy in patients with hematological malignancies. ClinicalTrials.gov includes 20 entries for clinical trials involving PIM-targeting drugs as of February 2024. The first to be tested in humans was Astex Pharmaceutical's 1st generation PIM inhibitor SGI-1776, which entered phase I trials for refractory prostate and refractory non-Hodgkin's lymphoma (NHL) (NCT00848601), and relapsed/refractory leukemias (NCT01239108). Despite showing efficacy, the drug was withdrawn owing to adverse cardiac events. AZD1208, developed by AstraZeneca, entered a phase I clinical trial for patients with acute myeloid leukemia (AML) in 2015 (NCT01489722), although this trial was terminated owing to a poor efficacy and safety profile. The same year AZD1208 was tested in a phase I trial for

advanced solid tumors and malignant lymphoma (NCT01588548), however the adverse events recorded during this trial sealed the fate for this drug and its primary purpose since has been as a research tool in a laboratory setting for PIM researchers. Incyte's pan-PIM inhibitor INCB053914 has completed phase I trials for relapsed/refractory diffused large B-cell lymphoma (NCT03688152) and is currently in phase I trials for relapsed/refractory multiple myeloma (MM) (NCT04355039), although the drug's performance in these trials is currently not public knowledge. INCB053914 has also entered combined phase I/II trials for patients with advanced hematologic malignancies (NCT02587598) and relapsed/refractory multiple myeloma (NCT04355039). Results are yet to be published for the latter, but in the former, response to INCB053914 was extremely limited (Patel *et al.* 2023a). Novartis' PIM inhibitor LGH447 has been tested in a number of indications in phase I trials including myelofibrosis (NCT02370706), relapsed/refractory MM (NCT01456689, NCT02144038, NCT02160951), and AML or high risk myelodysplastic syndromes (NCT01456689). In two of the relapsed/refractory MM trials, the frequency of hematologic adverse events was high, however the clinical benefit rate was shown to be approximately 25% (Raab *et al.* 2019; Iida *et al.* 2021). Other PIM inhibitors in phase I or I/II trials include: Shengke Pharmaceutical's ETH-155008 for AML and NHL (NCT05758610), B-cell NHL, chronic/small lymphocytic leukemia, and AML (NCT04840784) for which results are not yet available; Menarini's dual PIM/FLT3-targeting therapy SEL24/MEN1703 for AML (NCT03008187), with early data suggesting the drug has an acceptable safety profile (Martinelli *et al.* 2022) and; Sumitomo Pharma's TP-3654 in myelofibrosis (NCT04176198) and solid tumors (NCT03715504). In myelofibrosis, early data suggests that TP-3654 treatment results in a reduction in spleen volume and promising changes in patients' cytokine profiles, with a tolerable safety profile (El Chaer *et al.* 2023), while in the trial for solid tumors, there was no issue with dose-limiting toxicity and a maximum tolerated dose was not reached (Garrido-Laguna *et al.* 2020). In summary, in spite of early issues with safety, PIM inhibitors continue to be developed and tested mainly in hematologic cancers, with a few exceptions. The next generation of inhibitors appear to have an improved safety profile, and interest has shifted towards the potential of PIM inhibition in combination with other treatments.

2.3 Notch3

2.3.1 Notch signaling

In 1914, John S. Dexter observed a unique notch in the wings of a mutant in the Drosophila melanogaster strain that he was studying (Dexter 1914). Although, at the time, the existence and function of Notch proteins was unknown, Dexter had stumbled upon one of nature's most ancient and conserved signaling pathways. Later in the 20th century, it became clear that the mutant phenotype described by Dexter was the consequence of a Notch mutation, and his description of "perfect notched wings" inspired the name of the receptors in this protein family.

The Notch receptor family comprises four cell surface receptors (Notch1/2/3/4) that play pivotal roles in tissue development and homeostasis in all metazoan species. Notch receptors, and their ligands, are membrane proteins, and currently the model mechanism for canonical Notch signaling is as follows. When a Notch ligand, which is expressed on a neighboring cell, binds to the receptor and induces a conformational change, it thereby reveals an ADAM (A Disintegrin And Metalloproteinase) cleavage site which is predominantly cleaved by ADAM-10 and ADAM-17 (Brou *et al.* 2000; Mumm *et al.* 2000; Parks *et al.* 2000). This event, known as site 2 (S2) cleavage, precedes S3/S4 cleavage by gamma-secretase (De Strooper *et al.* 1999). Following these proteolytic cleavage events a truncated N-terminal intracellular domain of the Notch protein (NICD) is released into the cytoplasm and subsequently translocates to the nucleus where it can bind to C protein binding factor 1/Suppressor of Hairless/Lag-1 (CSL) and mastermind-like (MAML), forming a complex that regulates transcription (Fortini and Artavanis-Tsakonas 1994; Schroeter *et al.* 1998; Struhl and Adachi 1998; Wu *et al.* 2000) (Figure 2). In the absence of Notch, CSL is bound to transcriptional co-repressors and inhibits the transcription of Notch target genes, however, when NICD displaces these co-repressors, the resulting transcriptional complex activates target gene expression (Bray 2006).

In addition, Notch has been shown to signal via mechanisms that do not require Notch ligands or CSL. Any Notch signaling that deviates from the canonical pathway is known as non-canonical Notch signaling. A numerous and ever-growing list of non-canonical mechanisms are present in the literature (Andersen *et al.* 2012; Zhou *et al.* 2022), highlighting the fact that, in spite of some impressive breakthroughs in the Notch field, we have only scratched the surface of this complex and fascinating pathway. Moreover, it is important to remember that, in spite of overlap between members of the Notch family, in humans there are four distinct Notch receptors that possess unique signaling roles and different signaling capacities.

During development, Notch is a vital mediator between adjacent cells, enabling feedback mechanisms that facilitate differentiation and tissue specification. There are several reviews that detail the roles of Notch in, for example, neurogenesis (Engler *et al.* 2018), angiogenesis (Akil *et al.* 2021) and T cell development (Brandstadter and Maillard 2019), where the pathway plays an indispensable role. Indeed, in mice, knockout of Notch1 or Notch2 results in embryonic lethality (Swiatek *et al.* 1994; Conlon *et al.* 1995; Hamada *et al.* 1999), while knockout of Notch3 or Notch4 results in severe vascular developmental defects (Krebs *et al.* 2000; Domenga *et al.* 2004; James *et al.* 2014). Beyond development, Notch is important in tissue homeostasis. Notch assumes a critical role in tissue repair in both the liver (Köhler *et al.* 2004; Jörs *et al.* 2015; Lu *et al.* 2016) and skeletal muscle (Tran *et al.* 2013) and regulates the phenotype of vascular smooth muscle cells (Wang *et al.* 2003; Sweeney *et al.* 2004; Morrow *et al.* 2005; Proweller *et al.* 2005).

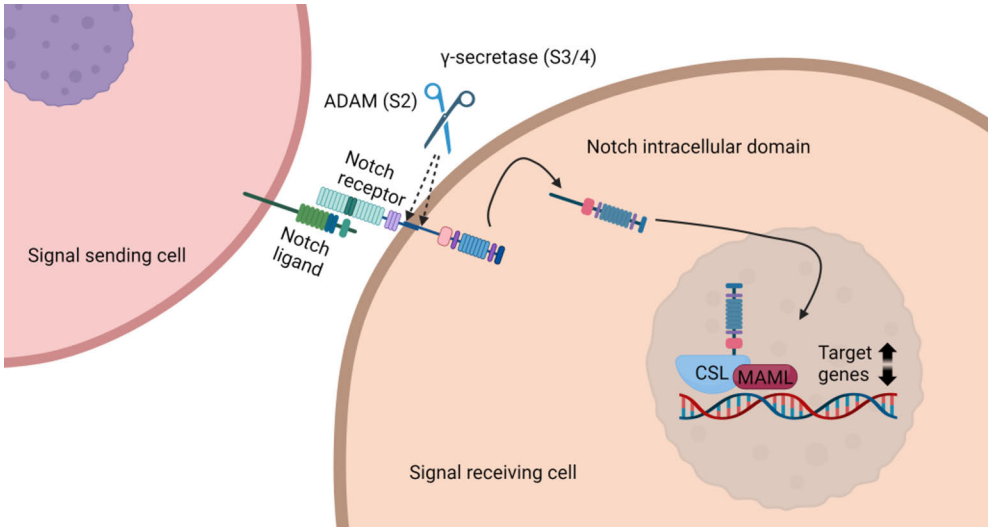


Figure 2: Canonical Notch signaling involves the interaction between a Notch receptor and its ligand, leading to receptor cleavage, release of the intracellular domain, and transcriptional activation of target genes. (Created with BioRender.com)

2.3.2 Notch post-translational modifications

To add further complexity to an already intricate signaling pathway, Notch post-translational modifications (PTMs) play a crucial role in fine-tuning its regulation.

It is becoming increasingly clear that Notch activity is modulated by PTMs of many different flavors. There are reports of Notch being the target of acetylation, glycosylation, hydroxylation, methylation, phosphorylation, SUMOylation, and ubiquitination (Antfolk *et al.* 2019). Depending on the specific PTM and target-site in question, these PTMs regulate protein stability and post-translational processing, function, localization, and interaction with other proteins. Indeed, research preceding my PhD in the Koskinen and Sahlgren labs showed that Notch1 and Notch3 are both PIM substrates (Santio *et al.* 2016a). The study showed that Notch1 is phosphorylated at serine-2162, in the second nuclear localization sequence (NLS) of the protein, and that as a consequence nuclear translocation is enhanced. This leads to increased Notch1-regulated transcriptional activity, which, in the context of this study, enhanced prostate cancer cell motility.

2.3.3 Notch in cancer

Given the multitude of processes linked to Notch, it is unsurprising that dysregulation of Notch signaling can result in many diseases. In cancer, the effects of Notch depend on the type of malignancy, the tumor microenvironment, the signal dose, the receptor paralog, and likely many other factors that remain unexplored. In short, the infamous catchall phrase "context-dependent", very much applies to Notch in cancer, meaning that it is essentially useless to try and generalize the effects of Notch to "cancer on", or "cancer off". This point is concretized rather effectively by Aster *et al.*, in their review of Notch in cancer (2017), in which Notch is linked to all 10 of the 2011 edition of cancer hallmarks (Hanahan and Weinberg 2011). Notch promotes 6 of the 10 cancer hallmarks, inhibits 3, and can promote or inhibit the remaining hallmark depending on the physiological context (Aster *et al.* 2017). In spite of these seemingly oxymoronic signaling outputs, Notch drugs for targeted cancer therapy have caught the imagination of many. The pathway consists of many sequential steps, and as such there are many different blocking strategies. γ-secretase inhibitors work under the premise that by preventing S3/S4 cleavage, one can prevent the translocation of active NICD to the nucleus, and thus prevent Notch-driven transcriptional regulation. These are the earliest forms of Notch inhibitors, many of which have entered clinical trials, and are used extensively in Notch research. These inhibitors do not come without their limitations. As well as being "pan-Notch" (S3/S4 cleavage is a requisite step for canonical signaling via all Notch receptor paralogs), these inhibitors can affect cleavage of more than 90 substrates (Majumder *et al.* 2021), which is a significant array of off-targets for a supposedly "targeted therapy". Nonetheless, the Notch field had cause for celebration at the tail end of 2023 when the γ-secretase inhibitor nirogacestat was granted FDA approval for use in desmoid tumors (U.S. Food and Drug Administration, 2023). As well as

being the first Notch-targeting drug to reach clinics, nirogacestat is the first targeted therapy for desmoid tumors. Other Notch targeting strategies include: γ-secretase modulation, which is achieved by molecules that reduce enzyme activity, usually via an allosteric mechanism (Golde *et al.* 2013); ADAM10/17 inhibition, which prevents S2 cleavage as a means of preventing Notch-driven transcriptional regulation; Notch receptor- or Notch ligand-targeting antibodies, which work by blocking signaling through specific paralogs, thereby offering increased specificity over γ-secretase and ADAM-targeting drugs; and transcriptional complex disrupters, which inhibit assembly of the Notch transcriptional complex, thereby suppressing Notch-driven transcriptional regulation without affecting non-canonical signaling mechanisms that occur outside the nucleus. Molecules from all these classes of Notch-targeting drugs are being or have been tested in clinical trials (Majumder *et al.* 2021).

2.3.4 Notch3 in breast cancer

The role of Notch3 in breast cancer has been challenging to elucidate, with some researchers believing that it is primarily oncogenic while others propose that it is a tumor suppressor. One study using mice that form tumors in response to cyclin D1 found that tumors that were high in Notch3 were more likely to resemble inflammatory breast cancer and more likely to metastasize (Ling *et al.* 2013). Another study in mice showed that Notch3 promotes proliferation of 4T1 murine mammary carcinoma cells by upregulating the CCL2/CCR4 signaling axis (Xiong *et al.* 2020). Meanwhile in breast cancer patients, a recent study looking at Notch3 and the Notch ligand Delta-like ligand 4 (DLL4) found that Notch3 and DLL4 expression was, on average, elevated, and that high expression levels of either correlated with a poor clinical outcome (Wang *et al.* 2023). Similar findings have been reported by researchers looking into the interplay between Notch3 and another Notch ligand, Jagged1 (Strati *et al.* 2017; Xue *et al.* 2017). In spite of this apparent interplay between Notch3 and its ligands, in a subset of basal breast cancers, Notch3 has also been shown to signal constitutively, independent of Notch ligands, and in this way support development and progression of the tumor (Choy *et al.* 2017). With regards to metastasis, Notch3 has been found to promote ER+ tumor cell colonizing lungs *in vivo*, and to promote TNBC metastasis to the brain, as well as supporting cancer cell self-renewal (Leontovich *et al.* 2018), and to promote metastasis to bone (Zhang *et al.* 2010).

