Thesis for doctoral degree (Ph.D.) 2019

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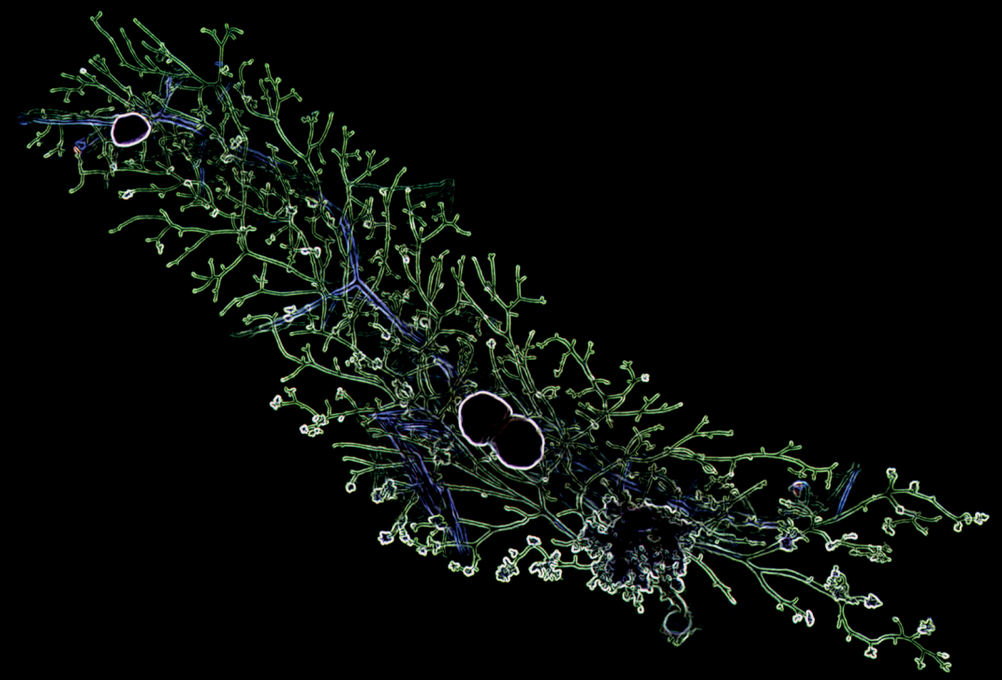

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PAK4 SIGNALING IN DEVELOPMENT AND CANCER

Tânia Costa

Stockholm 2019

Front cover depicts a mammary gland wholemount from a PyMT;PAK4$^{MEp-/-}$ mouse (adapted from Costa *et al.*, 2019). A tumor and numerous pre-malignant lesions are visible along the mammary epithelial tree that extends from the nipple to the limits of the fat pad.

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PAK4 SIGNALING IN DEVELOPMENT AND CANCER

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Para os meus pais e avós,
os meus primeiros mentores

“If I have seen further it is by standing on the shoulders of Giants.”

Isaac Newton

ABSTRACT

Our understanding of cancer biology has been evolving rapidly shaped by groundbreaking discoveries. We now understand that cancer is not one disease but many, and that tumors are not foreign objects in the human body but rather the result of changes in the previously normal tissues and organs. Thus, in order to ask fundamental questions and dissect the complexity of cancer it is essential to grasp how the healthy organs develop and function and the cellular and molecular mechanisms involved. The serine/threonine PAKs are signaling hubs with proven roles in development and disease. Specifically, they are important to several hallmarks of cancer. Thus, the family in general, and PAK4 in particular, is increasingly attracting the interest of the scientific community.

In this thesis I have explored the role of PAK4 in normal organ development and cancer. Novel mouse models with PAK4 depletion in the mammary gland and in the pancreas have been established and characterized in **Paper I** and **Paper II**. The absence of major tissue abnormalities upon PAK4 depletion in the mammary epithelium allowed me to use this model to study the role of PAK4 in tumorigenesis *in vivo*, in **Paper III**, and a counterpart mouse model with PAK4 overexpression in the mammary epithelium was also generated. These complementary *in vivo* setups showed that PAK4-overexpressing mammary glands occasionally developed mammary tumors while PAK4 abrogation impaired PyMT-driven mammary tumorigenesis. Extensive *in vitro* experiments, using state of the art techniques, then supported a model in which PAK4 confers selective advantages to cancer cells by overcoming the senescence barrier. This, in turn, constitutes a selective vulnerability of cancer cells that become susceptible to a senescence-like response upon PAK4 inhibition. The data presented also demonstrates a crosstalk between PAK4 and NF-κB signaling, and a direct interaction and phosphorylation site within the REL-homology domain of RELB is found to be relevant for tuning RELB-mediated transcription and cancer cell proliferation via C/EBPβ. Importantly, these findings were largely supported by correlations in clinical data and validated *ex vivo* in patient-derived cells, thus highlighting PAK4 as an attractive therapeutic opportunity in cancer.

Therefore, this thesis contributes to a better understanding of the mechanisms that govern breast tumorigenesis, with hopes that such knowledge will prove relevant in cancer prognosis and treatment.

RESUMO

O entendimento da biologia do cancro tem vindo a progredir rapidamente, esculpido por descobertas inovadoras. Atualmente entendemos que cancro não é uma única doença, mas sim uma multiplicidade de patologias, e que os tumores não são objetos estranhos no corpo humano, mas antes o resultado de alterações nos tecidos e órgãos outrora normais. Assim, para fazer face a questões fundamentais e dissecar a complexidade que o cancro apresenta, é essencial compreender o normal desenvolvimento e funcionamento dos órgãos e os mecanismos celulares e moleculares envolvidos. As serina/treonina cinases PAK ocupam um lugar central em cascatas de sinalização e têm importância demonstrada tanto no normal desenvolvimento como em situações patológicas. Especificamente, as PAKs são relevantes em várias vertentes do cancro. Por isso, o interesse da comunidade científica sobre esta família em geral, e sobre PAK4 em particular, tem vindo a aumentar.

Nesta tese, explorei o papel da PAK4 no desenvolvimento normal dos órgãos e em cancro. Modelos animais (ratinhos), previamente inexistentes, com deleção da PAK4 na glândula mamária e no pâncreas foram criados e caracterizados no **Artigo I** e no **Artigo II**. A ausência de anomalias no epitélio mamário após a deleção da PAK4 permitiu o uso deste modelo na investigação das funções que a PAK4 desempenha no processo de tumorigénese *in vivo*, no **Artigo III**, onde também foi gerado um modelo reverso: um ratinho com sobrexpressão da PAK4 no epitélio mamário. Essas estratégias complementares *in vivo* revelaram que as glândulas mamárias com sobrexpressão da PAK4 ocasionalmente desenvolviam tumores mamários, enquanto que a deleção da PAK4 atrasava o processo de tumorigénese mamária induzida pelo oncogene *PyMT*. Experiências *in vitro* usando tecnologia de ponta apoiaram um modelo no qual a PAK4 confere vantagens seletivas às células cancerígenas, transpondo a barreira anti-tumoral incutida pelo processo de senescência. Isso, por sua vez, constitui uma vulnerabilidade seletiva das células cancerígenas que se tornam suscetíveis a senescência perante inibição da PAK4. Os resultados apresentados também demonstram uma interligação entre a sinalização celular da PAK4 e do NF-κB e identificam uma interação e fosforilação no domínio de ligação ao DNA (domínio de homologia REL) do fator de transcrição RELB, que se revelou relevante para a regulação da transcrição mediada por RELB e para a proliferação de células cancerígenas via C/EBPβ. É importante salientar que estes resultados experimentais foram amplamente sustentados por correlações em dados clínicos humanos e validados *ex vivo* em células derivadas de pacientes com cancro de mama, destacando a proteína PAK4 como um potencial alvo terapêutico para o cancro.

Esta tese contribui assim para uma melhor compreensão dos mecanismos que governam o processo de tumorigénese das neoplasias da mama, na esperança de que este conhecimento se venha a relevar relevante no prognóstico e tratamento destas patologias.

LIST OF SCIENTIFIC PAPERS

I. Parisa Rabieifar*, Ting Zhuang*, **Tânia Costa**, Miao Zhao and Staffan Strömblad (2019). Normal mammary gland development after MMTV-Cre mediated conditional PAK4 gene depletion. *Scientific Reports* 9, 14436. https://doi.org/10.1038/s41598-019-50819-4

*Parisa Rabieifar and Ting Zhuang contributed equally.

II. Miao Zhao, Parisa Rabieifar, **Tânia Costa**, Ting Zhuang, Audrey Minden, Matthias Löhr, Rainer Heuchel and Staffan Strömblad (2017). Pdx1-Cre-driven conditional gene depletion suggests PAK4 as dispensable for mouse pancreas development. *Scientific Reports* 7, 7031. https://doi.org/10.1038/s41598-017-07322-5

III. **Tânia Costa**, Ting Zhuang, Julie Lorent, Emilia Turco, Helene Olofsson, Miriam Masia-Balague, Miao Zhao, Parisa Rabieifar, Neil Robertson, Raoul Kuiper, Jonas Sjölund, Matthias Spiess, Pablo Hernández-Varas, Uta Rabenhorst, Pernilla Roswall, Ran Ma, Xiaowei Gong, Johan Hartman, Kristian Pietras, Peter D. Adams, Paola Defilippi and Staffan Strömblad (2019). PAK4 suppresses RELB to prevent senescence-like growth arrest in breast cancer. *Nature Communications* 10, 3589. https://doi.org/10.1038/s41467-019-11510-4

CONTENTS

LIST OF ABBREVIATIONS

4-OHT	4-Hydroxytamoxifen
AID	Autoinhibitory domain
BLG	Beta-lactoglobulin
C/EBPβ	CCAAT-enhancer-binding protein beta
DDR	DNA damage response
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERBB2/HER2	Human epidermal growth factor receptor 2
FGF	Fibroblast growth factor
FGFR2	Fibroblast growth factor receptor 2
GBD	GTPase-binding domain
GTP	Guanosine-5'-triphosphate
HGF	Hepatocyte growth factor
HMEC	Human mammary epithelial cell
IGF1	Insulin-like growth factor 1
METABRIC	Molecular Taxonomy of Breast Cancer International Consortium
Mist1	Muscle, intestine and stomach expression 1
MMP	Matrix metalloproteinase
MMTV-LTR	Mouse mammary tumor virus long terminal repeat
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OIS	Oncogene-induced senescence
PAKs	p21-activated kinases
PAK4	p21-activated kinase 4
PBD	p21-binding domain
Pdx1	Pancreatic and duodenal homeobox 1
PTEN	Phosphatase and tensin homolog
Ptf1a	Pancreas associated transcription factor 1a
PyMT	Polyomavirus middle T
RHD	REL-homology domain
SA-β-gal	Senescence-associated beta-galactosidase

SASP	Senescence-associated secretory phenotype
SMA	Smooth muscle actin
SMS	Senescence messaging secretome
TCGA	The Cancer Genome Atlas
TEB	Terminal end bud
TDLU	Terminal ductal lobuloalveolar unit
TIMP	Tissue inhibitor of metalloproteinases
WAP	Whey acidic protein

1 INTRODUCTION

1.1 CELLS, TISSUES AND ORGANS

The human body contains over 10^{13} cells (Bianconi *et al.*, 2013) that can all be traced back to the fertilized egg (Xavier da Silveira Dos Santos and Liberali, 2019). During development, discrete populations of stem cells undergo progressive differentiation and, through multiple repeated cycles of cell division, various cell types ultimately organize into functional tissues and organs (Bryant and Mostov, 2008). As such, cells have been postulated as the "building blocks of life", that is, the basic unit of structure and function of living organisms (Schwann *et al.*, 1847).

Cells integrate intricate chemical and mechanical, intracellular and extracellular, local and systemic cues, and they reciprocally interact with each other and their environment (Bryant and Mostov, 2008; Xavier da Silveira Dos Santos and Liberali, 2019). This highly regulated behavior in space and time is critical to tissue homeostasis and function (Mayr *et al.*, 2019).

1.2 THE MAMMARY GLAND

1.2.1 The anatomy and physiology of the mammary gland

The mammary gland is an exceptional secretory organ that gave an entire class of animals its name, mammals (Peaker, 2002). Its primary function is lactation, the synthesis and secretion of nutritional and protective milk from the mother to the young, credited as essential for the evolutionary success of mammals (Peaker, 2002).