Considering Notch3 targeting, the literature contains some interesting reports. One study showed that Notch3 knockdown with small interfering RNA (siRNA) *in vitro* increases sensitivity of TNBC cells to inhibition with the epidermal growth factor receptor inhibitor gefitinib (Diluvio *et al.* 2018). Another interesting study looked into the therapeutic capacity of flavonoids obtained from litchi seeds and

found that the flavonoids decreased the viability and growth of breast cancer cells, and inhibited mammosphere formation by attenuating stem-like properties of the cells. Furthermore, in mice, litchi flavonoids inhibited stem cell-linked tumor initiation. The authors propose that the effects of litchi flavonoids are, at least in part, achieved by inhibiting nuclear translocation of Notch3 and thereby Notch3-driven transcriptional regulation (Liao *et al.* 2023). Notch3-targeting drugs have even been used in patients with advanced, pretreated ER+ breast tumors during a phase I clinical trial assessing PF-06650808, a Notch3-specific antibody-auristatin conjugate. In this study, PF-06650808 had a positive efficacy and safety profile in patients with Notch3-positive tumors (Rosen *et al.* 2020).

As previously alluded to, a number of other studies have pointed to a tumor suppressive role for Notch3 in the context of breast cancer. The earliest of these, which utilized a variety of cell lines, reported reduced Notch3 expression levels in cancerous breast and melanoma cells, and showed inhibition of tumor growth in samples where Notch3 levels were restored to normal expression levels (Cui *et al.* 2013). Since then, potential Notch3-linked tumor-suppressing mechanisms have emerged. For example, through transactivation of the tumor suppressor phosphatase and tensin homolog (PTEN), Notch3 has been shown to inhibit breast cancer cell proliferation and tumorigenesis (Zhang *et al.* 2021). Other proposed mechanisms of Notch3-linked suppression of breast cancer cell proliferation and tumorigenesis include: inhibition of the cell cycle regulator Mybl2 (Brahim *et al.* 2023); enhanced ERα expression in ER- cells, a phenomenon that also decreased EMT (Dou *et al.* 2017); and by regulating apoptosis and mammary epithelial cell expansion (Chung *et al.* 2022). Multiple studies have also reported that Notch3 signaling in breast cancer inhibits EMT. One study has pointed to the involvement of miRNAs in blocking Notch3 expression (Liang *et al.* 2018), while another reported that inhibitor of DNA binding 2 (Id2) upregulates Notch3 (Wen *et al.* 2018), with the consequences being an increase or decrease in EMT respectively. Multiple mechanisms for Notch3-controlled suppression of EMT have been put forward, including activation of Kibra-mediated Hippo/YAP signaling (Zhang *et al.* 2016), transactivation of glycogen synthase kinase-3-beta (GSK3β) (Chen *et al.* 2022), increasing STAT5A expression (Chen *et al.* 2023a), increasing GATA-3 expression (Lin *et al.* 2018), decreasing ZEB1 expression (Chen *et al.* 2024), and regulating tight/adherens junction positioning (Tan *et al.* 2019).

Taken together, these conflicting reports make it seem likely that the role of Notch3 in breast cancer is contingent on a multitude of physiological and environmental factors. This challenges the pervasive notion that cancer-associated genes serve as either “oncogene” or “tumor suppressor”. While for some genes, the effect is indeed binary, for others the reality is more complicated. With regards to Notch3-research and therapeutic targeting, it is important to keep this in mind.

2.4 LKB1

2.4.1 LKB1 in heath and disease

Liver kinase B1 (LKB1), also known as serine-threonine kinase 11 (STK11), is an unusual kinase in that inactivating mutations are associated with carcinogenesis, as opposed to activating mutations. As previously discussed, for Notch, the boundary between oncogene and tumor suppressor is blurred. For LKB1 the vast majority of reports label it a tumor suppressor that, in cancer, is inactivated. Like Notch, however, a phenotype associated with the gene was already described in the early 20th century, but the responsible gene was not characterized until far later. The first cases of Peutz-Jeghers syndrome (PJS), a disorder in which patients display hyperpigmentation on their lips and oral mucosa along with benign polyps in their gastrointestinal tract, were reported by JT Connor in 1895 and subsequently by J Peutz in 1921 (Beggs *et al.* 2010). However, it was not until the late 1990s that loss of LKB1 function, owing to mutations in STK11, would be pinpointed as the underlying cause (Hemminki *et al.* 1997; Jenne *et al.* 1998; Mehenni *et al.* 1998). These findings opened up an entirely new field of investigation; the biochemical and physiological functions of LKB1 were explored, and loss of LKB1 function has since been identified as a primary driver in certain types of cancer.

LKB1 phosphorylates multiple substrates, including 5' AMP-activated protein kinase (AMPK), which was first reported twenty years ago (Hawley *et al.* 2003; Woods *et al.* 2003; Shaw *et al.* 2004), and of LKB1's substrates, has been the most extensively studied (Figure 3). It was subsequently discovered that LKB1 also phosphorylates 12 other closely related kinases, all belonging to the AMPK-related kinase (ARK) family (Lizcano *et al.* 2004). AMPK's function is to inhibit energy expenditure in a cell, which it achieves by driving a cell into a catabolic state, promoting processes such as glucose uptake, fatty acid oxidation, mitochondrial biogenesis, and autophagy. Conversely, anabolic processes such as protein and lipid synthesis are limited, with the end result being net gain of adenosine triphosphate (ATP) as opposed to ATP consumption (Hardie *et al.* 2012). It is through AMPK that LKB1 primarily suppresses tumor formation and growth. As levels of AMP rise and ATP drop, AMPK is activated, and LKB1-mediated phosphorylation of the catalytic α subunit in AMPK enhances enzyme activity 100-fold (Suter *et al.* 2006). Thus, in LKB1-deficient settings, there is an enrichment of anabolic pathways, driving unnecessary and unrestrained biosynthesis, owing to a reduction in AMPK efficiency. This phenotype is consistent with our understanding of a cancer cell. LKB1 also regulates cell physiology through its other substrates, the ARK family. For example, LKB1 activates the microtubule affinity regulating (MARKs) and brain selective kinases (BRSKs) via phosphorylation, thereby altering cellular

microtubule dynamics, which in turn impacts cell polarity (Kojima *et al.* 2007; Nakano and Takashima 2012). Through the novel AMP related kinases (NUAKs), LKB1 has been shown to inhibit G1/S transition in the cell cycle by a mechanism that depends on cyclin-dependent kinase inhibitor 1A (CDKN1A) and TP53 (Zeng and Berger 2006; Esteve-Puig *et al.* 2014). Furthermore, LKB1-mediated phosphorylation of the salt-inducible kinases (SIKs) is another prominent route via which LKB1 regulates cellular metabolism; in the liver, SIK phosphorylation leads to a reduction in the anabolic process of gluconeogenesis (Patel *et al.* 2014).

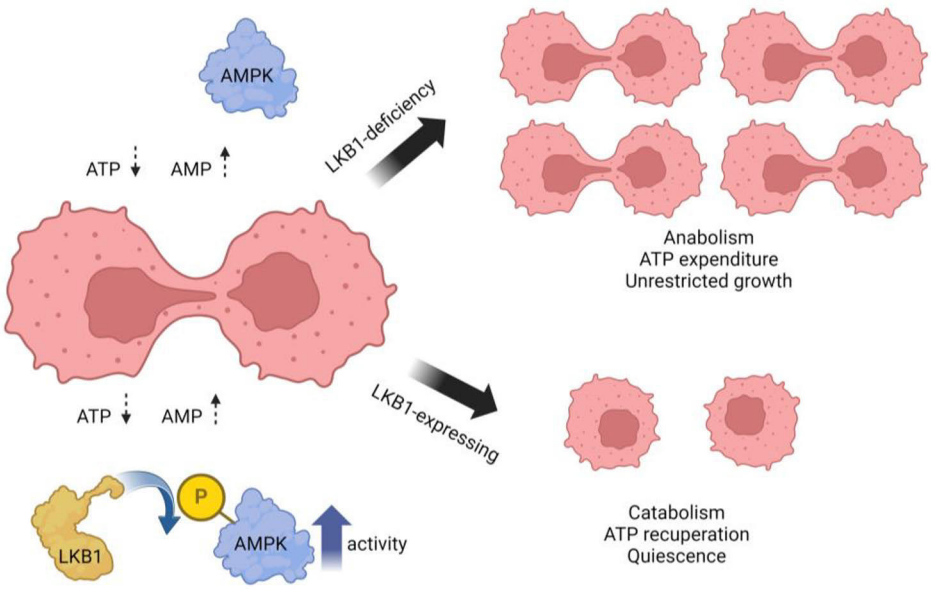


Figure 3: Liver Kinase B1 (LKB1) is primarily known for phosphorylating and thereby enhancing the activity of adenosine monophosphate-activated protein kinase (AMPK). AMPK drives a cell into a more quiescent phenotype. (Created with BioRender.com)

2.4.2 LKB1 the tumor suppressor

Somatic mutations in STK11 have been shown to be present in approximately 20% of cervical cancers (Wingo *et al.* 2009), and over 30% of lung adenocarcinomas (Ji *et al.* 2007; Matsumoto *et al.* 2007), a percentage that rises further to approximately 40% when non-small-cell lung cancer (NSCLC) is considered (Matsumoto *et al.* 2007). PJS is caused by a germline mutation and while rates of lung cancer can be detected amongst individuals with PJS at slightly elevated levels as compared to the rest of the population, in some respects it is surprising that the

risk of lunger cancer is not even higher. This may in part be explained by the fact that disease is autosomal dominant; patients possess a single mutated STK11 allele, and while one faulty allele is enough to cause PJS, two faulty alleles are generally required to drive oncogenesis. Moreover, cancer cells possess highly unstable and mutated genomes. In lung cancers with somatic LKB1 mutations, there is commonly co-occurrence of mutations in other cancer-associated genes, such as KRAS and TP53 (Caiola *et al.* 2018; Barta and McMahon 2019). Thus, although LKB1 is considered a tumor suppressor for good reason, deficiency is more likely to predispose an individual to developing certain types of cancer, rather than guaranteeing an individual develops cancer. Nonetheless, numerous LKB1-deficient tumor-forming mouse models, displaying tumors across many tissue types, have been described in the literature (Ollila and Mäkelä 2011).

2.4.3 LKB1-deficiency in breast cancer

PJS is known to increase breast cancer risk, with rates in women at aged 40 and 60 being 8% and 31% respectively (Hearle *et al.* 2006), which exceeds the rates observed in the general population. However, overall, it is unusual for breast cancer patients to have tumors displaying LKB1-deficiency. PJS itself is a rare disease, affecting approximately 1 in 120,000 births (Lindor and Greene 1998), and somatic mutations leading to breast cancer are far more likely to involve genes such as PIK3CA, ERBB2, GRB7, TP53, GATA, NOTCH2 (Harbeck *et al.* 2019). Initial findings suggested that LKB1 played no role in human breast cancer. The study in question reported that in 62 primary breast cancer cell lines, and 17 immortalized breast cancer cell lines, no somatic LKB1 mutations were detected (Bignell *et al.* 1998). Soon after this, however, another study reported that reintroducing LKB1 into breast cancer cell lines lacking LKB1 suppresses cell growth, and that in patient samples, low LKB1 expression correlated with higher histological grade, along with other unfavorable characteristics (Shen *et al.* 2002). Other *in vitro* studies have linked LKB1 loss in breast cancer to various adverse characteristics such as invasiveness and delayed DNA damage repair (Li *et al.* 2014; Wang *et al.* 2016). Likewise, LKB1 overexpression has been shown to drive breast cancer cells into acquiring a more epithelial phenotype, and to inhibit EMT, mammosphere formation, and expression of stem-cell-associated markers (Li *et al.* 2014; Avtanski *et al.* 2015).

Patient-based studies also paint a complex picture. One study reported that high grade HER2+ tumors frequently display LKB1 deficiency, a finding that they recapitulated in a subsequent *in vivo* study by showing that LKB1 loss in a HER2-enriched setting led to sporadic formation of metabolically active tumors, and that the severity of these tumors could be reduced through treatment with an mTOR

inhibitor along with 2-deoxy-D-glucose (Andrade-Vieira *et al.* 2013; Andrade-Vieira *et al.* 2014). One study including samples from almost 2000 patients and data pertaining to clinical outcome detected LKB1 in over 78% of patient samples but found no connection between LKB1 expression and various factors such as tumor size, lymph node status, and several biomarkers commonly associated with breast cancer (Syed *et al.* 2019). The authors did however find that for patients who had received endocrine therapy, their survival rate significantly improved if their tumors were positive for LKB1. Another study found that, without stratifying patients into separate molecular subtypes, LKB1 expression correlated with improved survival in breast cancers that had received chemotherapy (Nguyen *et al.* 2021). However, upon looking into the dataset in more detail, it became apparent that both the molecular subtype of the tumor and LKB1 substrate levels are important components of this complex mosaic. For example, NUAK2 expression is correlated with improved survival in ER- patients, however it correlates with reduced survival in ER+ patients (Nguyen *et al.* 2021). Meanwhile, another study looking into LKB1 expression, clinicopathologic factors, and clinical outcome amongst a cohort of Taiwanese breast cancer patients of various molecular subtypes detected improved survival amongst patients with LKB1-expressing HER2+ tumors, but not in any for breast cancer of any other molecular subtype (Chen *et al.* 2016b). Indeed, for other breast cancer subtypes, no clinicopathologic factors could be linked to LKB1 expression, except for a correlation that was detected between LKB1 and ERα expression. The potential link between ERα does not end here, indeed, it has been reported that LKB1 binds to ERα, and in doing so, enhances its transcriptional activity (Nath-Sain and Marignani 2009). This may help to explain why reports in the literature have differed when considering the role of LKB1 in ER+ and ER- breast cancers, and highlights the fact that, despite its "tumor suppressor" label, the truth of the matter is likely to be nuanced.

Another important question on the topic of LKB1 in breast cancer is, what is the importance of *in vitro* and *in vivo* studies if LKB1-deficiency rarely underpins breast cancer anyway? Breast cancer is heterogeneous, no two cases are the same, and by developing a more comprehensive understanding of the disease in all its forms we can hope to treat even those individuals that display a rare form of the disease with limited treatment options. Moreover, the broader societal impact and potential utility of basic research are often difficult to predict.

2.4.4 LKB1 post-translational modifications in breast cancer

PTMs regulate the function of LKB1 in various ways, and the literature contains reports of 4-hydroxynoneal adduction, acetylation, ADP-ribosylation, phosphorylation, prenylation, S-nitrosylation, sumoylation, and ubiquitination (Hu

et al. 2023). Broadly speaking, PTMs have been shown to affect LKB1 activity, subcellular localization, protein interactions, and stability. Phosphorylation, as is so often the case, has been studied in the most detail, and in the context of breast cancer two LKB1 phosphorylation events have been described. AKT phosphorylates LKB1 at serine-334, a PTM which has been reported in both MDA-MB-231 cells (a TNBC cell line) and in human embryonic kidney cells (Liu *et al.* 2012). In this instance, LKB1 phosphorylation promotes binding with 14-3-3, which results in LKB1 nuclear sequestration and consequently LKB1 activity being restricted. Cyclin D1 has been shown to phosphorylate LKB1 at serine-325 in MCF-10A cells that have been transformed with the oncogene NeuT (Casimiro *et al.* 2017). The authors also show that cyclin D1 knockdown with short hairpin RNA (shRNA) results in an increase in AMPK phosphorylation, and therefore hypothesize that by phosphorylating LKB1, cyclin D1 hinders LKB1 activity.

2.4.5 A more nuanced role for LKB1?

As a challenge to the dogma surrounding LKB1, the literature contains several examples of the LKB1-AMPK axis facilitating cancer hallmarks. For example, LKB1 overexpression and persistent activation facilitates cell survival when hepatocellular carcinoma cells are in a state of energy stress (Martínez-López *et al.* 2012; Lee *et al.* 2015). It has also been reported that LKB1 levels are enhanced in circulating tumor cells (CTCs), and that LKB1 mediates, at least in part, CTC intravasation (Trapp *et al.* 2017). Another study provides *in vitro* and *in vivo* evidence that a specific isoform of AMPK can contribute to colon tumor cell survival (Fisher *et al.* 2015). These reports beg the question - why is the role of LKB1 not as clear-cut as we initially thought? In their review, Ciccarese *et al.* (2019) discuss the idea that an LKB1-mediated switch to a less energetic phenotype may facilitate cancer cell survival under stress, such as energy or pharmacologically-induced stress. While a state of quiescence may serve to protect against intractable proliferation, it may promote survival under conditions when a cell may have otherwise perished, once again highlighting the need for "higher resolution" terminology when labelling proteins as either oncoproteins or tumor suppressors.

2.5 Estrogen receptor α

2.5.1 History

One-hundred years ago, Allen and Doisy reported that by injecting extracts of cow and pig ovaries into female rodents, they could prompt sexual activity (Fuentes

and Silveyra 2019). The responsible hormone in their extracts was, of course, estrogen, but it was not until the late 1950s that a receptor for this hormone would be discovered by Jensen (Jensen and Jacobson 1960; O'Malley and Khan 2013). Estrogen receptor α (ERα) would be cloned in the 1980s (Green *et al.* 1986; Greene *et al.* 1986), and estrogen receptor β (ERβ), the other estrogen nuclear receptor in humans, was cloned in the 1990s (Kuiper *et al.* 1996). We now know that estrogen signaling via ERα is essential for female reproductive maturation and the development of female secondary sex characteristics; however, breast cancer tumors that express ERα (ER+) depend upon the receptor for growth and progression. Approximately 80% of breast cancer tumors are ER+ (Giaquinto *et al.* 2022), and these can usually be treated effectively with endocrine therapies that work by blocking ERα signaling. However, the efficacy of estrogen blockers is limited in some patients due to intrinsic or acquired therapy resistance, as discussed in more detail in section 2.1.7.

2.5.2 Estrogens and their roles in the body

In conventional parlance, the term "estrogen" typically denotes estradiol, although this is something of a misnomer. An estrogen is in actual fact one of the four main naturally occurring female sex steroids, estrone (E1), estradiol (E2), estriol (E3), and estetrol (E4). The molecules are structurally identical but for a differing number of hydroxyl groups, a feature that informs the numbering used in their respective abbreviations. E1 serves mainly as a precursor to E2 and E3, is also a metabolite of E2, and a weak ERα agonist (Kuhl 2005). E3, also a weak ERα agonist, is synthesized in large quantities by the placenta during pregnancy, but outside of pregnancy is practically undetectable (Goodman 2003; Kuhl 2005). E4 is also only detectable during pregnancy owing to the fact that it is only synthesized by the liver of a developing fetus (Holinka *et al.* 2008). E2 is the strongest ERα agonist, and the primary workhorse in the estrogen signaling pathway (Kuhl 2005). E2 influences physiology in numerous ways, partly because ERα is expressed in most organs in the body, notably uterus, prostate, ovary, testes, epididymis, bone, breast, brain, and liver, as well as in white fat tissue (Dahlman-Wright *et al.* 2006). In females, ERα signaling regulates reproduction and the menstrual cycle, the maturation of breast tissue and sexual organs, and pregnancy-related changes in breast tissue architecture and function (Leung *et al.* 1976; Koos 2011; Yaghjyan and Colditz 2011; Hamilton *et al.* 2017). While these are some of the prominent and best-known examples of the estrogens' physiological roles, it is worth remembering that estrogen signaling can also regulate other bodily functions such as, somewhat surprisingly, male sexual function (Wibowo *et al.* 2011).

2.5.3 ERα signaling pathway

ERα is a type I nuclear receptor that is predominantly located in the cytoplasm of a cell in association with protein chaperones (Echeverria and Picard Didier 2010). E2, which can freely diffuse through plasma membranes (Oren *et al.* 2004), binds to ERα and in doing so liberates the receptor from its chaperone, enabling ERα to form a homodimer on which the nuclear localization sequence (NLS) is exposed, thereby promoting ERα translocation to the nucleus. In the nucleus, a complex of transcriptional regulators assembles with ERα, and in this way ERα drives the expression of certain genes, inhibits the expression of others, and ultimately influences cell physiology (Sever and Glass 2013) (Figure 4). This serves as a simple description of the canonical mechanism via which type I nuclear receptors signal.

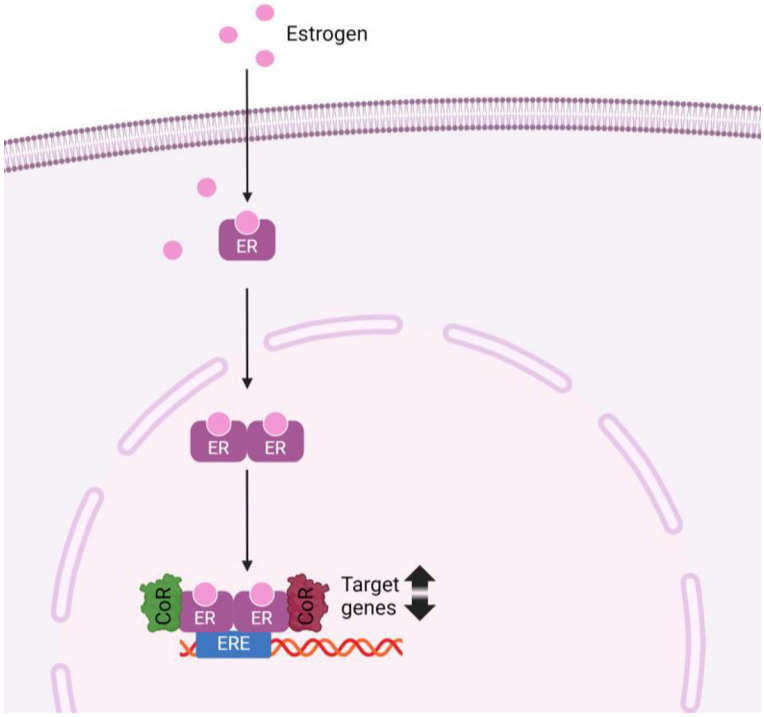


Figure 4: In canonical estrogen receptor (ER) signaling an estrogen molecule binds the receptor, which then dimerizes, translocates to the nucleus and binds estrogen response elements (ERE) on DNA to regulate target gene expression in cooperation with coregulators (CoR). (Created with BioRender.com)

2.5.4 ERα in breast cancer

As discussed in sections 2.1.4 and 2.1.6, estrogen signaling is inextricably linked to ER+ breast cancer. When one considers the roles of ERα in healthy tissue, it is unsurprising that dysregulation of the pathway can lead to tumorigenesis. In healthy individuals, ERα promotes changes in the tissue architecture of the breasts and female reproductive system, and these parts of the body are the most at risk of developing ERα-associated cancers. *In vitro*, ERα has been shown to stimulate breast cancer cell proliferation by upregulating expression of Hes-6 (Hartman *et al.* 2009), LRP16 (Zhao *et al.* 2005), and phosphatidylinositol-3-OH kinase (PI3K) (Lee *et al.* 2005), and by downregulating expression of retinoblastoma protein-interacting zinc-finger 1 (Gazzerro *et al.* 2006), and alkaline phosphatase (Guerreiro *et al.* 2007). ERα signaling has also been shown to promote breast cancer cell survival by downregulating prostate apoptosis response-4 (Casolari *et al.* 2011) and alkaline phosphatase (Guerreiro *et al.* 2007), regulate expression of the tumor angiogenesis factor vascular endothelial growth factor receptor-2 (Higgins *et al.* 2008), and contribute to tumor inflammation by upregulating prostaglandin E synthase (Frasor *et al.* 2008). Furthermore, ERα has been linked to the metastatic cascade in ER+ breast cancers, for example by suppressing Cap43 expression (Fotovati *et al.* 2006), upregulating myocardin-related transcription factor A (Zhang *et al.* 2013), increasing ezrin phosphorylation (Zheng *et al.* 2011), and disrupting tight junctions (Jiménez-Salazar *et al.* 2014). This is by no means an exhaustive list of the mechanisms through which ERα activity has been linked to cancer hallmarks in the context of breast cancer. The variety of targets and effects paints a complex picture, however given that ERα has been predicted to bind between 1,000 and 80,000 sites in the human genome (Vega *et al.* 2006; Lin *et al.* 2007; Bojcsuk and Bálint 2019), it is perhaps to be expected that the reported mechanisms are so diverse. Numerous studies have also pointed to a positive feedback loop in breast cancer cells, in which ERα signaling itself promotes the synthesis of E2. This is generally attributed to ERα directly upregulating or activating enzymes involved in the E2 synthesis pathway (Catalano *et al.* 2009; Shehu *et al.* 2011), or by causing the release of cytokines that themselves regulate the E2 synthesis pathway (To *et al.* 2014).

As discussed in previous sections, many proteins transcend the labels “tumor suppressor” and “oncoprotein”, and one can argue that this is also the case for ERα. Multiple studies provide examples of ERα inhibiting one or more cancer hallmarks in breast cancer. ERα has, for example, been shown to activate paired box 2 (PAX2), which in turn inhibits HER2 expression thereby reducing invasive behavior of luminal MCF-7 and ZR75-1 cells (Beauchemin *et al.* 2011), promote expression of integrin α5β1, which in turn reduces cell motility (Sisci *et al.* 2010), and induces apoptosis in estrogen-starved cells (Chen *et al.* 2015).