The number and positioning of mammary glands vary among mammals (Veltmaat *et al.*, 2013) but they are frequently present in pairs (*i.e.* 2 pairs in most primates but up to 7-10 pairs in pigs) (Rezaei *et al.*, 2016; Veltmaat *et al.*, 2013). Only rarely are mammary glands present in odd number (*i.e.* in some opossums) (Krause *et al.*, 2006). Male mammals usually have rudimentary mammary glands and nipples with the exception of male mice that lack nipples (Cardiff and Allison, 2012) and the male Dayak fruit bat that has lactating mammary glands (Francis *et al.*, 1994). The human females have two mammary glands that are enclosed within the breasts [**Figure 1**] while female rodents, specifically mice, have 10 mammary glands that are encased within mammary fat pads (Cardiff and Allison, 2012) [**Figure 2**]. Importantly, the organization of the murine mammary gland is fundamentally similar to the human counterpart, and the mouse is therefore a useful model to study mammary gland biology in health and disease (Cardiff and Allison, 2012).

The mammary gland is an elaborate network of continuously branched ducts that extends radially from the nipple, in an architecture that maximizes the surface area within a constrained volume and is thus common in organs whose function is to transport fluid (Gjorevski and Nelson, 2011) [**Figure 1**]. The two-layered mammary epithelial ductal tree is encased by a collagen-rich basement membrane and surrounded by a stroma containing adipocytes, fibroblasts, blood and lymph vessels, nerves and various immune cells (Pellacani *et al.*, 2019) [**Figure 1**]. Historically the stroma has been regarded as a single compartment but recent studies are dissecting and revealing distinct and important roles played by each of these cell populations and the overall microenvironment in normal mammary gland development and function (Polyak and Kalluri, 2010).

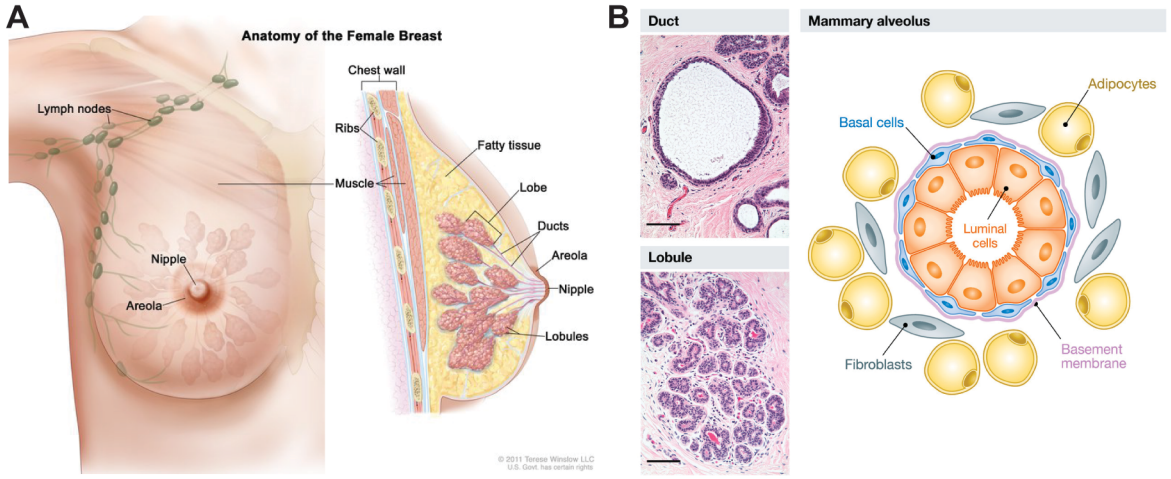

Figure 1 | Regional anatomy, macro- and microscopic structure of the adult human breast.
(A) Schematic diagrams of the mammary gland of an adult human female, located over the pectoralis major muscle of the chest. Mammary gland pyramidal lobules (also named TDLUs) are embedded in fibrous and adipose tissue and connect to a ductal system that extends radially from the nipple.
(B) Histological sections of a duct and a lobule (a cluster of alveoli) and schematic diagram of a mammary alveolus highlighting its assorted basic components. Scale bar 100 micrometers.

Panel A: "Breast Anatomy Female": For the National Cancer Institute © 2011 Terese Winslow LLC, U.S. Govt. has certain rights.
Panel B: From Pellacani *et al.*, 2019

Epithelial cells

The mammary epithelial ductal tree comprises one internal layer of luminal epithelial cells around an empty lumen and an outer layer of myoepithelial cells (also known as basal layer) in direct contact with the basement membrane (Pellacani *et al.*, 2019). The luminal epithelial cells that line the ducts are apically oriented, express keratins 8 and 18 and differentiate into milk-producing alveoli upon hormonal induction (Inman *et al.*, 2015). The basal myoepithelial cells express keratins 5 and 14 and α-SMA that mediates its contractile, smooth muscle-like properties, thereby facilitating the release of milk upon hormone-triggered contraction (Forsyth and Neville, 2009). Additionally, and consistent with the cyclic mammary gland remodeling

occurring over the lifetime of a female, several putative mammary stem and progenitor cell populations have been identified by cell-sorting experiments (Shackleton *et al.*, 2006; Stingl *et al.*, 2006; Visvader and Stingl, 2014).

Adipocytes

Fat-filled adipocytes are the largest population of cells within the mammary fat pad (Bartley *et al.*, 1981). Their role in mammary gland function has been largely overlooked, but recent studies indicate that adipocytes express several key ligands and receptors and are thereby thought to regulate epithelial growth, function and angiogenesis (Hovey and Aimo, 2010). Importantly, adipocytes with reduced lipid content are observed during pregnancy and lactation, suggesting that milk production is a metabolically demanding process that may benefit from this fat reservoir (Gregor *et al.*, 2013).

Fibroblasts

The mammary fibroblasts are found either embedded within the fat pad or in close proximity to the basal myoepithelium (Muschler and Streuli, 2010). Depending on their location, within fatty or collagenous environments, they display substantial differences in protein expression and are thus a heterogeneous cell population (Fleming *et al.*, 2008). Fibroblasts affect mammary branching morphogenesis through direct synthesizes and secretion of a number of growth factors, ECM components and various MMPs (Simian *et al.*, 2001; Wiseman and Werb, 2002). MMPs degrade the ECM and concomitantly facilitate the release of growth factors and cytokines (Simian *et al.*, 2001; Wiseman and Werb, 2002). Fibroblasts can thereby regulate epithelial cell features and phenotype by altering the composition, density and stiffness of the ECM (Luhr *et al.*, 2012).

Blood and lymph

The lymphatic network and the blood vasculature develop in close association with the mammary epithelial tree during puberty (Betterman *et al.*, 2012). Both vascular and lymphatic networks are vital during lactation for carrying nutrients and fluids into milk (Gjorevski and Nelson, 2011).

Immune cells

Various cells of the immune system can be found within the mammary gland stroma (Reed and Schwertfeger, 2010). Macrophages, eosinophils and mast cells have all been shown to regulate branching morphogenesis, in part, by altering the production of ECM and/or its organization near the growing epithelial ducts (Atabai *et al.*, 2007).

1.2.2 Mammary gland development

The mammary gland changes dramatically as it develops and it maintains a remarkable capacity to undergo continuous phases of remodeling and regeneration during adulthood (Cardiff and Allison, 2012). Throughout all these stages, cells of the mammary gland proliferate, differentiate or die, altering the glands architecture to fulfill its function (Inman *et al.*, 2015). In humans and mice alike, the development of the mammary gland can be split into embryonic, pubertal and adult stages, with the majority of mammary gland development occurring postnatally (Gjorevski and Nelson, 2011; Hennighausen and Robinson, 2005).

Embryonic mammary gland development

The embryonic development of the human mammary gland is not well documented though much has been inferred from studies of the cognate murine mammary gland (Veltmaat *et al.*, 2013). In mice, the embryonic mammary development begins during mid-gestation and happens between embryonic days 10.5 and 18.5 (Hens and Wysolmerski, 2005; Robinson, 2007). In all embryos (male and female), two ridges of multilayered ectoderm surface and form the milk lines. This is followed by the formation of five pairs of lens-shaped epithelial thickenings (placodes) that later sink into the mesenchyme forming mammary buds or anlagen (Sakakura *et al.*, 1976). The mammary buds then sprout and branch, transforming into rudimentary ductal structures whose growth is essentially stopped from embryonic day 18 until puberty (Hens and Wysolmerski, 2005). In male mice there is regression of this undeveloped tissue in response to androgens, while in male humans the connection to the nipple is kept (Cardiff and Allison, 2012).

Pubertal mammary gland development

During puberty, high levels of ovarian hormones drive the most prominent stage of mammary branching morphogenesis (Lyons *et al.*, 1958; Sternlicht, 2006). This is when the ends of the rudimentary ducts become multilayered epithelial structures, known as TEBs, where cap epithelial cells form an external layer that surrounds body epithelial cells (Hinck and Silberstein, 2005). TEBs are highly proliferative and lead the invading branch through the fat pad by successive elongation, bifurcation and lateral branching of the rudimentary ducts until the edge of the fat pad is reached (Silberstein and Daniel, 1982; Williams and Daniel, 1983) [**Figure 2**].

Adult mammary gland development

In adult females, the mammary epithelium and the surrounding stroma go through cyclical phases of remodeling and regeneration matching the hormonal changes of the menstrual cycle (Ramakrishnan *et al.*, 2002). Specifically, the side branches along the primary and secondary mammary epithelial ducts form and vanish during each cycle (Hennighausen and Robinson, 2005) [**Figure 2**]. Additionally, the mammary gland heavily changes during each cycle of pregnancy, lactation and involution, an ability that is maintained for several decades until menopause (Hennighausen and Robinson, 2005; Inman *et al.*, 2015). During pregnancy, the

luminal epithelium undergoes rapid proliferation in response to circulating prolactin and differentiates into milk-producing secretory alveoli (Brisken *et al.*, 1999; Oakes *et al.*, 2008). Throughout lactation, milk proteins are produced and secreted by luminal epithelial cells into the lumen. Upon oxytocin release triggered by the suckling infant, the myoepithelial cells contract prompting the milk to flow through the ductal tree to the nipple (Forsyth and Neville, 2009). Upon weaning, the stimuli for milk production are lost and vast apoptosis clears about 80% of the epithelium during involution, the process that returns the gland to a pre-pregnancy state (Walker *et al.*, 1989; Watson, 2006) [**Figure 2**].

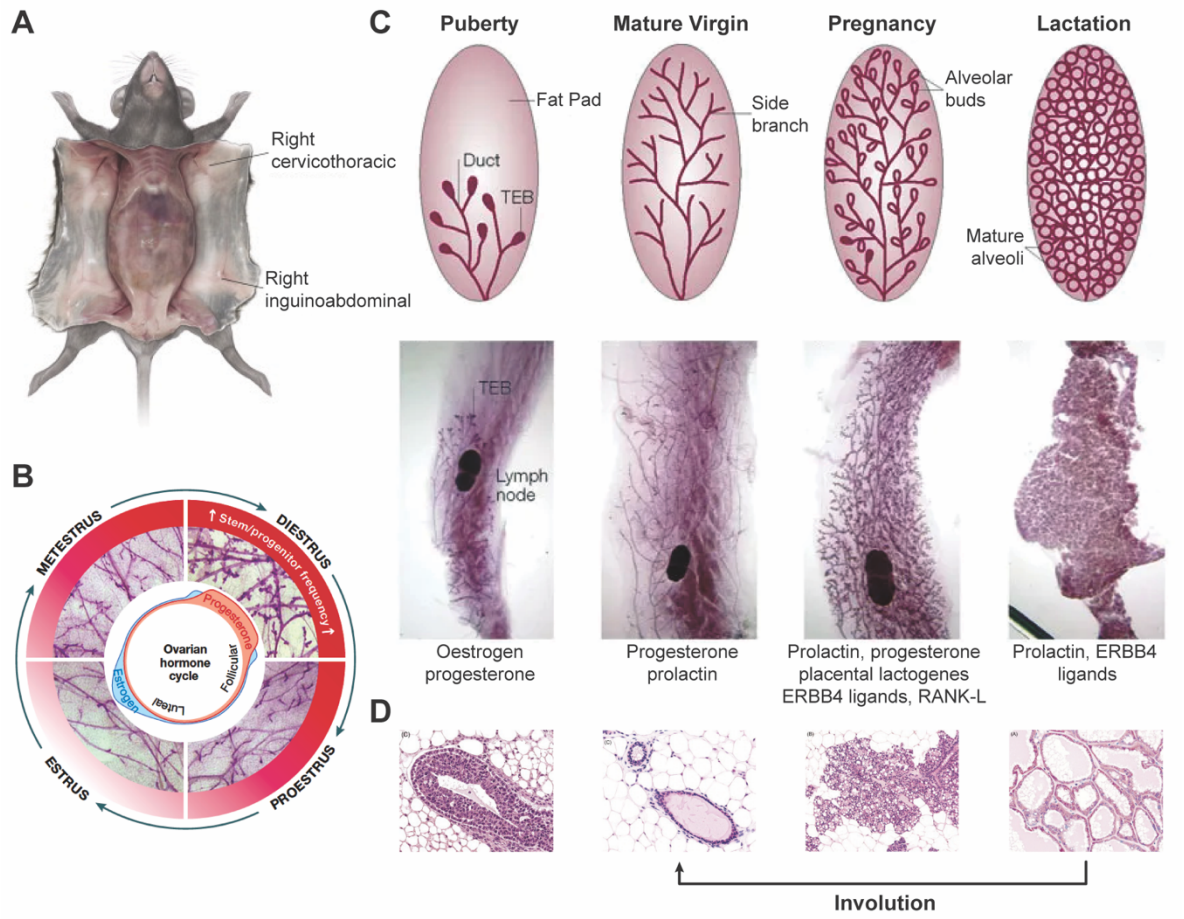

Figure 2 | Macro- and microscopic organization and development of the murine mammary gland.
(A) Illustration of the five pairs of bilaterally located murine mammary glands (three pairs positioned in the cervicothoracic region and two pairs situated in the inguinoabdominal area).
(B) Diagram emphasizing the gross morphological changes elicited by hormones during the murine estrous cycle.
(C) Schematic diagrams and representative mammary gland wholemounts showing the mammary gland architecture and key morphological structures and hormones at the indicated postnatal stages of development.
(D) Histological sections matching the developmental stages specified above to highlight a pubertal TEB, an organized adult duct, a lobule of a pregnant dam and milk-containing vacuoles of a lactating female. Upon weaning, the mammary gland regresses to a nearly pre-pregnancy state (involution).

1.2.3 Integrated signaling in mammary morphogenesis

Global endocrine hormones, such as estrogen and growth hormone secreted by the ovary and the pituitary, signal to mammary epithelial and stromal cells to convey the reproductive status of the system (Ramakrishnan *et al.*, 2002). These endocrine hormones set in motion the crosstalk between the epithelium and the stroma by activating a plethora of local paracrine signaling to induce proliferation, survival and branching (Hennighausen and Robinson, 1998). Local paracrine signals comprise several growth factors and their cognate receptors including EGF, HGF, IGF1, and FGFR2 (Coleman *et al.*, 1988; Kleinberg *et al.*, 2000; Lu *et al.*, 2008; Montesano *et al.*, 1991; Parsa *et al.*, 2008). Conversely, TGFβ serves as a negative regulator of mammary morphogenesis (Daniel *et al.*, 1996). In addition to growth factors and their receptors, MMPs display distinct, spatially localized profiles of expression and activity (Wiseman *et al.*, 2003). For instance, MMP14 is highly expressed in and around the TEBs while its inhibitor, TIMP3, is specifically downregulated at these sites (Szabova *et al.*, 2005); both the epithelial cells and the stroma express low levels of MMP9 (Wiseman *et al.*, 2003); MMP2 is least expressed at sites where lateral branching is likely to occur and MMP3 expression is readily detected in the stroma (Wiseman *et al.*, 2003). MMPs are key local regulators of mammary branching and patterning as indicated by several knockout mouse models (Sternlicht and Werb, 2001). Importantly, ECM degradation by MMP3 during involution, results in mammary epithelial cell death by anoikis (Lund *et al.*, 1996).

The mechanical features of the mammary cell microenvironment have been largely neglected in the past, but it is now increasingly evident that matrix stiffness influences the mammary epithelial phenotype (Levental *et al.*, 2009; Paszek *et al.*, 2005; Schedin and Keely, 2011). For instance, when mammary epithelial cells are grown on floating three-dimensional collagen gels, they self-organize into tubules but fail to do so if attached to collagen-coated culture dishes (with a typical stiffness ranging between 1 and 2 GPa) (Wozniak *et al.*, 2003). Similarly, mammary epithelial cells can produce milk proteins in soft ECM resembling the normal breast environment (typically 400 Pa) but not in more rigid contexts (Alcaraz *et al.*, 2008).

Thus, depending on the exact location of a cell within the epithelial tree, it will be subjected to a specific environment governing a context-dependent functional response.

1.3 THE PANCREAS

1.3.1 The anatomy and physiology of the pancreas

The pancreas is a combined endocrine and exocrine glandular organ that regulates systemic blood sugar levels through the secretion of hormones and participates in food digestion via secretion of digestive enzymes (Dintzis and Liggitt, 2012).

The pancreas of an adult human is solid, white-to-pink-colored, approximately 12–15 centimeters long, 2–9 centimeters wide, weights approximately 50–100 grams and lies in the upper left part of the abdomen, in close association with the upper duodenum (Bockman, 1993) [**Figure 3**]. Three macroscopically distinct parts can be identified in the human pancreas: a C-shaped head aligned with the upper curvature of duodenum; a tail that contacts the hilum of the spleen and a flat narrow body that extends almost horizontally under the stomach (Suda *et al.*, 2006). In mice, the pancreas is rather a diffusely distributed soft tissue (Dolensek *et al.*, 2015).

Two distinct compartments have been identified that relate to the pancreas function as an endocrine and exocrine glandular tissue (Bardeesy and DePinho, 2002) [**Figure 3**].

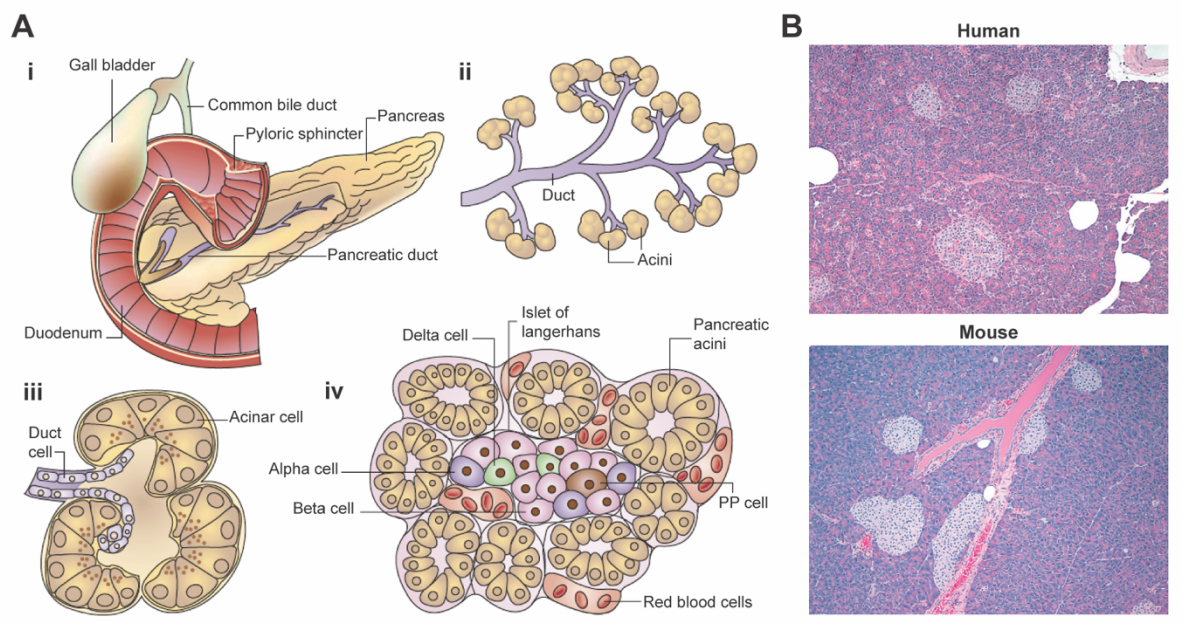

Figure 3 | Endocrine and exocrine pancreatic compartments and constituent cell types.
(A) Schematic macro- and microscopic organization of the adult human pancreatic compartments. **i.** The regional anatomy of the human pancreas. **ii.** Schematic wholemount view of the exocrine pancreas. **iii.** Cross-section highlighting exocrine cells. **iv.** Cross-section highlighting an endocrine Islet of Langerhans and its resident cells. **(B)** Histological sections of human and murine pancreatic tissues.

The exocrine pancreatic compartment makes up over 95% of the pancreatic tissue (Ellis *et al.*, 2017). In the exocrine pancreatic compartment, acinar cells secrete nutrient-digestive enzymes such as trypsin and amylase that are transported through a ductal epithelial system into the duodenum (Bardeesy and DePinho, 2002). The endocrine pancreatic compartment is organized in discrete islets of Langerhans that are composed of multiple cell types that secrete various hormones into the bloodstream (Bardeesy and DePinho, 2002). Specifically, glucagon is secreted by α-cells; insulin is secreted by β-cells; somatostatin by δ-cells; ghrelin by ε-cells and pancreatic polypeptide is secreted by γ [or PP]-cells (Pan and Wright, 2011; Shih *et al.*, 2013). Aberrant function of these cell populations has been linked to both endocrine pancreatic disorders (like diabetes mellitus and endocrine cancer) and exocrine diseases (such as pancreatitis and pancreatic adenocarcinoma) (Dunne and Hezel, 2015; Murtaugh and Keefe, 2015). The widespread prevalence of these diseases provides incentive for continued efforts to broaden our understanding of pancreatic biology that might ultimately pinpoint molecular targets for therapy (Dunne and Hezel, 2015; Murtaugh and Keefe, 2015)

1.3.2 Pancreas development

As with the study of the mammary gland, our understanding of pancreas organogenesis has relied on the mouse as a model to infer fundamental aspects (Jennings *et al.*, 2015).

The pancreas derives from dorsal and ventral buds that outgrow from either side of the primitive foregut endoderm (Zaret and Grompe, 2008). Induction of Pdx1 expression at embryonic day 8.5 is one of the first signs of pancreas development (Guz *et al.*, 1995), followed by Ptf1a expression at embryonic day 9.5 (Kawaguchi *et al.*, 2002). From embryonic days 9.5 to 12.5 cells express both Ptf1a and Pdx1 (Burlison *et al.*, 2008) and further differentiate to generate the cell lineages that ultimately originate all types of pancreatic cells that fulfill its different physiological roles (Bastidas-Ponce *et al.*, 2017; Benitez *et al.*, 2012; Stanger *et al.*, 2007).

1.4 CANCER

Cancer has occurred since antiquity (David and Zimmerman, 2010). The Edwin Smith Papyrus, written around 1600 B.C. in Ancient Egypt, provides some of the earliest descriptions of cancer (Breasted *et al.*, 1930) and evidences for the disease have been found in various archeological and paleontological specimens (David and Zimmerman, 2010). Specifically, an osteosarcoma diagnosed in a fossilized foot bone found in the Cradle of Humankind (the fossil-rich region of South Africa), stands as the earliest known cancer case and dates back to approximately 1.7 million years ago (Franklin *et al.*, 2016). Hippocrates, "the Father of Medicine", first named masses of cancer cells *"karkinos"*, the Greek word for crab around 400 B.C. (Hajdu, 2011). Later on, approximately 28-50 B.C., *"karkinos"* was translated to Latin, *"cancer"*, by the Roman physician Celsus (Hajdu, 2011). Around 130-200 A.D., the word *"oncos"* (meaning swelling in Greek) was used by the Greek physician Galen to describe tumors and this prefix is still prevalent in disease-related terms such as oncology or oncologist (Hajdu, 2011).

Our understanding of cancer, as its nomenclature, has also been evolving over time. Today, we no longer refer to cancer as one single disease, but rather as a large group of disorders (often also called malignant tumors or neoplasms) (Weinberg, 2006). We also consider cancer as a disease of malfunctioning cells that no longer create the form and function characteristic of a normal tissue and that disobey the rules of tissue formation and maintenance (Weinberg, 2006). Cancer cells deviate in behavior from their normal counterparts in a spectrum of *"in betweens"*, progressively away from normal and towards varying degrees of abnormality and aggressiveness (Weinberg, 2006). We are also more aware that tumors contain many cell types that interact and co-evolve with the cancer cells and the local environment, thereby contributing to the complexity of the disease (Hanahan and Weinberg, 2000, 2011).

Despite being an *"old"* disease (David and Zimmerman, 2010) that has been and continues to be extensively studied, fundamental questions linger unresolved and cancer continues to be a heavy burden worldwide. The estimates from the World Health Organization speak for themselves: worldwide, cancer is the second leading cause of death, accounting for approximately 9.6 million (1 in 6) deaths in 2018 and globally, it is expected that one in five men and one in six women will develop cancer during their lifetime, with 1 in 8 and 1 in 11, respectively, dying from the disease (Bray *et al.*, 2018). Thus, substantial efforts must be channeled into cancer research to get us better at preventing, diagnosing and curing cancer (Song *et al.*, 2018; Wild *et al.*, 2019).