2.5.5 ERα phosphorylation in breast cancer

To date, twenty-seven distinct phosphorylation sites have been identified on ERα (Hornbeck *et al.* 2015). The most studied of these are serine-118 (S118) and serine-167 (S167), and numerous studies have explored the link, if any, between phosphorylation at these residues and patient-response to endocrine therapy (Anbalagan and Rowan 2015). Reports have varied, depending on the context of the study, with patient data often seeming to contradict *in vitro* findings. S167, which sits in the N-terminal transactivation domain of the protein, is known to be phosphorylated by rsk1 (Joel *et al.* 1998), rsk2 (Clark *et al.* 2001), AKT (Campbell *et al.* 2001), CK2 (Arnold *et al.* 1994), S6K1 (Yamnik *et al.* 2009), IKKε (Guo *et al.* 2016), Aurora-A (Zheng *et al.* 2014).

Early *in vitro* studies suggested that S167 phosphorylation was a potential marker for ERα's sensitivity to agonists and antagonists and may play a role in ligand-independent signaling. One study showed that the PI3K/AKT pathway was instrumental in mediating the estrogenic effects of epidermal growth factor (EGF) and insulin-related growth factor I (IGF-1) in the absence of E2, however this was at a time when it was not known that AKT phosphorylates S167, and therefore the authors could not reliably speculate on whether it was the phosphorylation itself that potentiated ERα signaling or another PI3K/AKT pathway-linked effect (Martin *et al.* 2000). Soon after, Campbell *et al.* (2001) reported that S167 phosphorylation weakens the inhibitory effects of estrogen deprivation on ERα transcriptional activity and growth, and subsequent studies showed that PI3K/AKT inhibition has the opposite effect (Martin *et al.* 2003; Staka *et al.* 2005). During the same period, there were two reports of tamoxifen-resistant breast cancer cells displaying augmented phosphorylation of nuclear ERα at S118 and S167, as a consequence of MAPK and AKT activity, regulated by EGFR/HER2/IGF-1R (Nicholson *et al.* 2004; Shou *et al.* 2004). Following this, S167 was shown to enhance binding of ERα to DNA, however the authors also reported that S167 phosphorylation did not influence the affinity of the receptor for E2 or tamoxifen (Likhite *et al.* 2006). Another study showed that 40 S ribosomal S6 kinase 1 (p70S6K) phosphorylates ERα at S167 and contributes to its activation and breast cancer cell proliferation in that manner (Yamnik *et al.* 2009). There are also three similar reports concerning Aurora-A, IKKε, and DDX3X, all of which have been linked to increased S167 phosphorylation and tamoxifen resistance (Zheng *et al.* 2014; Guo *et al.* 2016; Pardeshi *et al.* 2022).

In patients, studies have mainly combined phospho-specific immunohistochemical stains and clinical data to determine whether the amount of ERα phosphorylation in breast cancer tumors correlates with patient survival and treatment response. Yamashita *et al.* (2005) concluded that phosphorylation at S167 was a good predictor of response to endocrine therapy and may serve as a good prognostic marker. Likewise, another study showed that in ER+ breast cancer

patients S167 phosphorylation was linked to favorable characteristics such as low tumor grade, improved relapse-free survival, and overall survival, and that the activity of kinases that phosphorylate S167 correlated with better prognoses. The authors concluded that S167 phosphorylation may serve as a predictive marker for patient response to endocrine therapies (Jiang *et al.* 2007). Another study looking at samples from metastatic breast cancer patients who had been treated with AIs reported that S167 phosphorylation correlated with increased progression-free survival (Motomura *et al.* 2010). In their study, Chen *et al.* (2013) concluded that phosphorylation at S118, but not S167, correlated with resistance to tamoxifen, while another study in postmenopausal breast cancer patients showed that phosphorylation at S167 was in fact a positive marker for disease-free survival and favorable response to endocrine therapy (Ishida *et al.* 2018).

Despite our incomplete understanding of the interplay between kinases and site-specific modifications in driving therapy resistance, the combined use of kinase inhibitors and anti-estrogens is gaining traction as a treatment strategy in endocrine-therapy resistant patients. There are a number of clinical trials ongoing that combine these modalities and in some cases, it is being adopted as a standard of care. The CDK 4/6 inhibitors abemaciclib, palbociclib, and ribociclib have all been shown to improve survival in postmenopausal ER+, HER2- breast cancer patients (Hortobagyi *et al.* 2022; Johnston *et al.* 2023; Watanabe *et al.* 2023). A CDK 4/6 inhibitor in combination with an antiestrogen is becoming a standard line of care for this class of breast cancer, and the combination is being explored for other breast cancer indications as well (Campone *et al.* 2022). It is interesting to note here, as touched on in section 1.2.7 of this thesis, that abemaciclib doubles up as a pan-PIM kinase inhibitor (Gelbert *et al.* 2014; Litchfield *et al.* 2020). Similarly, the mTOR inhibitor everolimus is becoming an increasingly common choice to use in combination with endocrine therapy for patients with ER+, HER2- tumors. It has proven to be particularly effective as a second or third line of treatment (François-Martin *et al.* 2023). In USA, the dual inhibitor lapatinib, which targets HER2/neu and EGFR, has been approved for use in combination with letrozole in advanced metastatic ER+ HER2+ breast cancer patients since 2010, where the combination is more efficacious than use of letrozole alone (Riemsma *et al.* 2012).

3 Aims

Broadly, the purpose of this thesis was to explore the effects of PIM kinases in luminal A breast cancer. I considered their involvement in cancer cell biology and addressed the question, is it rational to block these pathways as a therapeutic strategy? In the compilation of studies, I explored PIM-mediated phosphorylation of three substrates (Figure 5): (1) phosphorylation of Notch3 at serine-1672; (2) LKB1 at serine-334, and; (3) ERα at serine-167.

Study I: Does PIM-mediated phosphorylation of Notch3 support luminal A breast cancer cell growth and viability?

In a previous study from our research group, Notch1 was validated as a PIM substrate, and while phosphodeficient Notch1 was shown to suppress luminal A breast cancer cell tumorigenicity, wild-type (WT) and phosphomimicking Notch1 were shown to support tumorigenicity (Santio *et al.* 2016a). In the same study, Notch3 was also shown to be a PIM substrate, however the phosphorylation site and impact of this PTM on Notch signaling were not explored. In study I, we aimed to identify the PIM target site, determine its effect on Notch signaling, and explore the consequence of Notch3 phosphorylation on the growth and viability of cancer cells.

Study II: How do PIMs regulate LKB1 activity? Is it rational to use PIM inhibitors to treat LKB1-deficient tumors?

PIM 1/2/3 triple knockout (TKO) mouse embryonic fibroblast cells had previously been shown to have enhanced levels of AMPK phosphorylation (Beharry *et al.* 2011), suggesting that PIMs play a role in regulating this energy-sensitive metabolic pathway. We decided to determine if PIMs exert their effects on AMPK via an intermediary, namely LKB1, which is the primary AMPK-phosphorylating kinase. Owing to LKB1's well-documented role as tumor suppressor, we decided to explore this relationship in the context of cancer and consider the possibility of PIM inhibition as a therapeutic strategy for LKB1-deficient tumors.

Study III: Do PIMs regulate ERα signaling? Do they play a role in facilitating endocrine therapy resistance?

In luminal A breast cancer cells PIMs are upregulated following treatment with E2 (Malinen *et al.* 2013; Santio *et al.* 2016a), however prior to this study nothing was known about PIM-mediated regulation of the ERα signaling pathway. As AKT and PIMs share many substrates, and ERα is an AKT substrate, we decided to investigate the possibility of PIM-mediated ERα phosphorylation. Furthermore, we hoped to shed light on the importance of PIMs in ERα signaling and assess the role, if any, of PIMs in endocrine therapy resistance.

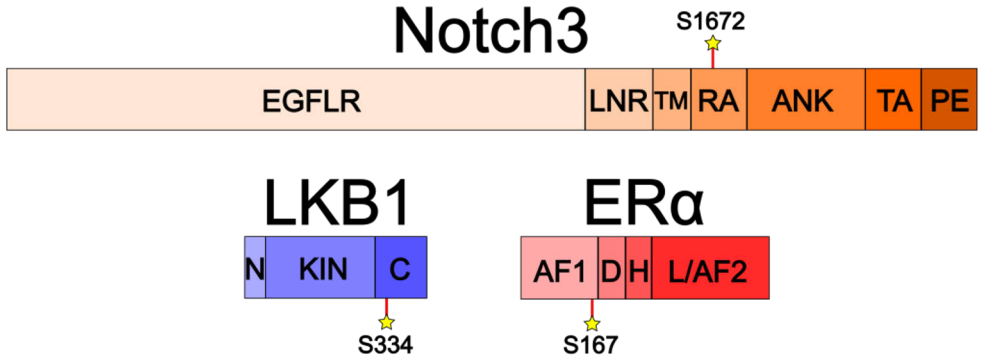


Figure 5: Schematic of novel PIM substrates and their domains. Stars highlight the serine that is phosphorylated by PIMs in each instance.

AF1	activation function 1
ANK	ankyrin repeats
C	C-terminus
D	DNA binding domain
EGFLR	epidermal growth factor like repeats
H	hinge
KIN	kinase domain
L/AF2	ligand binding domain and activation function 2
LNR	LIN-12-Notch repeats
N	N-terminus
PE	proline, glutamic acid, serine, and threonine-rich domain
RA	RBP-J associated molecule (RAM) domain
TA	transactivation domain
TM	transmembrane domain

4 Materials and Methods

4.1 Materials and methods used in the articles of this thesis

A summary of the most pertinent materials and methods used in the studies in this thesis are outlined in **tables 2 – 8**. A more comprehensive description of the relevant methods can be accessed by referring to the primary literature sources.

Table 2: Eukaryotic cell lines used in the studies and their origin. Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) or Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin. RPMI-1640 medium was further supplemented with 1 X MEM Non-Essential Amino Acids (Gibco, #11140050; ThermoFisher Scientific) and 1 X sodium pyruvate (Gibco, #11360070; Thermo Fisher Scientific). In experiments requiring estrogen-starvation in article III, cells were cultured in phenol red-free DMEM (Gibco™ #21063029; Thermo Fisher Scientific), supplemented with charcoal stripped FBS (Gibco™ #A3382101; Thermo Fisher Scientific), plus L-glutamine and penicillin/streptomycin at the same concentration as for the other media used.

Cell line	Species	Type	Tissue origin	Article
FDC-P1	Mouse	Myeloid progenitor	Bone marrow	II
HeLa	Human	Human papillomavirus-related cervical adenocarcinoma	Cervix	I, II
MCF-7	Human	Invasive breast carcinoma of no special type	Pleural effusion	I, II, III
PC-3	Human	Prostate adenocarcinoma	Bone	II, III
T-47D	Human	Invasive breast carcinoma of no special type	Pleural effusion	I, III

Table 3: *Escherichia coli* strains used, purpose, and culture conditions. Cells were cultured in Luria Broth (LB) supplemented with kanamycin (50 µg/ml) or ampicillin (100 µg/ml), depending on the resistance cassette of the construct in use. Clones were stored on LB-agar plates with appropriate antibiotics at +4°C for up to one month, or in antibiotic-free LB + 15% v/v glycerol for long-term storage at -80°C.

Strain	Purpose	Conditions		Article
BL21	Recombinant protein production	+30 – 37°C	180 RPM	I, II, III
DH5α	Molecular cloning and plasmid isolation	+37°C	200 – 250 RPM	I, II, III

Table 4: Site-directed mutagenesis primers. Genes are of human origin unless stated otherwise. F, forward; R, reverse.

Gene	Target mutation	F/R	Sequence	Article
ESR1	S167>A	F	CTTCCCTTGTCATTGGTAGCGGCCAATCTTTCTCTGCC	III
	S167>A	R	GGCAGAGAAAGATTGGCCGCTACCAATGACAAGGGAAG	III
	S167>E	F	CATACTTCCCTTGTCATTGGTTTCGGCCAATCTTTCTCTGCCACC	III
	S167>E	R	GGTGGCAGAGAAAGATTGGCCGAAACCAATGACAAGGGAAGTATG	III
LKB1	S334>A	F	CACCACAGTCATGGCGCGCCACCGGTCC	II
	S334>A	R	GGACCGGTGGCGCGCCATGACTGTGGTG	II
	S428>A	F	GCTTGCAGGCCGCCAGCCGGCGG	II
	S428>A	R	CCGCCGGCTGGCGGCCTGCAAGC	II
Notch3 mouse	S1673>A	F	CGAAAGCGAGAGCACGCGACCTTGTGGTTCCCAGAGGGTTTTGC	I
	S1673>A	R	GCAAAACCCTCTGGGAACCACAAGGTCGCGTGCTCTCGCTTTCG	I
	S1673>E	F	GGCGAAAGCGAGAGCACGAGACACTATGGTTCCCTGAGG	I
	S1673>E	R	CCTCAGGGAACCATAGTGTCTCGTGCTCTCGCTTTCGCC	I
	S2064>A	F	CTGGGACCAAGAAGGCTAGAAGGCCACCCGGGAAGACCG	I
	S2064>A	R	CGGTCTTCCCGGGTGGCCTTCTAGCCTTCTTGGTCCCAG	I

Table 5: Antibodies. CST, Cell Signaling Technology; NB, Novus Biologicals; PLA, proximity ligation assay; SA, Sigma-Aldrich; SC, Santa Cruz Biotechnology; WB, western blot.