1.4.1 Breast cancer

According to the World Health Organization, breast cancer is the most commonly diagnosed cancer in women worldwide (24.2 % of all new cancers) and it is also the leading cause of cancer death in women (15 %) (Bray *et al.*, 2018).

Substantial advances have been made in our understanding of breast cancer over the past 50 years and women diagnosed with breast cancer today, who can access adequate treatment, face a much lower mortality risk compared to the past (Hayes, 2019). However, this is still not the case after metastatic spread (Tevaarwerk *et al.*, 2013). The understanding that breast cancers are biologically different and, as such, amenable to a semi-personalized treatment, is largely behind the relatively successful management of local breast cancer (Hayes, 2019). Dated classification of breast cancers was based on positivity for the hormone receptors for estrogen and progesterone (nearly 70 % of cases), expression of ERBB2/HER2 (nearly 15 % of patients), or negativity for all three markers and thus named triple-negative (the remaining 15 %) (Waks and Winer, 2019). More recently, five major subtypes of breast cancer have been identified (Perou *et al.*, 2000; Sorlie *et al.*, 2001) discernible by the expression of 50 genes (PAM50) as basal-like, luminals A and B, normal-like and ERBB2/HER2 (Parker *et al.*, 2009). Moreover, even more refined clusters of breast cancer have been derived (Curtis *et al.*, 2012).

Breast cancer is often diagnosed on a screening mammogram or by detection of a palpable mass in the breast or axillary region and it is generally non-metastatic at diagnosis (Waks and Winer, 2019). Clinical management of early stage breast cancers aims to eradicate the primary tumor and prevent its recurrence with surgical resection, postoperative radiation and systemic therapies determined by the tumor subtype (Waks and Winer, 2019). Patients carrying tumors that are positive for hormone receptor usually benefit from systemic endocrine (anti-estrogen) therapy that counteracts the estrogen-supported tumor growth. Depending on the menopausal status, systemic endocrine treatment consists of tamoxifen (that competes with estrogen for binding the receptor) or aromatase inhibitors such as anastrozole, exemestane, and letrozole (that inhibit the conversion of androgens to estrogen and thus reduce circulating estrogen levels) (Ignatiadis and Sotiriou, 2013). Patients with tumors expressing ERBB2 may benefit from treatment with the humanized monoclonal antibody directed against HER2 (trastuzumab) or small-molecule tyrosine kinase inhibitors (Gingras *et al.*, 2017). The choice of the systemic therapy, the treatment schedules and the combination of multiple agents is still a matter of ongoing research to optimize clinical outcome and minimize the adverse effects that often accompany the therapy (Ponde *et al.*, 2019; Richman and Dowsett, 2019). Additionally, specific criteria help to pre-emptively identify women at high risk of developing breast cancer (Tharmapalan *et al.*, 2019).

Heterogeneous expression of molecular markers has also been noted within the same tumor and discordance between primary tumors and their metastases has also been observed (Lindstrom *et al.*, 2012). Thus, both inter- and intratumor heterogeneity are pronounced features of breast cancers that complicate the clinical management of the disease (Zardavas *et al.*, 2015).

1.4.2 Hallmarks of cancer

Back in the year 2000, Douglas Hanahan and Robert Weinberg proposed the hallmarks of cancer: a list of six common traits or capabilities shared by cancer cells that facilitate the growth of tumors and their metastatic spread (Hanahan and Weinberg, 2000). A decade later, the list of hallmarks was updated (Hanahan and Weinberg, 2011). Their publications also featured the categorical role played by the microenvironment in tumorigenesis and provided a remarkable framework that helped the scientific community to rationalize the abysmally complex biology of cancer [**Figure 4**].

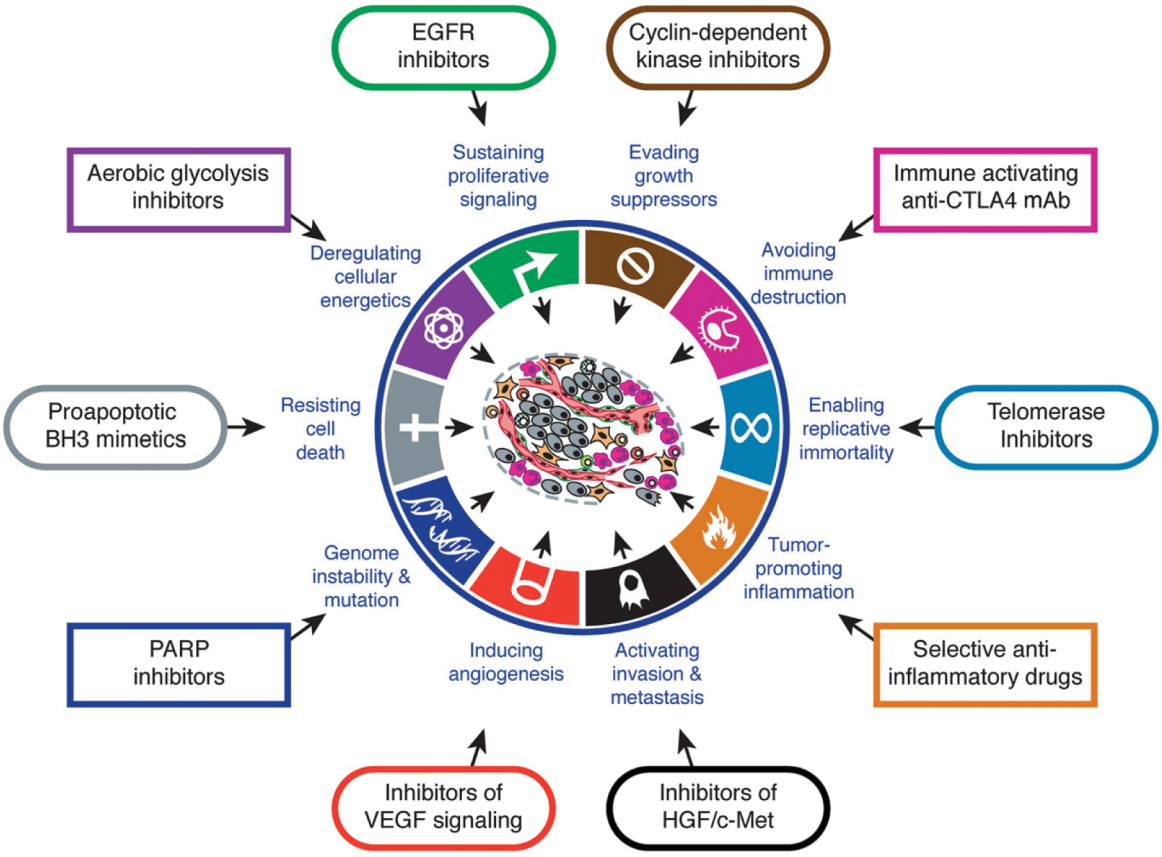

Figure 4 | The hallmarks of cancer according to Douglas Hanahan and Robert Weinberg.
Schematic diagram showing the 6 core hallmarks **(1-6)**, the 2 emerging hallmarks **(a-b)** and the 2 enabling characteristics **(A-B)** shared by cancer cells. Key features of each represent potential therapeutic opportunities. **(1) Sustaining Proliferative Signaling**. The first hallmark poses that cancer cells are self-sufficient in growth signals, *i.e.* they can stimulate their own growth. **(2) Evading growth suppressors.** Cancer cells are insensitive to inhibitory signals that might otherwise stop their growth. **(3) Resisting cell death.** Cancer cells are able to evade programmed cell death mechanisms such as apoptosis. **(4) Enabling replicative immortality.** Cancer cells express telomerase that confers them with limitless replicative potential; they can multiply virtually indefinitely. **(5) Inducing angiogenesis.** Cancer cells stimulate blood vessel growth for nutrient replenishment. **(6) Activating invasion and metastasis.** Cancer cells can locally invade the tissue and spread to noticeably distant sites. **(a) Deregulating cellular energetics.** Cancer cells display abnormal metabolic pathways. **(b) Avoiding immune destruction.** Cancer cells can evade elimination by the immune system. **(A) Genome instability and mutation**, that generate diversity. **(B) Tumor-promoting** inflammation, that promotes several of the hallmarks of cancer.

1.5 CELLULAR SENESCENCE

In 1961, Leonard Hayflick and Paul Moorhead coined the term "cellular senescence" when describing that primary normal human fibroblasts have a limited proliferative capacity (of about 50 to 60 population doublings) when propagated in culture (Hayflick and Moorhead, 1961). At that time, their work was rejected by a prominent journal and their observation was considered an artifact by a scientific community devoted to the dogma that cultured cells replicate indefinitely if provided the proper milieu *in vitro* (Shay and Wright, 2000). Eventually, the Nobel Prize–winning discovery of telomerase and telomere shortening (Greider and Blackburn, 1985, 1989; Szostak and Blackburn, 1982) provided an explanation to the so-called "Hayflick limit". It is now recognized that proliferating normal cells that lack telomerase expression undergo progressive telomere erosion with every cell division that ultimately exposes an uncapped chromosome end triggering a permanent DDR (Shay, 2016). This establishes "replicative senescence" or "premature senescence", that halts the proliferation of these damaged cells (Shay, 2016). Senescence has since been assumed to contribute to aging, but this was only attested when selective elimination of p16INK4A-positive senescent cells delayed ageing-associated disorders (Baker *et al.*, 2011) and extended the lifespan of mice (Baker *et al.*, 2016). Subsequently a wide range of cellular stressors / triggers including persistent DNA damage caused by cytotoxic agents, epigenomic alterations and oxidative stress have been shown to evoke a phenotypically similar senescence response (Collado and Serrano, 2010).

Strikingly, normal cells also responded to oncogene activation by undergoing senescence, a phenomenon known as OIS (Serrano *et al.*, 1997). Similarly, loss of tumor suppressors such as PTEN or the activation of classic cell cycle inhibitors such as p16INK4A also induced a senescence response (Collado and Serrano, 2010). However, the physiological relevance of OIS was questioned until the identification of senescent cells in pre-malignant / benign states (such as the nevus or colon adenoma) and their scarcity in subsequent established tumors (melanoma or the colon carcinoma, respectively) (Braig *et al.*, 2005; Chen *et al.*, 2005; Michaloglou *et al.*, 2005). These studies therefore suggested that the senescence program can potently prevent cancer by acting as a major barrier to tumorigenesis *in vivo* (Collado *et al.*, 2005; Narita and Lowe, 2005) [**Figure 5**]. The capacity to overcome senescence has since been postulated as a crucial step in the progression from pre-malignant to malignant, but it remains to be elucidated if tumors arise as a consequence of a true senescence bypass or escape (Braig and Schmitt, 2006).

Cellular senescence is thus a general stress-inducible process that imposes a proliferative arrest on damaged cells and complements apoptosis in the maintenance of tissue homeostasis (Rodier and Campisi, 2011). Given that tumors often present with defective apoptotic machinery, senescence is considered an additional safeguard tumor suppressing mechanism (Rodier and Campisi, 2011). Additionally, senescent cells remain viable and can either be cleared by the immune system or accumulate in the tissues (Munoz-Espin and Serrano, 2014) supporting the notion of senescence as an example of antagonistic pleiotropy: a beneficial process that helps

preventing cancer at young age but that becomes detrimental later in life, contributing to age-related decline (Campisi, 2005). However, recent studies showed that cellular senescence also plays fundamental roles, mediated by the secretome, during embryonic development (Munoz-Espin *et al.*, 2013), in cellular reprogramming / plasticity (Banito *et al.*, 2009; Mosteiro *et al.*, 2016) and in tissue repair and regeneration (Demaria *et al.*, 2014), thereby suggesting senescence as a broad tissue remodeling process both in normal physiology and pathology (Munoz-Espin and Serrano, 2014; Rhinn *et al.*, 2019).