Target	Manufacturer	Product code	Dilution and application	Article
ACTB	CST	#3700	1:1000-5000 (WB)	I, II, III
ACTB	CST	#4970	1:1000-5000 (WB)	I, II, III
ACTB	SA	D13K4803	1:20 0000 (WB)	I
activated NOTCH1	Abcam	ab8925	1:1000 (WB)	I
AKT	CST	#9272	1:2000 (WB)	II
AMPKα	CST	#2793	1:1000 (WB)	II
cleaved NOTCH3	CST	#C2211	1:1000 (WB)	I
Duolink® In Situ Orange Starter Kit	SA	DUO92102	1:5 (PLA)	I, II
ERα	SC	F-10	1:1000 (WB)	III
Flag	SA	F1804	1:500-1:1000 (WB)	I, II, III
Flag	SA	F7425	1:500 (PLA)	II
full-length NOTCH3	CST	#2889S	1:1000 (WB)	I
GFP	CST	#2956S	1:1000 (WB)	I
Goat anti-rabbit	CST	#7074	1:5000 (WB)	I, II, III
His-tag	CST	#12698	1:1000 (WB)	II, III
Horse anti-mouse	CST	#7076	1:5000 (WB)	I, II, III
Lamin A	SA	L2193	1:5000 (WB)	I
Lamin A/C	CST	#4777	1:1000-5000 (WB)	II
LKB1	CST	#3047	1:1000 (WB)	II
Notch3	Abcam	ab23426	1:500 (PLA)	I
Notch3	SC	A-6	1:500 (PLA)	I
Phospho-AKT (Ser473)	CST	#4060	1:2000 (WB)	II
Phospho-AMPKα (Thr172)	CST	#2325	1:1000 (WB)	II
Phospho-ERα (Ser167)	CST	#64508	1:1000 (WB)	III
Phospho-Ser	Abcam	ab9332	1:1000 (WB)	III
Phospho-Ser/Thr (RXXS*/T*)	CST	#9614	1:1000 (WB)	I, II, III
PIM1	Abcam	ab7577	1:10000 (WB)	I
PIM1	CST	#2907	1:500 (WB)	I, II
PIM1	Merck	MABC553	1:500 (PLA)	II
PIM1	NB	H00005292-M16	1:500 (PLA)	I
PIM1	SC	12H8	1:500 (WB)	I, II, III
PIM1	SC	19F7	1:500 (WB)	I
PIM2	CST	#4730	1:500 (WB)	I, II, III
PIM3	CST	#4165	1:500 (WB)	I, II, III
TFF1	Abcam	ab92377	1:1000 (WB)	III
TFF1	CST	#15571	1:1000 (WB)	III
β-tubulin	CST	#86298	1:1000-5000 (WB)	I, II

Table 6: DNA constructs. Inserts are of human origin unless stated otherwise.

Tag	Backbone	Insert	Additional mutants	Article
-	pSpCas9(BB)-2APuro (PX459)	sgRNA targeting *LKB1*, *PIM1/2/3*		II
	pUCIDT (Amp)	*Notch3* mutant template		I
Flag	pFlag-CMV-2	*ESR1*	S167A S167E	III
	pFlag-CMV-2	*LKB1*	S334A	II
	p3xFLAG-CMV-7.0	*Notch3* intracellular domain (ICD) mouse	S1673A S1673E	I
GFP	pEGFP-C1	*ESR1*		III
	pcDNA™6.2/NEmGFP-DEST	*LKB1*		II
	pEGFP-C1	*Notch3* ICD mouse	S1673A S1673E	I
	pEGFP-C1	*PIM1* mouse		III
	d1EGFP	*RBPJ*		I
	pSpCas9(BB)-2AGFP (PX458)	sgRNA targeting *RBPJ*, *NOTCH1/3*, *LKB1*, *PIM1/2/3*		I, II
GST	pGEX-6P-1	*LKB1*	S334A	II
	pGEX-4T-3	*Notch1* ICD mouse		III
	pGEX-6P-3	*Notch3* ICD mouse	S1673A S2064A	I
	pGEX-6P-1	*PIM1*	K67M	I, II, III
	pGEX-6P-1	*PIM2*		I, II, III
	pGEX-6P-1	*PIM3*		I, II, III
His	pRFSDuet-1	*ESR1*	S167A	III
	pRFSDuet-1	*LKB1*	S334A S428A	II
	pcDNA3.1/V5-His-C	*PIM1*		I, II, III
	pcDNA3.1/V5-His-C	*PIM2*		III
	pcDNA3.1/V5-His-C	*PIM3*		III
	pSMT3	*RBPJ*		I
mCardinal	pGloSensor-22F (cAMP)	*Notch3* mutant template		I
RFP	pTag-RFP-N	*PIM1*		I, II

Table 7: Inhibitor molecules. DMSO, dimethyl sulfoxide; EtOH, ethanol; MeOH, methanol.

Short name	Formal name	Diluent	Target	Article
AZD-1208	(5Z)-[[2-[(3R)-3-amino-1-piperidinyl][1,1'-biphenyl]-3-yl]methylene]-2,4-thiazolidinedione	DMSO	Pan-PIM	I, II
Cycloheximide	4-{(2R)-2-[(1S,3S,5S)-3,5-Dimethyl-2-oxocyclohexyl]-2-hydroxyethyl}piperidine-2,6-dione	MeOH	Eukaryotic 60S ribosome subunit (Protein synthesis)	I
DHPCC-9	1,10-dihydropyrrolo[2,3-α]carbazole-3-carbaldehyde	DMSO	Pan-PIM	I, II
PIMi	unpublished	DMSO	Pan-PIM	III
PF-03084014	(2S)-2-[(6,8-difluoro-1,2,3,4-tetrahydronaphthalen-2-yl)amino]-N-(1-{1-[(2,2-dimethylpropyl)amino]-2-methylpropan-2-yl}-1H-imidazol-4-yl)pentanamide	DMSO	γ-secretase (Notch pathway)	I
Tamoxifen	(Z)-2-[4-(1,2-Diphenylbut-1-enyl)phenoxy]-N,N-dimethylethanamine	EtOH	ERα (Selective estrogen receptor modulator)	III

Table 8: Experiments and techniques. Techniques were performed *in vitro* unless stated otherwise.

Experiments and procedures	Applied techniques	Article
Cell proliferation rate	IncuCyte® S3 live-cell analysis	II
Cell viability	alamarBlue™	III
	MTT assay	III
Clinical dataset analysis	kmplot.com "Kaplan–Meier Plotter"	I
(in silico)	R software analysis	I
DNA transfer	Bacterial transformation	I, II, III
	Mammalian transfection	I, II, III
Genome modification	CRISPR/Cas9 gene editing	I, II
Molecular cloning	Bacterial cell culture	I, II, III
	Plasmid isolation	I, II, III
	Site-directed mutagenesis	I, II, III
Protein expression	Western blot	I, II, III
	Fluorescence lifetime imaging microscopy (FLIM)	I, II
	Immunoprecipitation (IP)	I, II, III
	Isothermal titration calorimetry (ITC)	I
	Proximity ligation assay (PLA)	I, II
Protein localization	Laser scanning confocal microscopy (LSCM)	I
	Nuclear/cytoplasmic fractionation	I
Protein phosphorylation	*In vitro* kinase assay (IVK)	I, II, III
Protein production	Bacterial cell culture	I, II, III
	Recombinant protein isolation	I, II, III
Structural biology analysis	Molecular modelling *(in silico)*	I
	X-ray crystallography	I
Transcriptional activity	Luciferase assay	I, III
	qPCR	I, III
Tumour growth	Chick chorioallantoic membrane (CAM) model	I, II
(in vivo)	Orthotopic mouse model	I

4.2 Methods not included in articles

4.2.1 Prime editing to generate knock-in cell lines

Prime editing (Anzalone *et al.* 2019) was used in an attempt to generate knock-in cell lines with a stable mutation of serine to alanine at Ser-167 (S167A) on the amino acid sequence of the endogenously expressed *ESR1* gene. The pegFinder (Chow *et al.* 2021) (http://pegfinder.sidichenlab.org/) online tool was used to design prime editing guide RNA (pegRNA). A gBlock® gene fragment (Integrated DNA

Technologies, Coralville, Iowa, USA) was then ordered containing this sequence. This fragment was digested with BbsI and ligated into the BsaI-HF-digested pU6-pegRNA-GG-acceptor (Addgene #132777) (Table 9). To increase the efficiency of the process, a nick was also made on the non-edited strand. pegFinder (Chow *et al.* 2021) was used to design a single guide RNA (sgRNA) for this purpose. Single-strand oligonucleotides containing this sequence were ordered from Integrated DNA Technologies (Coralville, Iowa, USA), they were annealed, and ligated into BsmBI-v2-digested BPK1520 (Addgene #65777) (Table 9). Cells were then transfected with pU6-pegRNA-GG-acceptor and BPK1520, both containing the designed inserts, and pCMV-PE2-P2A-GFP (Addgene #132776) which encodes GFP-tagged Cas9 H840A nickase-reverse transcriptase fusion protein (Table 9, 10). The GFP-tag enabled single-cell sorting by GFP using an SH800 Sony Cell Sorter (Sony Biotechnology, San Jose, CA, USA). 3 days after transfection, single cells were sorted into 20% FBS cell culture medium in 96-well plates and allowed to proliferate until wells were approximately ~80% full. At this point, cells were detached and transferred to a new plate, while 20% of the cell suspension was put aside for genomic DNA extraction and PCR-based screening. Genomic DNA extraction and PCR amplification were performed by using Mouse Direct PCR Kit (B40013; Bio-Connect, TE Huissen, The Netherlands) according to the manufacturer´s protocol, with PCR annealing temperature set to 60 °C and extension time to 1 min (Table 11). Positive clone cultures were expanded and negative clones were discarded. Hits during screening were then sequenced by Sanger sequencing (EZ-Seq, Macrogen Europe, Netherlands) to confirm that the desired mutation had occurred in the correct location.

Table 9: Constructs for prime editing

Construct	Purpose	Addgene accession #	A gift from
pU6-pegRNA-GG-acceptor	After inserting a suitable pegRNA sequence, this construct: a) guides Cas9 nickase to a protospacer adjacent motif (PAM) close to site to be mutated b) encodes template sequence for reverse transcription that includes the intended mutation.	132777	David Liu (Anzalone et al. 2019)
BPK1520	After inserting a suitable sgRNA sequence, this construct guides Cas9 nickase to a separate site, which improves the efficiency of template incorporation into the genome.	65777	Keith Joung (Kleinstiver et al. 2015)
pCMV-PE2-P2A-GFP	This construct encodes a Cas9 nickase-reverse transcriptase-GFP fusion protein. The nickase mediates the single-stranded break at the target PAM, the reverse transcriptase reverse transcribes pegRNA into DNA for incorporation into the genome, the GFP enables successfully transfected cells to be sorted according to fluorescence.	132776	David Liu (Anzalone et al. 2019)

Table 10: Prime editing transfection conditions. K, thousand; M, million.

Cell line	MCF-7	T-47D
Plate	6-well plate	10cm plate
Seeding density	200 K	1.5 M
pCMV-PE2-P2A-GFP (ng)	1500	6125
pU6-pegRNA-GG-acceptor_ERS167A-insert (ng)	500	2045
BPK1520_ERS167A-insert (ng)	166	678

Table 11: Primers used for screening. Single cell clones were screened by PCR using the following primers targeting *ESR1* genomic DNA. Both primers were used as sequencing primers on different occasions. The resulting amplicon is 507 bp in length. F, forward; R, reverse.

F/R	Sequence
F	ACAGACGGCAAGAGGTAATG
R	TCCTGAGATCCTGTCTCTTCTC

4.2.2 pU6-pegRNA-GG-acceptor insert

pU6-pegRNA-GG-acceptor was digested with BsaI-HF, and a BbsI-digested gBlock® with sticky ends was ligated in. The following gBlock® was designed and purchased from Integrated DNA Technologies (Coralville, Iowa, USA).

ccttttg**gaagac**ctcacc**g**CCAGGGTGGCAGAGAAAGATGTTTTAGAGGCTAGAAATAGCAA

GTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGGTAG

CGGCCAATCTTTCTCTGCCACCttttggg**gtcttc**gagctag

In red and plain – sequences to increase efficiency of restriction digestion by BbsI

In **red and bold** – the restriction enzyme recognition sites for BbsI

In red and underlined and adjacent to the **BbsI recognition site** – the cut site, leaving sticky ends (2/6)

In black and plain – sticky end for cloning into pU6-pegRNA-GG-acceptor vector

Bold g – guanine required for efficient initiation of U6 promoter signal

In blue – pegRNA spacer to guide Cas9 H840A nickase

In brown – pegRNA scaffold as described in Anzalone *et al.* (2019)

In green – pegRNA extension template for targeted mutation of AGT to GCT

In green and underlined – reverse complement of GCT, this is the alanine codon that replaces the serine codon in the knock-in cell line.

4.2.3 BPK1520 insert

The following sequences were designed using the pegFinder online tool (Chow *et al.* 2021) (http://pegfinder.sidichenlab.org/) and ordered as single-stranded oligonucleotides from Integrated DNA Technologies (Coralville, Iowa, USA).

Forward: caccgACTTCCCTTGTCATTGGTAG

Reverse: aaacCTACCAATGACAAGGGAAGTc

In lower case are sticky ends to enable ligation into digested BPK1520 vector

In UPPER CASE are reverse complementary sequences that guide Cas9 H840A nickase to site that is close to editing site

The single-stranded oligonucleotides were annealed by mixing in ddH2O to a concentration of 10 μM for each respective oligonucleotide and were next heated to 95°C for 5 minutes followed by ramp down cooling to 10°C at a rate of -5°C/minute. BsmBi-v2 was used to digest BPK1520, and the annealed double-stranded oligonucleotide product with sticky ends was then ligated into the digested vector.

4.2.4 PCR product handling

Following PCR on genomic DNA extracts, BsrI was used to digest the amplicon. The intention here was to discern between WT and S167A amplicons following agarose gel electrophoresis. While the WT amplicon is digested into two fragments (190 and 317 bp) by BsrI, S167A is not recognized (Table 12).

Table 12: Description of PCR products in WT vs. S167A following incubation with restriction enzyme BsrI. BsrI recognition site is defined as: sense → 5' ACTGGN/ 3' & antisense → 5' TGAC/CN 3'. The bases recognized by the enzyme are underlined below. The cut site is indicated by a ^. The green AGT is the Ser-167 codon. The blue GCT is the mutated codon for alanine.

Amplicon	Sequence	Digested	Fragment length(s) (bp)
WT	…ATTGG**C^C**AGTACCAA…	Yes	190 & 317
S167A	…ATTGGCCGCTACCAA…	No	507

5 Results and Discussion

5.1 Biochemical and physiological consequences of Notch3 phosphorylation

5.1.1 PIMs phosphorylate Notch3 at S1672

Notch3, along with Notch1, was previously shown to be a PIM substrate in *in vitro* kinase assays (Santio *et al.* 2016a), however Notch3 phosphorylation was not explored further in that study. In **Study I**, Landor *et al.* (2021), our *in vitro* kinase (IVK) assays with recombinant proteins provided compelling evidence that PIMs phosphorylate mouse Notch3 protein at S1673 (I: Fig. 2A), corresponding to S1672 on the human protein. Next, immunoprecipitation assays, laser scanning confocal microscopy (LSCM), fluorescence lifetime imaging microscopy (FLIM), and proximity ligation assays (PLA) were performed to confirm that PIMs colocalize with, interact with and phosphorylate Notch3 in MCF-7 luminal A breast cancer cells (I: Fig. 2D & 3). This discounted the possibility that the phosphorylation we detected in IVK assays was purely a biochemical phenomenon that occurs upon mixing the proteins in a test tube.

Despite numerous examples of Notch1 phosphorylation in the literature (Hornbeck et al. 2015), Notch3 phosphorylation has been studied less. EGFR has previously been reported to promote Notch3 phosphorylation at an unknown tyrosine residue in HCC2429 lung non-small cell carcinoma (Arasada *et al.* 2014). The authors included a large amount of circumstantial evidence to indicate that EGFR is the responsible kinase, however, given that the study did not include IVK assays they rightly conclude that Notch3 is a "direct or indirect substrate". Our study, therefore, provided the first detailed characterization of a Notch3 phospho-modification, where site, responsible kinase, and functional consequence were presented. Interestingly, S1672 falls within the following sequence (–RRKREHSTL–), which does not fully align with the PIM consensus sequence (Peng *et al.* 2007). Since the study, no further Notch3 PTMs have been reported.

5.1.2 Phosphorylation hinders canonical Notch3 signaling

Our study provided a description of how phosphorylation at a single residue can efficiently and elegantly disrupt a biomolecular process. PIM phosphorylates Notch3 in its CSL/RBPJ-associated molecule (RAM) domain. This domain plays a crucial role in canonical signaling as it facilitates binding with CSL, which is the DNA-binding component of the Notch transcriptional complex (Kovall and Hendrickson 2004). *In silico* molecular modelling predicted that phosphorylation at S1672 results in a confirmational twist (I: Fig. S3D), which likely disrupts Notch3-CSL binding. Our collaborators confirmed this by performing isothermal titration calorimetry experiments with Notch3 peptides, where they showed that Notch3-RAM binds CSL with an efficiency of approximately 10-fold less than Notch1-RAM, and that if the same Notch3-RAM peptide with an additional phosphorylation at S1672 was used, no binding could be detected (I: Fig. 4D). Further evidence for this line included an inability to crystallize/immunoprecipitate phosphorylated Notch3-RAM/phosphomimicking (SE) Notch3 in complex with CSL (I: Fig. 5A).

As expected, phosphorylated Notch3's ability to promote Notch target gene expression is substantially compromised. The Notch3 SE mutant promoted CSL transactivation in luciferase assays with an efficiency of approximately 10% in comparison to Notch3 WT (I: Fig. 5B-C), while PIM inhibition with DHPCC-9 improved CSL transactivation when Notch3 was overexpressed (I: Fig. 5D). The luciferase assays on cells subjected to exogenous Notch activation and the qPCR assays on Notch target gene expression paint a slightly more complicated picture. In WT and Notch3 knockout (N3KO) MCF-7 cells, Notch activity in response to challenge with recombinant ligands was diminished when PIM was inhibited, while in Notch1 knockout (N1KO) cells, PIM inhibition did not hamper Notch activity (I: Fig. 5E). These differences are likely explained by the presence or absence of Notch1, upon which PIM is known to have a stimulatory effect (Santio *et al.* 2016a). Notch1 likely plays a more prominent role in these cells, which may in part be explained by Notch1's stronger affinity for CSL (I: Fig. 4D). In qPCR assays, phosphodeficient (SA) Notch3 was in most cases a more robust activator of HES1 and HEY1 expression than WT Notch3 (I: Fig. 5F-G). A notable exception was HEY1 expression in WT cells, which was upregulated to an equal extent by WT and SA Notch3 (I: Fig. 5F), however HEY1 expression appears to be more dependent on Notch1 expression, as in N1KO cells, HEY1 expression drops to approximately 10% of what is seen in WT cells. SA Notch3 is likely a more potent transactivator of CSL owing to the fact that it is no longer a target for PIMs, meaning that phosphorylation-linked steric hindrance between Notch3-RAM and CSL is not an issue. Interestingly, Arasada *et al.* (2014) reported that EGFR-mediated phosphorylation of an unknown tyrosine also disrupts Notch3 canonical signaling. In their luciferase assays, kinase-

deficient EGFR promoted Notch activity. Likewise, in qPCR assays, use of the EGFR inhibitor erlotinib caused an increase in HES1 and HEY1 expression in non-small cell lung cancer cells. These findings are similar to our own, albeit linked to phosphorylation at a different residue. A potential study in which the unknown tyrosine residue is identified and characterized is low hanging fruit for Notch researchers. Owing to the lack of tyrosine residues in the Notch3-RAM domain, it is likely that the mechanism is entirely different, which is an exciting prospect.

5.1.3 CSL-independent Notch3 signaling is tumorigenic in luminal A cells

According to our mouse experiments, orthotopic N3KO MCF-7 xenografts form smaller tumors (I: Fig. 1C). This suggests that broadly speaking, Notch3 supports cancer progression in these cells. Likewise, we have seen similar results for heterotopic N3KO MCF-7 xenografts in the chick chorioallantoic membrane (CAM) model, although the data are unpublished. The CAM assays in study I focused on Notch3 phosphomutants, where it was clear that under estrogenic conditions, SA Notch3 is less tumorigenic. This was demonstrated in MCF-7 cells at both endogenous and enhanced levels of the protein (I: Fig. 6A, 6D), and in Notch3 overexpressing T-47D cells (I: Fig. 6B).

Our findings indicate that in the context of two widely used luminal A breast cancer cell lines, the enzyme-substrate relationship between PIMs and Notch3 promotes cancer. This appears to be a consequence of non-canonical Notch signaling owing to the fact that phosphorylated Notch3 bypasses CSL, however the precise details underpinning this mechanism were not explored in our study. In the future, a more thorough dissection of this mechanism is warranted. Our data offer few clues as to what could be involved, however, given that SA tumors grow at a similar rate to WT tumors in the absence of additional estrogen, the mechanism may intersect with estrogen signaling.

Dou *et al.* (2017) showed that orthotopic Notch3-overexpressing MDA-MB-231 xenografts grow more slowly than their WT counterparts, which runs slightly contrary to our orthotopic mouse experiment, however, given that MDA-MB-231 cells are an ER- breast cancer cell line the data are only superficially comparable. An interesting aspect of the paper, however, is the reported interplay between Notch3 and ERα, which may be relevant for potential non-canonical Notch3 signaling mechanisms involving PIM. The paper reported that Notch3 promotes the expression and activity of ERα, which is in part due to the presence of CSL-binding elements in the ESR1 promoter. How these findings relate to our own is unclear, however they

are likely a piece of a larger puzzle linking these molecular pathways. Hopefully, future studies into Notch3, ERα and PTMs will start to arrange these fragments into a more coherent whole. We also remain ignorant to the effects of S1672 phosphorylation in human patients with luminal A breast cancer. Given the time and funding, the author would enjoy the opportunity to explore this aspect further by developing a phospho-specific antibody, probing patient tissue samples with the antibody, and subsequently comparing Notch3 phosphostatus with various clinical parameters.

Taken together, study I implies that in this context, PIM inhibition would be therapeutically beneficial. Notch3's serine-1672 residue is like a fulcrum; phosphorylation at the residue serves as a switch, pushing the balance one way or the other, thereby promoting a more or less aggressive cancer phenotype. This discovery may help to explain the conflicting findings with regards to the role of Notch3 in ER+ breast cancers. In my mind's eye I can imagine that in non-phosphorylated Notch3 contexts we have tumor suppression, however, in kinase-enriched settings, the protein is phosphorylated and the function of the protein switches entirely. While this is likely to be a simplification, it is surely an important thought to consider in the future. In some senses the idea implies that, unless you can effectively control for PTMs on your protein of interest during your experiments, you cannot reliably compare data across studies.

5.2 Biochemical and physiological consequences of LKB1 phosphorylation

5.2.1 PIMs phosphorylate LKB1 at S334

In **Study II,** Mung *et al.* (2021), we provided clear evidence that LKB1 is a PIM substrate, and that PIM-dependent phosphorylation occurs at S334. We presented IVK assays, which showed that PIMs phosphorylate LKB1 in a test tube environment and that S334 is the primary target residue (II: Fig. 3A-E). Immunoprecipitation and FLIM assays in turn were used to show that PIMs interact with and phosphorylate LKB1 at the target site in MCF-7 and PC-3 cells (II: Fig. 3F-G, Fig. 4A-B).

Interestingly the PIM target serine, S334, falls within the following sequence (–DRWRSMTV–), which does not fully align with the reported PIM consensus sequence (Peng *et al.* 2007). Prior to this study, LKB1 phosphorylation had been reported at 15 separate residues: 8 serines, 5 threonines, and 2 tyrosines (Hu *et al.*

2023), and S334 is amongst these. The residue has previously been reported to be phosphorylated by AKT in MDA-MB-231 cells and human embryonic kidney (HEK) cells (Liu *et al.* 2012) However, our paper is the first instance in which PIMs have been shown to phosphorylate this residue, and the third instance of LKB1 phosphorylation being identified in breast cancer cells after Liu *et al.* (2012) and Casimiro *et al.* (2017). Moreover, given that the former study utilized a TNBC cell line, and the latter used a transformed derivative of the ER- MCF-10A cell line, our finding is likely the first example of LKB1 phosphorylation in luminal A breast cancer cells.

5.2.2 PIMs inhibit LKB1 activity

We first noticed a significant increase in the level of AMPK phosphorylation upon treating cells with PIM inhibitors (II. Fig. 1A-C). Similar results were obtained from PIM 1/2/3 triple knock-out (TKO) cells (II: Fig. 2D). Conversely PIM overexpression led to a sharp decrease in AMPK phosphorylation (II: Fig. 2D-E). Given that AMPK is a prominent LKB1 substrate, these findings led us to speculate that LKB1 may be involved as a mediator. After confirming that LKB1 is a PIM substrate (II: Fig. 3A-E), we looked at the functional consequence of phosphorylation on LKB1. The phosphodeficient (SA) LKB1 mutant was a more potent inducer of AMPK phosphorylation in PC-3 cells than WT LKB1, however in MCF-7 cells there was no difference between the two (II: Fig 4C). In both cell lines the activity of WT LKB1 is enhanced by using a PIM inhibitor, while PIM inhibition has no effect on the activity of SA LKB1 (II: Fig. 4C). This served as reliable evidence for the fact that S334 phosphorylation is the prominent mechanism by which PIMs regulate LKB1 activity. Further evidence that PIMs regulate LKB1 action on AMPK came when we evaluated AMPK phosphorylation levels in TKO cells, LKB1 knockout (LKB1KO) cells, and combined TKO/LKB1KO (TKOLKB1KO) cells. AMPK phosphorylation is enhanced in TKO cells; however, AMPK phosphorylation is similar in LKB1KO cells and TKOLKB1KO cells (II: Fig. 5C), suggesting that the phenotype we observe in TKO cells is due to enhanced activity of LKB1.