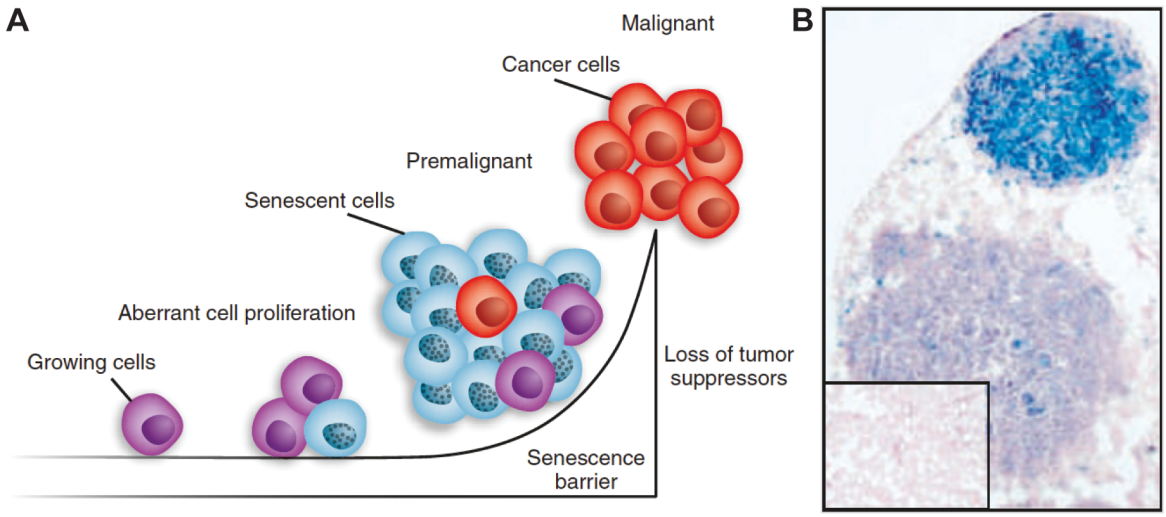

Figure 5 | Oncogene-induced senescence as a barrier to tumorigenesis.
(A) Schematic diagram illustrating how the OIS barrier may be overcome. Upon oncogene activation, normal cells undergo an initial phase of aberrant cell proliferation (purple cells) that is followed by the establishment of senescence, specifically OIS (blue cells). If intact, the senescence program can restrict the growth of the pre-malignant lesion. However, additional events may disable the senescence program (*i.e.* loss of tumor supressors), and cells may overcome the senescence barrier and form malignant tumors (red cells). **(B)** Histological lung section showing abundant SA-β-gal activity (blue) in a pre-malignant KRAS-driven lung adenoma (top) in sharp contrast with a largely negative malignant lung adenocarcinoma (bottom). The inset represents the negative control.

1.5.1 The senescent phenotype

The phenotype of senescent cells is heterogeneous as diverse triggers can provoke it and various mechanisms can enforce it (Hernandez-Segura *et al.*, 2018). Importantly, the traditional view of cellular senescence as a static state has now been challenged and senescence starts to be perceived as a dynamic process (Hoare *et al.*, 2016; Schmitt, 2016).

To date, there are no senescence-specific and universal markers (Sharpless and Sherr, 2015). Instead, senescent cells are often characterized by multiple features including a flattened morphology *in vitro*, a durable proliferative arrest accompanied by the 'gold-standard' increased activity of lysosomal SA-β-gal (Dimri *et al.*, 1995) and a plethora of secreted pro-inflammatory cytokines and chemokines (referred to as SASP or SMS) (Acosta *et al.*, 2008;

Coppe *et al.*, 2008; Kuilman *et al.*, 2008). Various other potential senescence markers have been proposed over the years including the loss of LAMIN B1 (that compromises the integrity of the nuclear membrane) and associated presence of chromatin fragments in the cytoplasm, overexpression of anti-apoptotic BCL-2 family members (conferring apoptosis resistance) and metabolic changes (Hernandez-Segura *et al.*, 2018).

1.5.2 Pro-senescence therapies and the two-hit synthetic lethal strategies

The secretome of senescent cells reinforces the senescence-associated growth arrest via an autocrine loop and may, in a paracrine way, induce senescence in surrounding cells, recruit the immune system for self-clearing or *i.e.* promote cancer in neighboring cells (McHugh and Gil, 2018). So, in essence, cellular senescence plays primarily an advantageous tumor-suppressive role but, in the long term, senescent cells are potentially detrimental due to SASP-related chronic inflammation and the risk of neoplastic conversion of nearby cells (Faget *et al.*, 2019). Thus, researchers are attempting two-hit synthetic lethal strategies that first induce a senescence response in tumors followed by selective elimination of these cells (Dorr *et al.*, 2013). These strategies have shown some success elegantly demonstrated by the laboratories of Van Deursen using the 'INK-ATTAC' transgenic mouse (Baker *et al.*, 2011) and Campisi using the p16-3MR mouse (Demaria *et al.*, 2017; Demaria *et al.*, 2014). Additionally, a very active branch of research works towards the development of senolytics, small-molecule compounds that can selectively eliminate senescent cells (Wang *et al.*, 2019). Examples include ruxolitinib and dasatinib (tyrosine kinase inhibitors) and ABT-263 (inhibitors of the anti-apoptotic BCL2 family) but their true usefulness in clinical settings remains to be seen (Zhu *et al.*, 2015).

1.5.3 NF-κB signaling in senescence

The NF-κB family of transcription factors is composed of five subunits (NFKB1, NFKB2, RELA, RELB and C-Rel) that form homo and heterodimers and regulate the expression of a wide spectrum of pro-inflammatory genes (Zhang *et al.*, 2017).

NF-κB signaling has traditionally been differentiated into canonical / classical and non-canonical / alternative depending on the subunits actively involved (Cildir *et al.*, 2016). In canonical NF-κB signaling, RELA-p50 heterodimers accumulate in the nucleus following the cleavage of NFKB1 into active p50 form. The processing of NFKB2 into p52 and assembly of RELB-p52 complexes characterizes the non-canonical NF-κB pathway (Perkins, 2012).

NF-κB (specifically canonical, RELA-mediated) acts a master regulator of the SASP (Chien *et al.*, 2011; Jing *et al.*, 2011) as many of the SASP components are bona fide NF-κB targets genes (Acosta *et al.*, 2008; Coppe *et al.*, 2008; Kuilman *et al.*, 2008).

1.6 PAK SIGNALING IN DEVELOPMENT AND CANCER

1.6.1 The PAK family

PAKs were first discovered in 1994 by Ed Manser in the laboratory of Louis Lim when screening for proteins that interacted with GTP-bound Rac in rat brain (Manser *et al.*, 1994). Since then, a total of six family members have been identified in mammals (PAKs 1 to 6) that are evolutionarily conserved (Kumar *et al.*, 2017). Mammalian PAKs distribute across two subgroups based on sequence homology and structural differences: the first subgroup includes PAKs 1–3 and the second subgroup comprises PAKs 4–6 (Chan and Manser, 2012). All PAKs display a GTPase- (or p21-) binding domain (GBD / PBD) at the N-terminus and a serine/threonine kinase domain at the C-terminus half that share approximately 50% homology among the family members (Eswaran *et al.*, 2008; Radu *et al.*, 2014). Despite the similarity among PAK gene sequences and their core structures, PAKs exhibit distinct mechanisms of activation where group I PAKs have an AID and require activation, whereas group II PAKs contain an AID-like pseudosubstrate domain and are constitutively active (Kumar *et al.*, 2017) [**Figure 6**].

Our understanding of the biology of PAKs has been greatly influenced by discoveries regarding the founding members of the family subgroups, PAK1 (Manser *et al.*, 1994) and PAK4 (Abo *et al.*, 1998), that are also the most widely studied PAKs (Dart and Wells, 2013). A large body of literature has shown that PAKs can localize in several subcellular compartments and exert their functions through their kinase activity and/or as scaffold-adaptor proteins (Kumar *et al.*, 2017). PAKs receive numerous extracellular and intracellular signals and convey the message via several PAK interacting proteins, downstream substrates and genomic targets, ultimately leading to a phenotypic response (Radu *et al.*, 2014). PAKs have different expression patterns in different tissues (Rane and Minden, 2019) and have both overlapping and unique substrates (Kumar *et al.*, 2017) [**Figure 6**]. Likewise, they have overlapping and unique functions as supported by gene knockout studies. For instance, PAK1 knockout mice are viable and fertile while PAK2 knockout mice are embryonically lethal (Arias-Romero and Chernoff, 2008) and PAK3 knockout mice exhibit mental retardation (Meng *et al.*, 2005). Additionally, PAK4 knockout mice die in early embryonic developmental stage (E11.5) due to heart and neural tube defects (Qu *et al.*, 2003) while PAK5, PAK6 and double PAK5/PAK6 knockout mice are all viable and fertile (King *et al.*, 2014). This thus suggests that PAK2 and PAK4 functions cannot be fully compensated for by other members of the PAK family.

Understandably, abnormalities in PAK signaling disrupt cellular homeostasis and impact cellular functions, with consequences in a vast number of human diseases that span from neurological disorders, to cardiac disease and cancer (Kumar *et al.*, 2017).

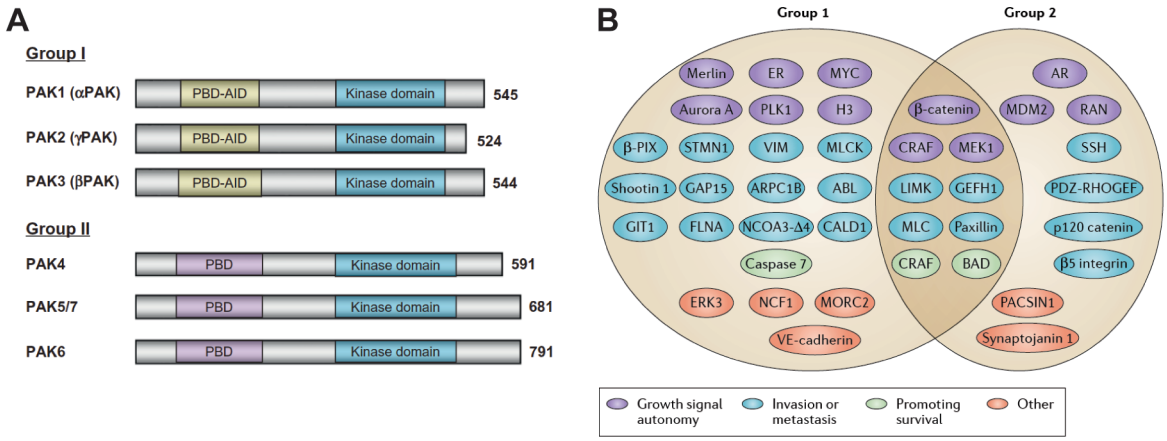

Figure 6 | The family of p21-activated kinases

(A) Schematic diagram of the structural domains of group I and II PAKs. All PAKs share a high degree of similarity at the N-terminus and kinase domain but subgroups show distinct autoinhibitory domains.

(B) Illustration summarizing recognized PAK substrates and their roles in cancer hallmarks. While there is some overlap between the groups, subgroup-specific substrates have been identified.

Panel A: Reprinted from Advances in Cancer Research, 130, R. Kumar, D.-Q Li, PAKs in Human Cancer Progression: From Inception to Cancer Therapeutic to Future Oncobiology, Pages 137-209, Copyright (2016), with permission from Elsevier.

Panel B: Reprinted by permission from Springer Nature: Nature Reviews Cancer PAK signaling during the development and progression of cancer, Maria Radu, Galina Semenova, Rachelle Kosoff, Jonathan Chernoff, COPYRIGHT (2013)

1.6.2 PAK4 and the hallmarks of cancer

A large body of literature shows that PAKs-mediated signaling is vital to a variety of hallmarks of cancer (Radu *et al.*, 2014) [**Figure 6**]. The same applies to PAK4. Succinctly, PAK4 promotes anchorage independent growth (Callow *et al.*, 2002; Qu *et al.*, 2001), protects cells from certain apoptotic stimuli (Gnesutta and Minden, 2003; Gnesutta *et al.*, 2001) and has a well proven role in the regulation of cell adhesion and migration namely, through interaction with αvβ5 integrin to regulate adhesion dynamics (Li *et al.*, 2010a; Li *et al.*, 2010b; Zhang *et al.*, 2002). Moreover, several studies provide evidence to suggest that PAK4 is involved in controlling several aspects of proliferation (Dart and Wells, 2013; Rane and Minden, 2019) and contributes to drug-resistance (Moon *et al.*, 2015; Zhuang *et al.*, 2015).

1.6.3 Clinical relevance of PAK4 in cancer

Human cancer cell lines originated from various tissues frequently display PAK4 overexpression (Callow *et al.*, 2002). The same is true for the more tumorigenic cell lines of the MCF10A progression series (So *et al.*, 2012). Importantly, PAK4 overexpression has been reported in several human tumors. These include a small set of human breast cancer specimens (Bi *et al.*, 2016; He *et al.*, 2017; Liu *et al.*, 2010), pancreatic cancer (Tyagi *et al.*, 2014), ovarian cancer (Siu *et al.*, 2010), prostate cancer (Park *et al.*, 2018), gallbladder cancer (Kim *et al.*, 2008), gastric cancer (Ahn *et al.*, 2011; Kobayashi *et al.*, 2016), endometrial cancer (Siu *et al.*, 2015), gliomas (Kesanakurti *et al.*, 2012) and hepatocellular carcinoma (Xu *et al.*, 2016).

In some instances, PAK4 overexpression has been correlated to *PAK4* gene amplification such as in pancreatic (Chen *et al.*, 2008; Kimmelman *et al.*, 2008) and ovarian cancers (Davis *et al.*, 2013). Even though the *PAK4* gene sits in a chromosomal region (19q13.2) frequently amplified in basal-like breast cancers (Yu *et al.*, 2009), *PAK4* amplification was only detected in about 2 % of TCGA breast tumors (Kumar and Li, 2016).

Notably, high levels of PAK4 expression correlate to poor patient prognosis in ovarian cancer (Siu *et al.*, 2010), non-small cell lung cancer (Cai *et al.*, 2015; Wang *et al.*, 2016), gastric cancer (Kobayashi *et al.*, 2016), endometrial cancer (Siu *et al.*, 2015), prostate cancer (Park *et al.*, 2018) and in endocrine-positive breast cancer patients (Li *et al.*, 2019; Santiago-Gomez *et al.*, 2019; Zhuang *et al.*, 2015).

1.6.4 Inhibitory molecules targeting PAK4

Given the established links between PAK4 and cancer, great efforts are ongoing to develop PAK4 inhibitory molecules to be used as either first line or adjuvant cancer treatment (Rane and Minden, 2019).

Nearly a decade ago, Pfizer developed PF-3758309, an ATP-competitive inhibitor that potently binds PAK4, inhibits PAK4-dependent phosphorylation of GEFH1 and reduces proliferation and anchorage-independent growth across a panel of cancer cells of diverse origin (Murray *et al.*, 2010). Despite designed to specifically target PAK4, PF-3758309 showed broad activity towards other PAKs and other members of the kinome (Murray *et al.*, 2010). Unfortunately, it failed to progress past an early-phase human clinical trial due to undesirable pharmacokinetic characteristics, namely poor bioavailability with accompanying lack of tumor response and adverse side effects (Thillai *et al.*, 2018).

Compound 17 (also known as GNE-2861) is a potent inhibitor developed by Genentech that demonstrates selectivity for group II PAKs (Staben *et al.*, 2014). It reduces viability, decreases migration and invasion and enhances tamoxifen-sensitivity (Zhuang *et al.*, 2015). Disappointingly, this compound also shows poor bioavailability that may be due to poor permeability and/or high efflux (Rudolph *et al.*, 2015).

A number of additional inhibitors of PAK4/PAKs activity have been reported. LCH-779944 has yet to be tested *in vivo* but it has been shown to inhibit PAK4 kinase activity and reduce cell proliferation and invasion (Rane and Minden, 2019).

More recently, a novel class of PAK4 allosteric modulators (KPT-8752 and KPT-9274) has been presented that reduce PAK4 protein levels, rather than only targeting PAK4 kinase activity, most likely by binding to and destabilizing the protein (Abu Aboud *et al.*, 2016; Rane *et al.*, 2017). Given the kinase-independent functions reported for PAK4 and the fact that the protein is often overexpressed in cancer, such compounds could prove useful in a cancer setting. KPT-9274 is currently in phase I clinical trial (NCT02702492).

2 AIMS

General aim

Signaling by PAK kinases regulates central aspects of tissue homeostasis. The research articles included in this thesis contribute novel evidence towards a better understanding of the role of PAK4 in normal mammary gland and pancreatic function and in breast cancer, provide novel transgenic mouse models to study PAK4 signaling in health and disease and propose original mechanistic insight.

Specific aims

Paper I: To develop a novel transgenic mouse model with conditional PAK4 depletion in the mammary epithelium and to characterize its phenotype.

Paper II: To develop a novel transgenic mouse model with conditional PAK4 depletion in the pancreas and to characterize its phenotype.

Paper III: To elucidate novel functions and signaling mechanisms of PAK4 in breast cancer.

3 METHODOLOGICAL CONSIDERATIONS

Detailed descriptions of the materials and methods can be found in the corresponding papers. This section rather brings up some general considerations, advantages and limitations regarding the experimental models used in this thesis.

3.1 TOOLKIT FOR STUDYING DEVELOPMENT AND DISEASE

A variety of tools are available to the present-day scientist to modulate and study protein/cellular function *in vitro*, *in vivo* and *ex vivo* and several experimental model systems are currently available to study developmental and cancer biology.

The various experimental models range from *in vitro* to *in vivo* setups and largely vary in complexity. One important aspect is that models should be seen as complementary, with each model presenting benefits and weaknesses when used to address specific questions. It is thus important to identify the optimal model for the right purpose, making compromises, and to acknowledge that reductionist approaches are often needed to untangle and understand the contributions and interactions of the various components to a level that wouldn't be achievable *i.e.* in more physiological *in vivo* settings.

For example, in **Paper III**, we have used various animal models (transgenic mice and xenografts), primary and established cancer cell lines, patient-derived cells and even recombinant proteins to test hypotheses using laboratory techniques that ranged from more traditional and reductionist (such as radioactive kinase assays) to state-of the art and global methods (such as exome and RNA-Sequencing). Additionally, clinical datasets were used to identify correlations, with hopes that such a broad approach would be better to unravel potential roles of PAK4 in breast cancer.

3.1.1 Cell lines

The first human cancer-derived cell line, HeLa, was established in 1951 from cervical cancer cells harvested from Henrietta Lacks (Masters, 2002). Ever since, a large number of cancer cell lines have been established from many tumor types and various panels of cancer cell lines per tumor type have been assembled (such as the National Cancer Institute NCI-60 panel) (Shoemaker, 2006). Cancer cell lines have been traditionally grown on artificial 2D monolayers on plastic, substantially missing important features of the complex tumor microenvironment, and thus constitute an obviously reductionist model system of cancer. Nevertheless, cell lines are relatively easy to propagate and manipulate in the laboratory, low cost and therefore remain widely used and an important source of information.

It is important to keep in mind that clonal evolution and positive selection continues in culture leading to cell divergence over time, which helps to explain why different stocks of widely used cancer cell lines are highly heterogeneous in their genome, transcriptome and response to therapies (Ben-David *et al.*, 2018). Thus, it is of utmost importance to control the origin, culture growth conditions, the cumulative passaging of cells and eventual contaminants (*i.e.* mycoplasma) to minimize their influence in experimental outcomes. Additionally, when plausible, experiments should be carried out in a microenvironment that better mimics the original cell context. To this end, systems that have been developed include 3D organoid cultures, the use of various ECM coatings and substrates of varied stiffness or modulation of oxygen to more physiological levels.

To overcome some of the limitations of cancer cell lines, scientists have been adopting patient-derived cancer models that are closer to the patient and thus more likely to reflect the patient's disease (Hidalgo *et al.*, 2014). However, these models are likely more heterogeneous and still subjected to the same evolutionary pressures during propagation.

Normal cells, cancer cell lines of various origins and patient-derived cells have been used in **Paper III** to test *in vitro* and *ex vivo* effects of PAK4 depletion or overexpression and to identify the molecular mechanisms involved. Detailed characteristics of the breast cancer cell lines used can be found in the **Supplementary Table 1** of **Paper III**.

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3.1.2 Mouse models

Despite the ethical concerns that involve experiments with laboratory animals, the study of tissue development and tumorigenesis commonly employs mouse models where cells of interest are within a more natural environment. All three papers included in this thesis extensively relied on mouse models to derive new knowledge. Fortunately, the use of laboratory animals in Sweden requires well-founded ethical permission and obeys to very high standards of animal welfare. Reducing the number of required animals and minimizing their potential suffering should nevertheless always be in the mind of the researcher.

Cell line-derived models

The most commonly used mouse models in basic and translational cancer research rely on the inoculation into mice of *in vitro* expanded cancer cells (Gengenbacher *et al.*, 2017). Murine cancer cells can be allografted into syngeneic, immunocompetent mice while human cancer cells can be xenografted into immunocompromised mice. Depending on the route of inoculation, tumors then form relatively fast and synchronously subcutaneously (thus more easily monitored), orthotopically (better mimicking tumor growth in its original organ) or systemically (if cells are intra-peritoneally, intravenously or intracardially injected for studying metastatic disease). The use of a standard cell line may present as advantageous by resulting in

a more homogenous response within treatment groups, thus facilitating comparisons and conclusions. Additionally, cancer cells can be manipulated *in vitro* prior to inoculation or established tumors can be subjected to treatments. Breast cancer cells with stable PAK4 knockdown or control cells were xenografted onto the back of immunodeficient mice in **Paper III**, to validate *in vivo* the previously acquired *in vitro* data.

Genetically engineered mouse models

Genetically engineered mouse models inform on the biological role of genes in a physiological context and rank as the second most commonly used mouse model in cancer research (Gengenbacher *et al.*, 2017).

The most common strategies for generating genetically engineered mice include transgene overexpression and conventional or conditional gene knockin and knockout. The latter were only possible due to the isolation of mouse embryonic stem cells and the groundbreaking discovery of homologous recombination (Capecchi, 2001; Evans, 2001; Smithies, 2001).

In conventional knockouts, the gene of interest is inactivated by disrupting its open reading frame thus blocking its expression or, alternatively, by deleting critical exons for gene function. Constitutive knockout may cause embryonic lethality, if the gene is essential during development, and impede further investigation beyond a specific embryonic stage. This is the case in mice with constitutive PAK4 knockout (Qu *et al.*, 2003). To overcome this problem, conditional mouse models were generated based on the Cre/loxP system (that relies on site-specific recombinase) and allow a spatially and temporally controlled gene expression (Kim *et al.*, 2018). This strategy has been employed to study PAK4 *in vivo* after embryonic day 11.5 (Nekrasova and Minden, 2012; Tian *et al.*, 2009; Tian *et al.*, 2011).

This thesis contributes three novel PAK4 mouse models: two conditional knockouts with PAK4 depletion in the mammary epithelium (**Paper I**) and in the pancreas (**Paper II)** and a mouse model with PAK4 overexpression in the mammary epithelium (**Paper III**).

MMTV-LTR, WAP, BLG and cytokeratin 14 are promoters commonly used to drive expression of a gene in the murine mammary epithelial cells. Among these, MMTV-LTR is the most frequently used promoter given its activity in both virgin and lactating females (Vargo-Gogola and Rosen, 2007). This was also the reason why the MMTV-LTR promoter was chosen to express Cre and PAK4 in the mammary epithelium in **Paper I** and in **Paper III**, respectively. It is important to remember that MMTV-LTR expression is sensitive to steroid hormones and peaks during lactation (Taneja *et al.*, 2009) which may be important when interpreting data derived from models with MMTV-driven gene expression. Apart from targeting expression to the mammary epithelium, MMTV-driven expression is often detected in salivary glands, seminal vesicles, skin, erythrocytes, B cells and T cells while little background was observed in the lung, kidney, liver and brain tissues (Henrard and Ross, 1988).

Thus, caution may be needed if expression in any of these additional organs can constitute a confounding factor.

Several transcription factors have been used to create pancreas-specific mouse models. They include cytokeratin 19, nestin, elastase, Mist1, Ptf1a (or p48) and Pdx1 (Magnuson and Osipovich, 2013). The Pdx1 promoter was chosen in **Paper II** to drive Cre expression in pancreatic tissues given its early expression in embryonic development (E8.5), thus targeting both endocrine and exocrine pancreatic cells.

Genetically engineered mouse models have significantly contributed to our understanding of breast cancer initiation and progression because they represent cancer as an evolutionary process where cancer cells develop and grow within their local and systemic environment (Vargo-Gogola and Rosen, 2007). However, it is important to emphasize that none of the currently available models can completely reproduce the complex environment of a human tumor and thus completely recapitulate the disease (Anisimov *et al.*, 2005).

Commonly used mouse models of breast cancer overexpress Erbb2/neu (Guy *et al.*, 1992b; Muller *et al.*, 1988) or the PyMT (Guy *et al.*, 1992a) oncogenes under the MMTV-LTR promoter. The PyMT model develops focal mammary tumors with 100 % penetrance, short latency and resemble, to some extent, the biological markers and stages seen in the progression of human breast cancer (Lin *et al.*, 2003). Males also develop mammary tumors albeit with a longer latency (Guy *et al.*, 1992a). The PyMT model was chosen in **Paper III** to study the effects of PAK4 abrogation in mammary tumorigenesis because PAK4 was found overexpressed in PyMT-driven tumors.