Given that SA LKB1 was a more potent inducer of AMPK phosphorylation in PC-3 cells than WT LKB1, however in MCF-7 cells there was no difference between the two, it may be interesting to compare the basal level of LKB1 phosphorylation in these cells. If basal LKB1 phosphorylation is higher in PC-3 cells, it suggests that LKB1 is in a less active state in these cells. As a result, overexpression of WT LKB1 would have a less pronounced effect on AMPK phosphorylation compared to MCF-7 cells. This could be due to PIM or other, as-yet-unidentified kinases.

At least one other kinase is known to phosphorylate S334, namely AKT, which has been reported to promote nuclear sequestration of LKB1 by phosphorylating this residue (Liu *et al.* 2012). The authors showed that phosphorylation-dependent binding of 14-3-3 to LKB1 restricts cytoplasmic translocation and consequently its activity in the cytoplasm is limited. While study II similarly demonstrates that PIMs restrict LKB1 activity through phosphorylation at the same residue, we found no evidence to suggest that this was a consequence of altered subcellular localization. LKB1WT and S334A show a similar cellular distribution when overexpressed (II: Fig. S6A). Similarly, cells lacking PIM or treated with PIM inhibitors display no differences in terms of LKB1 compartmentalization (II: Fig. S6B-C), suggesting that PIMs inhibit LKB1 activity via a distinct mechanism. It has previously been reported that phosphorylation at S325 and S428 by ERK and RSK disrupts binding between LKB1 and AMPK, and therefore reduces LKB1-mediated AMPK phosphorylation and activation (Zheng *et al.* 2009). Cyclin D1-mediated phosphorylation of S325 is also known to inhibit LKB1 activity, however the underlying mechanism in this study was not explored (Casimiro *et al.* 2017). S325, S334 and S428 all sit within the C-terminal domain, and given the proximity between S325 and S334, it seems possible that PIM-mediated phosphorylation at S334 may also lead to impaired binding between LKB1 and AMPK. This hypothesis would explain our findings in study II and would be an interesting channel of enquiry in the future.

5.2.3 Tumorigenicity is reduced in luminal A cells when PIMs and LKB1 are knocked out

In the *in vitro* proliferation assays that were performed with an IncuCyte® S3 live-cell analysis system (Essen BioScience, Ltd., UK) the pan-PIM inhibitor DHPCC-9 restricted the proliferation rate of both WT and LKB1KO MCF-7 cells (II: Fig. 5A). Somewhat surprisingly, the proliferation rate in TKO MCF-7 cells was not reduced as compared to WT cells (II: Fig. 5B). LKB1KO MCF-7 cells also proliferated at a rate similar to WT cells (II: Fig. 5A-B). In subsequent experiments, the proliferative capacities of TKO and LKB1KO cells were reflected in the sizes of tumors formed by these cells when xenografted onto the CAM (II: Fig. 5D).

As discussed in section 2.2.4, PIMs on their own are often not enough to promote oncogenesis; there are numerous other oncogenic programs in MCF-7 cells that underpin its malignant nature. Nonetheless, there is a clear discrepancy between the pan-PIM inhibitor and TKO cells. This may be due to factors such as signal rewiring in the TKO cells to negate their PIM-deficiency or off-target DHPCC-9 effects; the inhibitor may target other oncogenic pathways with reasonable efficacy or hit a

broad range of targets at high concentrations. This would be somewhat surprising, as our group has previously seen that 10 µM DHPCC-9 is not cytotoxic in cells with normal levels of PIM protein (Santio *et al.* 2010). In the future it would be interesting to see whether combined knockdown of all PIM family member transcripts by RNA interference affects proliferation in a manner that is more akin to genome editing or pharmacological inhibition. The results we obtained for LKB1KO cells shows that LKB1-deficiency does not invariably lead to cancer formation or promote a more aggressive phenotype, as discussed in section 2.4.2. On the other hand, LKB1KO was sufficient to promote a more proliferative phenotype in PC-3 cells (II: Fig. 5D). We speculate that this is a consequence of combined deficiency of both PTEN and LKB1 in these cells. PTEN is a known tumor suppressor (Lee *et al.* 2018), and shortly before the publication of study II it was shown in mice that LKB1-deficiency alone is not sufficient to promote prostate cancer formation, however in combination with PTEN heterozygosity, LKB1-deficiency frequently leads to sporadic formation of aggressive prostate cancer (Hermanova *et al.* 2020).

We observed an interesting phenomenon when we started studying the proliferative capacity and tumorigenicity of TKOLKB1KO cells. In these cells, AMPK phosphorylation is compromised to an even greater extent than in LKB1KO cells (II: Fig 5C), which one assumes may promote a more aggressive metabolic state. However, amongst our panel of MCF-7 cells, these derivatives grew significantly more slowly than WT cells and the individual TKO and LKB1KO derivatives (II: Fig. 5B, 5D). This may serve as an interesting example in which the LKB1-AMPK axis is in fact beneficial for cancer cells, protecting them from environmental stresses or metabolic overdrive by balancing their energy demands, which is an idea that was discussed in section 2.4.5. Indeed, in their review, Vara-Ciruelos *et al.* (2019) posit the idea that although AMPK activity suppresses cancer formation, following oncogenesis AMPK may enhance cancer cell survival, for similar reasons as discussed in section 2.4.5. This line of thinking matches up well with our data concerning the behavior of TKOLKB1KO cells.

Taken together, this study suggests that in tissues where PIMs are aberrantly overexpressed, they have an inhibitory influence over the LKB1-AMPK axis, and that PIM inhibition may help to restore the activity of this axis. By restoring the activity of this axis, it may be possible to reduce the risk of tumorigenesis occurring in healthy tissues. This, however, is not a therapeutically realistic scenario, as it implies that we would make use of prophylactic PIM inhibitors to reduce the risk of cancer formation. In the unusual case that a patient displays LKB1-deficient breast cancer, our results in study II imply that PIM inhibition may in fact be a rational

therapeutic strategy, as LKB1KO cell proliferation and tumor volume were reduced by PIM inhibition or ablation (II: Fig. 5A-B, 5D).

5.3 Biochemical and physiological consequences of ERα phosphorylation

5.3.1 PIMs phosphorylate ERα at S167

When we looked at ERα's primary protein structure and compared this to the PIM kinase consensus phosphorylation sequence, R-X-R-H-X-S (Peng *et al.* 2007), we identified a likely phosphorylation site with the sequence R-E-R-L-A-S*. The serine corresponds to S167, which sits in the N-terminal transactivation domain of the protein and is known to be phosphorylated by rsk1 (Joel *et al.* 1998), rsk2 (Clark *et al.* 2001), AKT (Campbell *et al.* 2001), CK2 (Arnold *et al.* 1994), S6K1 (Yamnik *et al.* 2009), IKKε (Guo *et al.* 2016), Aurora-A (Zheng *et al.* 2014). In **Study III**, Eccleshall *et al.* (under review), we confirmed that ERα is a novel PIM substrate and that S167 is indeed the prominent target residue (III: Fig. 1). This was achieved by performing IVK assays, immunoprecipitation, and western blotting in experiments using phosphodeficient ERα S167A and PIM inhibition. A pertinent question here relates to the PIM consensus sequence. Is it time to update our definition of the PIM kinase consensus sequence? Are the kinases more promiscuous than we give them credit for? Yet again, as seen in studies I and II, the target serine on ERα falls within a sequence that does not fully align with Peng and colleagues' consensus sequence that is invariably cited in the PIM kinase literature (Peng *et al.* 2007).

5.3.2 PIMs support ERα signaling

ERα activity was initially explored by using an estrogen response element (ERE) luciferase reporter construct. By overexpressing different ERα S167 phosphomutants in ERα-naïve PC-3 cells we could determine the effects of ERα S167 phosphorylation on ERα activity in the absence of endogenous ERα. Phosphomimicking ERα was moderately more active than WT and phosphodeficient forms of the protein, however this difference was insignificant (III: Fig. 2E). PIM1 overexpression enhanced the activity of WT ERα (III: Fig. 2C, 2D), while PIM inhibition caused a modest reduction in ERα activity in MCF-7 cells and a slight but insignificant decrease in ERα activity in T-47D cells (III: Fig. 2B). The reduced effect in T-47D cells may in part be explained by the lower level of PIM expression in these cells, a feature that we observed in study I (I: Fig. S5B). We detected the most striking difference in ERα activity when we compared MCF-7 TKO cells to WT cells. The ERα activity in TKO cells was drastically reduced (III: Fig. 2A). We

obtained similar results when we performed qPCR and western blotting to check for levels of estrogen-inducible genes. These are significantly downregulated in TKO cells (III: Fig. 3A) and can be upregulated by overexpressing PIM1 (III: Fig. 3C).

Taken together, these data suggest that PIMs are important regulators of ERα activity. Given that ERα activity is hindered so drastically in TKO cells, but that S167 phosphomutants behaved similarly in luciferase assays, it seems likely that PIMs regulate ERα activity via additional mechanisms alongside S167 phosphorylation. ERα signaling is known to promote PIM expression in luminal A cells (Malinen *et al.* 2013; Santio *et al.* 2016a), however study III shows for the first time that regulation occurs in the opposite direction as well. This hints at a system of signaling feedback between the two proteins, that would be exciting to explore in a future study.

Given that the phosphorylation site is known to be phosphorylated by at least 7 other enzymes, there are already numerous reports relating to how this PTM influences ERα activity. S167A phosphomutants have been shown to have reduced transcriptional activity as compared to WT cells in experiments with baby hamster kidney fibroblasts (Joel *et al.* 1998; Clark *et al.* 2001), COS-1 cells (Campbell *et al.* 2001), HeLa cells (Shah and Rowan 2005) and MCF-10A cells (Guo *et al.* 2016), and in HEK cells ERα transcriptional activity has been shown to correlate with S167 phosphorylation levels (Yamnik *et al.* 2009). Indeed, S167A was shown in one study to bind more weakly to the promoter of *TFF1*, an estrogen-inducible gene (Shah and Rowan 2005), while S167 phosphorylation by AKT has been shown to slightly enhance binding of ERα to EREs in DNA (Likhite *et al.* 2006). These mechanisms likely rely upon the recruitment of coactivators, owing to the fact that S167 sits in ERα's activation function 1 (AF1) domain. Shah and Rowan (2005) showed that Gal-steroid receptor coactivator 1 (SRC-1) and cAMP binding protein (CBP) stabilize interactions between ERα and DNA, however they did not show that interactions between ERα and these coactivators are dependent on S167 phosphostatus. Likhite *et al.* (2006) showed that S167 phosphorylation increases affinity for SRC-3, but only in the presence of E2. Finally, it is important to remember that ERα is phosphorylated on at least twenty-seven residues (Hornbeck *et al.* 2015), and it is therefore likely that ERα function is dependent on a mosaic of phosphorylation patterning.

5.3.3 No evidence for PIMs promoting endocrine therapy resistance

We could find no evidence to support the notion that PIMs would protect MCF-7 cells under conditions of estrogen deprivation or inhibition. In MTT viability assays, ERα S167A or S167E overexpression did not enhance or reduce the viability of cells under estrogen-deprivation or following treatment with E2 (III: Fig. 4A), suggesting that in this limited context S167 phosphorylation confers no advantage to cells. Similarly, in alamarBlue™ viability assays, although PIM overexpression improved the overall viability of MCF-7 TKO cells, it did not enhance WT cell viability, nor improve the viability of tamoxifen-treated cells (III: Fig. 4B). Indeed, upon developing a tamoxifen-resistant strain of MCF-7 and T-47D cells we noted that PIM levels were in fact downregulated in these cells as compared to non-resistant cells (III: Fig. 4C-D), further implying that PIMs have a limited effect in this context. We were somewhat surprised by these data because four separate studies have proposed that S167 phosphorylation promotes endocrine therapy resistance, at least *in vitro* (Campbell *et al.* 2001; Zheng *et al.* 2014; Guo *et al.* 2016; Pardeshi *et al.* 2022).

Most previous studies have explored the impact of different ERα phosphomutants by looking at the biomolecular and physiological consequences of overexpression in both ERα-expressing and ERα-naïve settings. Only one study, to the author's knowledge, has explored the physiological effect of ERα S167 phosphodeficiency at an endogenous level (Huderson *et al.* 2012). In the study, MCF-7 cells were stably transfected with shRNA that reduced endogenous ERα levels, and then stably transfected with WT and ERα S167A. While levels of the reintroduced protein were similar to endogenous levels in WT cells, genomic ESR1 knockdown was incomplete, which means that the results must be considered while remaining aware of their potential limitations. Interestingly, in the study Huderson *et al.* (2012) showed that in terms of viability and apoptosis, S167A ERα conferred no advantage or disadvantage over WT ERα upon treatment with E2 or antiestrogens, which is similar to what we observed (III: Fig. 5a). Interestingly, ERα-S167A-expressing cells had a more invasive and migratory phenotype, proliferated more quickly, were smaller and less complex, and demonstrated altered ERα-regulated gene expression. Our plan was to explore ERα sensitivity to agonism and antagonism and put this into a physiological context by generating ERα S167A knock-in mutants through genome editing. These mutants would have had the advantage of expressing endogenous ERα with no background expression of the WT form of the protein. Unfortunately, our knock-in method yielded little success, as discussed in section 5.3.4.

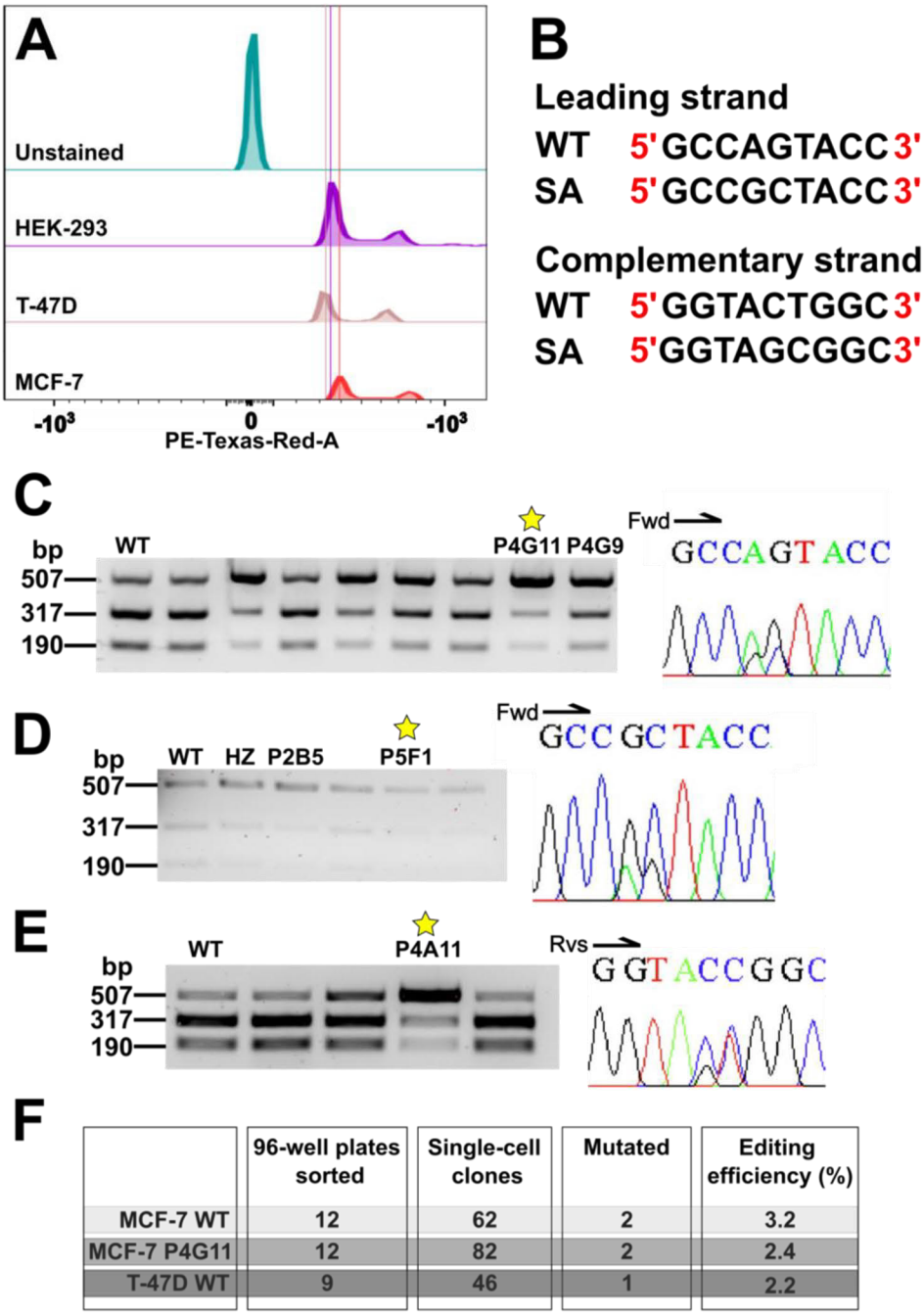
The role of S167 phosphorylation in therapy resistance remains divisive; the conclusions that can be drawn, depending on the study in question, clash profoundly.

What drives this difference? First and foremost, it is important to remember that *in vitro* 2D models of cancer are limited in their capacity to reliably recapitulate the complexity of cancer in a patient. It is possible that key molecular drivers of resistance are relevant *in vitro* only, and that their effect is nullified or even reversed in the context of a patient tumor. Secondly, while considering PTMs and development of therapy resistance, one must be careful in labelling hyperphosphorylation of a specific residue as a causal factor. This kind of claim requires careful experimental design. Hyperphosphorylation in therapy resistance may be the switch that drives this new phenotype, but it may also be a secondary signature relating to the enrichment of other growth factor and/or kinase signaling pathways that are themselves facilitating therapy escape. Finally, a common weakness in *in vitro* studies is the use of systems that rely on overexpression. Artificially high levels of a protein can introduce biological artefacts and may drive a cell to an unrealistic physiological extreme. Moreover, in the context of phosphomutant overexpression, it is common for the endogenous wildtype protein to persist at low levels, even after a mutant form of the protein has been introduced to the system. These factors can cloud reality, and lead to extrapolations that do not accurately reflect the precise physiological balance in a living organism.

5.3.4 Heterozygous mutants

In this part of the project, we used prime-editing, a modified CRISPR/Cas9 approach (Anzalone *et al.* 2019), in an attempt to generate an endogenous ERα serine-alanine knock-in at serine-167, however we were only able to generate heterozygous mutants. We designed and cloned constructs for this purpose, and, following transfection and cell sorting, waited for approximately 4 weeks before screening. MCF-7 cells are hypertriploid and contain three copies of the *ESR1* gene (Figure 6A). For MCF-7 cells we were able to obtain a monoallelic mutant heterozygote (Figure 6C), which was then transfected again and sorted to obtain a biallelic mutant heterozygote in which two *ESR1* alleles contained the desired serine-alanine mutation (Figure 6D), while the third remained unchanged. For T-47D cells, which are diploid, we obtained a heterozygote that possessed one mutated allele (Figure 6E). Further attempts to edit the remaining allele in these cells were unfortunately unsuccessful. This is likely due to the fact that the editing efficiency we achieved with these prime editing constructs was very low (Figure 6F). It seems unlikely that homozygous S167A would be non-viable. Many studies have looked into S167 phosphorylation already, and it is reasonable to expect that if the serine was such a critical part of ERα function and breast cancer cell viability, this would already have been reported. Given the time and resources, I would have relished the opportunity

to design and implement altered prime-editing constructs to obtain the homozygous mutants that we were aiming for.



F

	96-well plates sorted	Single-cell clones	Mutated	Editing efficiency (%)
MCF-7 WT	12	62	2	3.2
MCF-7 P4G11	12	82	2	2.4
T-47D WT	9	46	1	2.2

Figure 6: Unsuccessful attempt to generate full homozygous serine-167 to alanine (SA) mutations of MCF-7 and T-47D cells using prime-editing. **A)** Propidium iodide staining followed by flow cytometry was used to compare DNA content across cell lines. The increased DNA content measured in MCF-7 cells is due to their hypertriploid status. **B)** Comparison between leading and complementary strands at the desired edit site. The relevant serine codon is AGT on the leading strand and ACT on the complementary strand, which is mutated to the alanine codon GCT on the leading strand and AGC on the complementary strand. In **C, D,** and **E,** PCR was performed using primers flanking the edited region. The resulting amplicon was then either treated with BsrI to cleave WT amplicons into 2 fragments of unequal size or sent for sanger sequencing. **C)** Relevant results for 1st generation of MCF-7 prime-editing, from which two, monoallelic mutant heterozygotes were obtained. **D)** Relevant results for 2nd generation of MCF-7 prime-editing, from which two, biallelic mutant heterozygotes were obtained. **E)** Relevant results for T-47D prime-editing, from which a single heterozygote was obtained. **F)** Description of the success rates achieved with the prime-editing method of generating knock-in mutants.

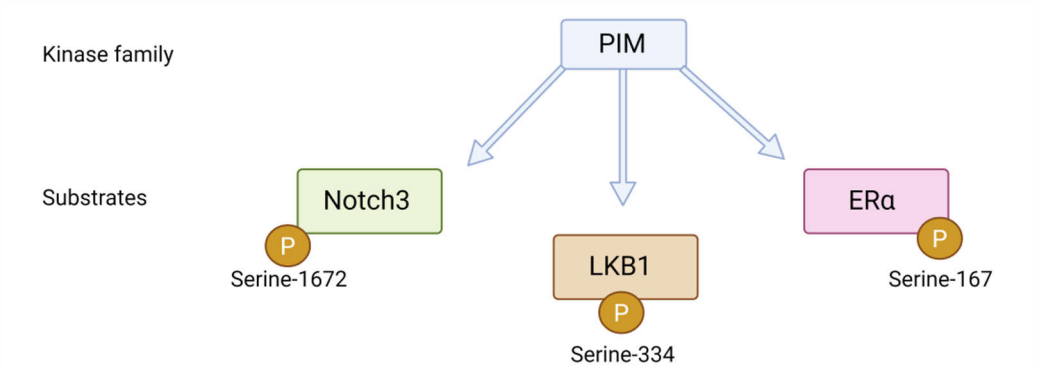
6 Conclusions

6.1 Inhibiting PIM kinases – simple switch, complex results

In light of their constitutive activity and direct involvement in proliferation and cell survival, it is no wonder that PIM kinases (PIMs) have been associated with numerous malignancies in contexts where they are overexpressed. Early attempts to develop PIM inhibitors faltered when the compounds entered clinical trials, however the current generation of inhibitors appear to have improved safety profiles. There is real optimism that these might help patients in the future, particularly when the inhibitors are used in combination with other therapies.

This thesis investigated the relationship between PIMs and three substrates, two of which were entirely novel, in the context of luminal A breast cancer (Figure 7). As well as defining the target phosphorylation site in each respective substrate, I have described the relevant impact of PIMs on the signaling of these substrates. In study I, PIMs were found to inhibit Notch3 canonical signaling by phosphorylating a site that mediates binding to the transcriptional coregulator CSL, thereby disrupting Notch-dependent transcriptional regulation. Despite this, phosphorylated Notch3 supports tumor growth, albeit by an unknown mechanism. In study II, PIMs were found to inhibit LKB1 enzymatic activity. This explains why, in PIM1/2/3 triple knockout (TKO) cells, the LKB1 substrate AMPK is hyperphosphorylated. Furthermore, the proliferation of LKB1 knockout (LKB1KO) cells and their tumorigenic capacity were suppressed by knocking out or inhibiting PIMs. In study III, PIMs were shown to support estrogen receptor α (ERα) signaling via a mechanism including, but not limited to, receptor phosphorylation. While there is some evidence that PIM overexpression improves the viability of TKO cells under basal conditions, there was no evidence to indicate that PIMs would protect breast cancer cells from estrogen deprivation or inhibition. All things considered; it is easy to appreciate the versatility of PIMs. This thesis includes examples of PIMs phosphorylating a cell surface signaling protein, a regulatory enzyme in metabolism, and a nuclear hormone receptor protein.

In studies I and III, PIMs were shown to support oncoprotein activity, while in study II PIM inhibited tumor suppressor activity. A logical conclusion to draw would be that, given the effects documented in this thesis, PIM inhibition has the potential to benefit some luminal A breast cancer patients. This statement does not come without caveats, however. In writing this thesis and throughout my PhD work I have come to realize just how complex and intertwined intracellular signaling pathways are, and how efficiently cancer cells can rewire to negate intended therapy effects. Moreover, it is important to remember the contextual nature of molecular signaling. Despite the functions described in the experimental part of this thesis, Notch3 and ERα are known to act as tumor suppressors in some instances, and LKB1 is known to support cancer cell survival in some contexts. In conclusion, it is imperative that we carefully consider these factors moving forward, striving to treat breast cancer with safer, smarter, and more effective techniques.



Other kinases	None	AKT	Aurora-A, AKT, CK2, IKKε, rsk1/2, S6K1
Molecular effect	Canonical signalling disrupted	Catalytic activity inhibited	Differential transcriptional regulation
Effect on MCF-7 cell function	Enhanced tumor growth in the presence of estrogen	On its own, no prominent change - may serve as one of many risk factors	No impact on viability or sensitivity to antiestrogens

Figure 7: Schematic describing the most prominent findings of the studies included in this thesis. (Created with BioRender.com)

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