It is well established that different genetic murine backgrounds have an impact on the resulting PyMT-driven phenotype (Davie *et al.*, 2007). The original PyMT model in the FVB/N background (Guy 1992) is often used because of the short tumor latency it exhibits and the very high incidence of metastasis in the lung. However, the PyMT model obtained after continued backcrossing into the C57Bl/6J strain has a much longer latency. This strain seems to be less susceptible and more resistant to PyMT-driven mammary tumorigenesis (Davie *et al.*, 2007). The PAK4 conditional knockout model developed in **Paper I** had been backcrossed to the C57Bl/6J strain and, as such, the PyMT model chosen in **Paper III** matched the strain background of the PAK4 conditional knockout mouse. Conversely, the FVB/strain was the choice to create in **Paper III** the MMTV-PAK4 mice, to enhance the possibility of detecting mammary tumors.

4 RESULTS AND DISCUSSION

Constitutive PAK4 depletion in mice results in embryonic lethality by day E11.5 due to heart defects (Qu *et al.*, 2003). PAK4-null mice also showed neuronal defects and abnormal vascularization (Qu *et al.*, 2003). Therefore, models of conditional PAK4 knockout in various tissues have since been developed (Nekrasova and Minden, 2012; Tian *et al.*, 2009; Tian *et al.*, 2011).

In **Paper I** and **Paper II** two mouse models with conditional PAK4 depletion in the mammary gland and pancreas, respectively, were created and the consequences of PAK4 depletion were analyzed.

In **Paper I**, PAK4$^{fl/fl}$ mice (Tian *et al.*, 2009) were crossed with MMTV-Cre (line D) (Wagner *et al.*, 1997) to target Cre expression to the mammary epithelium and consequently deplete PAK4 expression in mammary epithelial cells. The phenotype of the resulting MMTV-Cre;PAK4$^{fl/fl}$ mice (referred to as PAK4$^{MEp-/-}$) was then compared to MMTV-Cre mice (PAK4$^{MEp+/+}$) because Cre expression has been show to disrupt physiological processes, including lactation (Robinson and Hennighausen, 2011).

As judged by immunohistochemistry, Cre expression was readily detected in over 90% of mammary epithelial cells, in agreement with the substantial reduction in PAK4 expression observed in both luminal and myoepithelial compartments of the mammary ducts. Even though the approach was largely successful, it is important to notice the incomplete penetrance of Cre expression and that some (albeit few) mammary epithelial cells retained PAK4 expression. This was likely a consequence of the stochastic nature of transgene expression characteristic of this model, leading to a previously recognized mosaic pattern of expression (Wagner *et al.*, 2001; Wagner *et al.*, 1997).

Mammary glands harvested from PAK4$^{MEp-/-}$ mice largely resembled those of control, PAK4$^{MEp+/+}$, mice with regards to ductal elongation and branching during postnatal mammary gland development. Consistently, markers of cell proliferation (Ki67-positivity) and invasion (MMPs expression and activity) were detected to a similar extent in both genotypes.

Importantly, the body weight of the offspring of both PAK4$^{MEp-/-}$ and PAK4$^{MEp+/+}$ dams was identical at weaning, suggesting that PAK4$^{MEp-/-}$ mice were able to efficiently nurse their progeny. This observation was also coherent with the identical morphologies of mammary tissues harvested on the second day of lactation.

Altogether, the data suggests that PAK4 is dispensable for the development and function of the murine mammary gland. That contrasts with the previously known role of PAK1 whose depletion in mammary epithelial cells impairs lobuloalveolar development and cell differentiation (Wang *et al.*, 2003).

In **Paper II**, PAK4$^{fl/fl}$ mice (Tian *et al.*, 2009) were crossed with Pdx1-Cre mice (Hingorani *et al.*, 2003) to target Cre expression to early pancreatic progenitors and attain PAK4 depletion in both exocrine and endocrine pancreatic compartments. Immunoblot analyses of whole pancreas lysates revealed the success of this approach given that PAK4 expression was below the detection limit in samples harvested from mice with conditional PAK4 knockout.

Pancreatic tissues harvested from conditional PAK4 knockout mice largely resembled their wild-type counterparts. Histomorphological and immunohistochemistry-based analyses of the exocrine pancreas revealed the expected distribution of ductal and acinar structures, appropriately labeled by anti-cytokeratin 19 and anti-amylase antibodies, respectively. Likewise, analyses of the endocrine pancreatic compartment revealed equal numbers of randomly scattered islets of Langerhans and no difference was observed in islet size between the groups. Additionally, the various cell types were present in the right proportion and localization in the islets: insulin-stained β-cells were abundant and formed the core of the islets that were delimited in the periphery by glucagon-stained α-cells.

Importantly, no discernible differences were detected between the two genotypes regarding body weight at various ages and mice of both genotypes displayed identical glucose induction and clearance curves as assessed with a glucose tolerance test, suggesting that PAK4 depletion does not affect the unchallenged glucose regulatory function.

These results are similar to those obtained with PAK5, PAK6, and double PAK5/PAK6 knockout mice (Furnari *et al.*, 2013, 2014; Nekrasova *et al.*, 2008) but contrast with the previously known roles for PAK1 and PAK3 in pancreatic function. Specifically, PAK1 depletion impaired glucose homeostasis with defects observed in the secretion of insulin and atypical clearance of glucose (Wang *et al.*, 2011) and glucose tolerance after high fat diet was altered as a result of PAK3 depletion (Piccand *et al.*, 2014).

Thus, conclusions of **Paper I** and **Paper II** support the idea that different PAK family members fulfill distinct functions throughout organ development (Kelly and Chernoff, 2012). Furthermore, the lack of overt phenotypes in these new conditional mouse models with PAK4 depletion offered the exciting possibility of using these models to study the role of PAK4 in disease settings. Given the common frequency of breast cancer diagnoses and associated lethality (Bray *et al.*, 2018), the role of PAK4 in breast cancer was explored in **Paper III**.

A potential role for PAK4 in breast cancer has been suggested in the literature (Rane and Minden, 2019) but, until now, the hypothesis remained untested *in vivo*. To address it, a mouse model with PAK4 overexpression in the mammary epithelium was established using the MMTV-LTR promoter in an inbred FVB/N strain. Mammary glands of virgin MMTV-PAK4 mice were phenotypically indistinguishable from their wild-type counterparts until the age of 6 months, when signs of hyperplasia and mammary lesions became apparent. By the age of 20–24 months (approximately equivalent to 56–69 years old humans (Flurkey *et al.*, 2007)), 25 % of MMTV-PAK4 mice developed palpable mammary tumors while all age-matched wild-type FVB/N females remained tumor-free. It is important to highlight that the incidence

of mammary tumors upon PAK4 overexpression was analyzed in cohorts of virgin, nulliparous females, thus contrasting with studies where tumor incidence is analyzed after multiple pregnancy and lactation cycles to maximize MMTV-driven transgene overexpression that peaks during lactation under hormonal influence (Taneja *et al.*, 2009). The relatively low penetrance and long latency of tumors developed in mice overexpressing wild-type PAK4 is comparable to the phenotype exhibited upon overexpression of a catalytically active PAK1 mutant that caused mammary tumors in 20% of transgenic females (Wang *et al.*, 2003; Wang *et al.*, 2006). Both results suggest that PAK4 and PAK1 act as relatively weak mammary oncogenes and are likely facilitators of tumorigenesis driven by other oncogenes. This hypothesis was corroborated by the occasional G12C and G13D activating RAS mutations detected by whole exome sequencing in mammary tumors harvested from MMTV-PAK4 mice and is in line with inhibited tumorigenesis in a KRAS-driven transgenic mouse with PAK1 deletion (Chow *et al.*, 2012). Given the high prevalence of oncogenic RAS mutations in approximately 30% of all human cancers and in over 90% of pancreatic cancers (Prior *et al.*, 2012) and the limited success in pharmacologically targeting mutant RAS (O'Bryan, 2019), PAKs may be attractive therapeutic targets for RAS-driven tumors.

The PyMT transgenic breast cancer mouse model (Guy *et al.*, 1992a) overexpressed PAK4 and thus emerged as a suitable system to study PAK4 depletion in mammary tumorigenesis. The PyMT model does not harbor PAK4 amplification (Rennhack *et al.*, 2017) but the oncoprotein binds to and co-opts several oncogenic signaling pathways including the RAS and PI3 kinase pathways, which are thus candidates for causing PAK4 overexpression in this model (Lin *et al.*, 2003). To that end, the mouse model with conditional PAK4 depletion in the mammary epithelium created in **Paper I**, was crossed with MMTV–PyMT mice to generate cohorts of MMTV–PyMT;MMTV-Cre;PAK4$^{fl/fl}$ (PyMT;PAK4$^{MEp-/-}$) and MMTV–PyMT;MMTV-Cre (PyMT;PAK4$^{MEp+/+}$) control mice. By 12 weeks of age, PyMT;PAK4$^{MEp-/-}$ females exhibited significantly less lesions and hyperplasia than the control cohort. Consequently, mammary tumors were first palpable considerably later in PyMT;PAK4$^{MEp-/-}$ females which, in turn, matched their extended overall survival.

Interestingly, male mice also develop PyMT-driven mammary tumors that exhibit a slower kinetics of tumor initiation and progression compared to the accelerated female model (Guy *et al.*, 1992a). Importantly, the differences in tumor latency prompted by PAK4 abrogation were even better discerned in this male model, further supporting the important impact of PAK4 in mammary tumorigenesis. While breast cancer is common in females and is universally studied in females, male breast cancer is a rare disease. Male breast cancer in human usually presents at later and more advanced stages and, as a consequence, survival rates are lower for men and have not improved over the recent years as female outcomes have (Anderson *et al.*, 2010). Thus, there is an obvious importance of also studying male breast cancer.

At this stage, it was important to recognize the complexity and the caveats inherent to the model system used and to evaluate how the experimental outcome may have been affected. PyMT-driven tumors could develop in all ten murine mammary glands simultaneously; often multiple

tumors arose per gland, until eventually, a faster/dominant tumor reached the humane endpoint determined by the ethical application (and that was used as a proxy for overall survival). Adding to this already heterogeneous model, MMTV-driven Cre expression often results in a mosaic pattern of target gene depletion as previously identified in **Paper I** and in the literature (Pylayeva *et al.*, 2009; Wagner *et al.*, 1997). Furthermore, given that Cre and PyMT expression is not directed to the exact same cells, stochastically, some cells could co-express Cre and PyMT while other cells could express only Cre, PyMT or none of the transgenes. Given the strong nature of the PyMT oncogene, cells with PyMT expression would very likely be the only cells that contribute to tumors. To grasp the extent of Cre-mediated recombination and the efficiency of PAK4 depletion, PAK4 and Cre expression was analyzed in the dominant tumors of both cohorts at the humane experimental endpoint. Immunoblot analyses of whole tumor extracts and immunostaining of tumor sections with an anti-PAK4 antibody (only suitable to detect high PAK4 expression) revealed substantial, although incomplete, PAK4 depletion in tumors harvested from the PyMT;PAK4$^{MEp-/-}$ group. Additionally, to evaluate the spatial distribution of the remaining PAK4 expression and the discernment of hypothetical discrete subpopulations of tumor cells with differential PAK4 expression, *in situ* hybridization (RNAScope) and a semi-quantitative scoring system to detect low and high PAK4 mRNA levels in murine mammary tissues was set-up. Results of *in situ* hybridization revealed that PAK4 expression was lower in early lesions but readily detected in late-stage control tumors (PyMT;PAK4$^{MEp+/+}$) that also presented small cell sub-populations with undetectable PAK4 (such cells are likely unaffected by Cre-mediated PAK4 depletion and may have contributed to the tumors that arose in PyMT;PAK4$^{MEp-/-}$ mice). Importantly, *in situ* hybridization analyses revealed substantial PAK4 expression remaining in tumor areas of PyMT;PAK4$^{MEp-/-}$ mammary tissues, indicating that PAK4 depletion was incomplete and suggesting that remaining PAK4 may have contributed to tumors. Thus, the effect of PAK4 ablation in mammary tumorigenesis was likely underscored in this model system. Consistently, a mosaic pattern of Cre expression was identified that was particularly obvious in mammary tumors derived from PyMT;PAK4$^{MEp-/-}$ mice where Cre expression was substantially lower.

The prominent effect of PAK4 abrogation in reducing hyperplasia and lesions in 12 weeks old mammary glands and the selectivity against Cre-positive (thus PAK4 depleted) cells in late-stage tumors suggested that PAK4 potentially acts at an early stage of tumorigenesis, when senescence (specifically OIS) acts as a recognized barrier to early tumor development (Collado *et al.*, 2005; Narita and Lowe, 2005). Given that tumors harvested from MMTV-PAK4 mice often displayed oncogenic RAS mutations, the hypothesis that PAK4 may facilitate RAS-driven cancers by overcoming the senescence barrier was tested using an established model where human mammary epithelial cells harbor inducible H-RAS-V12 and display a typical OIS growth arrest upon 4-OHT treatment (Borgdorff *et al.*, 2010). Indeed, upon PAK4 overexpression, H-RAS-V12-induced HMECs retained some proliferative capacity, suggesting that PAK4 overexpression confers a selective advantage to cells and offering an explanation for the high PAK4 expression levels often seen in cancer. Conversely, a senescence-like response (characterized by growth arrest accompanied by additional

senescence-associated features such as a flat and enlarged cellular morphology, increased SA-β-gal activity, and senescence-associated gene expression changes) was restored in a large panel of cancer cells subjected to siRNA-, shRNA- or CRISPR/Cas9-mediated PAK4 inhibition *in vitro*. Importantly, evidence of a senescence-like response upon PAK4 inhibition was observed in three *in vivo* settings. First, SA-β-gal activity was compared in mammary tissues harvested from 8–11 weeks old PyMT;PAK4$^{MEp-/-}$ and PyMT;PAK4$^{MEp+/+}$ mice. Although technically challenging (due to the inherent heterogeneity of the model discussed above and given the high abundance of senescence at this stage, as it is known for other cancers (Collado *et al.*, 2005), a modest increase in SA-β-gal activity was found in mammary lesions of PyMT;PAK4$^{MEp-/-}$ mice, as compared to control. Then, PyMT-driven tumors treated with a PAK4 inhibitor (PF-03758309) that was previously shown to block the growth of several tumor xenografts (Murray *et al.*, 2010) also exhibited increased in SA-β-gal activity. Finally, shPAK4-expressing MCF7 breast cancer cells xenografted onto the back of immunodeficient mice resulted in smaller tumors that were also highly positive for SA-β-gal activity as compared to shControl-expressing tumors. These observations suggest that epithelial cancers of various origins, as gliobastomas (Cosset *et al.*, 2017; Franovic *et al.*, 2015), require PAK4 to avoid senescence, an idea that is also consistent with the generalized view of PAK4 addiction in cancer (Radu *et al.*, 2014).

Transcriptome analyses by RNA-Sequencing of Hs578T and BT-549 breast cancer cell lines, 72 hours after siControl- or siPAK4-transfection, revealed a large number of differentially expressed NF-κB family members (namely NFKB1, NFKB2 and RELB) and NF-κB response genes that was consistent with the known role of NF-κB (canonical NF-κB signaling via RELA) in senescent phenotypes (Chien *et al.*, 2011; Vaughan and Jat, 2011). Interestingly, RELB, a noncanonical NF-κB subunit whose role in senescence has remained largely unknown (Iannetti *et al.*, 2014), was upregulated at mRNA and protein level upon PAK4 knockdown and, it was functionally required for growth arrest upon PAK4 depletion in Hs578T breast cancer cells. Furthermore, both endogenous and overexpressed PAK4 and RELB could be reciprocally co-immunoprecipitated from cancer cell extracts, which suggested their interaction in living cells. Additionally, radioactive kinase assays and mass spectrometry-guided mapping identified PAK4 as a RELB kinase that phosphorylates the residue serine 151 (RELB-Ser151). Importantly, expression of phospho-mimicking RELB-S151E (Serine → Glutamic Acid substitution) did not affect Hs578T cell proliferation while wild-type RELB expression readily reduced it, thus consistent with the results presented above. RELB-Ser151 is evolutionarily conserved among species, sits in the DNA-binding RHD of RELB and is directly bound to the DNA backbone via a hydrogen bond as observed in the crystal structure of the complex RELB-DNA (PDB ID: 3DO7) (Fusco *et al.*, 2009). Our computational modeling of the potential effect of Ser151 phosphorylation predicted a weakened binding of RELB to DNA that was validated in an ELISA-based comparison of phospho-mimicking RELB-S151E (Serine → Glutamic Acid substitution), which exhibited defective binding to cognate κb sites, as compared to phospho-null RELB-Ser151A (Serine → Alanine substitution)

and to wild-type RELB. In agreement, overexpression of PAK4 in breast cancer cells also inhibited the binding of RELB to DNA while the knockdown of PAK4 increased it.

Given the important role of post-translational modifications in regulating the transcriptional activity of NF-κB subunits (Baud and Collares, 2016), we further explored consequences of RELB-Ser151 phosphorylation by comparing RELB-S151E to wild-type RELB in a qPCR array designed to include senescence-associated genes that were differentially expressed upon PAK4 knockdown in breast cancer cells as determined by RNA-Sequencing. C/EBPβ, whose role in senescence has been previously demonstrated (Hoare *et al.*, 2016), emerged as the top altered hit. Importantly, C/EBPβ is upregulated upon PAK4 knockdown and co-knockdown of C/EBPβ and PAK4 partially rescued the proliferative arrest induced by PAK4 depletion. Given the recognized roles and feedback loops of NF-κB, C/EBPβ and NOTCH1 signaling (Hoare *et al.*, 2016), it remains to be elucidated if and how these effects are mediated by the SASP and PAK4. Nevertheless, this data added RELB-Ser151 to the short list of known RELB phosphorylation sites (Baud and Collares, 2016) and presented RELB-Ser151 as a site that bears important consequences for tuning RELB transcriptional activity, target gene expression and consequently, for the regulation of cancer cell proliferation.

Importantly, the *in vitro* and *in vivo* findings presented in **Paper III** were largely supported by correlations in patient-derived clinical datasets, in particular the large METABRIC breast cancer cohort that includes RNA, DNA and clinical data for approximately 2000 breast cancer patients (Curtis *et al.*, 2012). PAK4 overexpression was found in breast tumors as compared to normal breast tissues in the METABRIC dataset and validated in two independent datasets of breast cancer (TCGA, 2012; Zhao *et al.*, 2004). Noticeably, PAK4 overexpression was found across all breast cancer subtypes regardless if stratified according to the PAM50 signature (Parker *et al.*, 2009) or the IC10 integrative clusters (Curtis *et al.*, 2012). PAK4 overexpression was associated with poor patient prognosis, specifically shorter disease-specific survival and overall survival. Furthermore, we also found that only approximately 2 % of breast cancer patients carried tumors harboring PAK4 amplification that was nevertheless clinically relevant given that these patients tended to exhibit worse clinical outcome. This is in agreement with the previous notion that PAKs overexpression in breast cancer is likely due to mRNA upregulation (Kumar and Li, 2016). On the mechanistic side, a positive correlation between PAK4 expression and the proliferative score of tumors was observed while PAK4 expression was negatively correlated with an NFKB signature and RELB expression specifically in breast tumors but not in normal breast. Also, the HER2-positive breast cancer patients exhibiting $PAK4^{low}/RELB^{high}$ expression exhibited a better prognosis. Interestingly, this breast cancer subtype shows the highest PAK4 expression and we also detected a positive correlation between HER2 signaling and PAK4 expression in the same dataset.

Finally, patient-derived breast cancer cells were susceptible to growth arrest upon PAK4 knockdown *ex vivo* while primary, non-immortalized, HMECs did not show signs of growth arrest despite further reduction of their low PAK4 levels. This substantiates the concept that PAK4-addicted cancer cells may be selectively targeted.

5 CONCLUSIONS

This thesis contributed novel PAK4 mouse models that invite the study of the role of PAK4 in the physiology and diseases afflicting the mammary gland and the pancreas *in vivo*. Additionally, this thesis comprehensively addressed the role of PAK4 in breast cancer and uncovered a novel function and mechanism by which PAK4 expression in oncogene-activated cells overcomes the senescence barrier and promotes mammary tumorigenesis. Importantly, this selective advantage conferred by PAK4 in early stages of tumorigenesis then becomes a selective vulnerability (an "Achilles heel") of established cancers, where a senescence-like response can be restored upon PAK4 inhibition, via a mechanistic axis linking PAK4 to the noncanonical NF-κB signaling subunit RELB and C/EBPβ. This work thus offers an additional reason for the frequent PAK4 overexpression in cancer and provides further incentive to the assessment of PAK4 as therapeutic strategy in cancer settings.

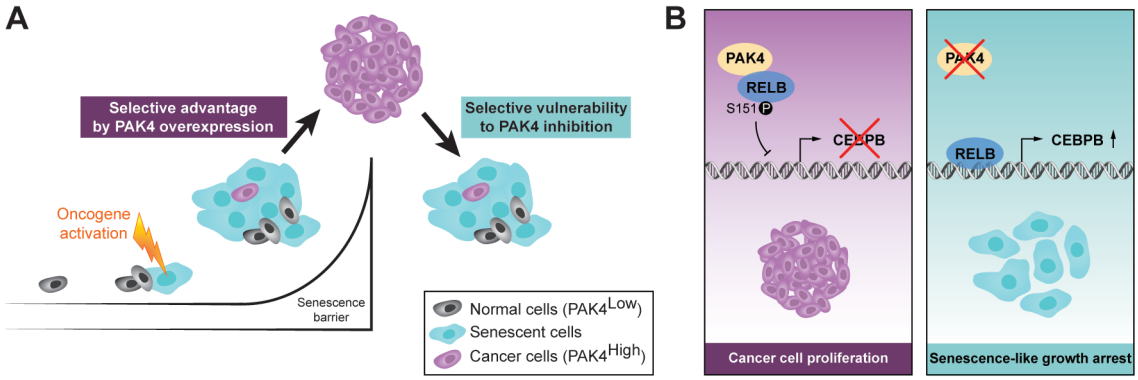

Figure 7 | Schematic summary models for the novel role of PAK4 in breast cancer.
(A) Normal, non-immortalized, human mammary epithelial cells (that express low PAK4 levels, grey cells) normally undergo senescence imposed by oncogenic mutations (OIS, blue cells). In a context of PAK4 overexpression, oncogene-activated cells are able to overcome the OIS barrier and selectively contribute to tumor masses (hence the high PAK4 expression often found in tumors, purple cells). These tumor cells are addicted to PAK4 expression and PAK4 inhibition leads to a restored senescence–like phenotype, a selective vulnerability that may be clinically exploited for cancer therapy.
(B) Mechanistically, PAK4 directly interacts with RELB and phosphorylates RELB–Ser151. In a context of PAK4 abundance (*i.e.* cancer, purple cells), this impairs the binding of RELB to DNA and the transcription of senescence regulators including C/EBPβ, resulting in cancer cell proliferation. Upon PAK4 inhibition, RELB accumulates, which culminates in the expression of C/EBPβ and the induction of a senescence-like growth arrest (blue cells).

Panel A: Inspired by Narita and Lowe, 2005
Panel B: From Costa *et al.*, 2019

6 FUTURE PERSPECTIVES

The coming years will likely warrant a better understanding of fundamental aspects of cancer biology and its core cellular and molecular mechanisms. Additionally, evolving technologies will contribute to our understanding of cancer beyond what we can currently grasp.

Zooming in on the PAKs, nearly twenty-five years have passed from the time when they were first cloned to the current understanding of PAKs as clinically relevant signaling hubs affecting many of the hallmarks of cancer. The substantial evidence that has been gathered by others and in this thesis strongly supports the PAK family members (and specifically PAK4) as suitable targets for cancer therapy. It is therefore anticipated that the scientific community will continue to devote attention to PAK4 and the other PAK family members to better grasp how they signal and function in cancer *versus* normal cells; how their activity is regulated by auto-regulatory mechanisms, intracellular and extracellular cues; their list of known substrates and upstream regulators is likely to increase and unknown feedback loops will likely be exposed. Moreover, there is still a lot to uncover regarding the overlapping and distinct roles for the various PAKs in health and disease and a better understanding of the co-dependence and co-expression of PAKs will likely come about. From a clinical perspective, the usefulness of isoform-specific PAK inhibitors and even PAN-PAK inhibitors as therapeutic targets remains to be ascertained. We will soon learn the outcome of an ongoing clinical trial and additional trials will probably be announced given the ongoing efforts towards the development of better PAK(4) inhibitors.

It is my conviction that empowered with a better understanding of basic biology, we will be better able to clinically tackle cancer and reduce its mortality, a hope that is shared by the World Health Organization and by all of us who have, have had or personally know or have known someone with cancer.

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