# Cancer and Therapy: A look into stemness and the tumour microenvironment

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## A look into stemness and the tumour microenvironment

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## A look into stemness and the tumour microenvironment

Carmen Rodriguez-Cupello

LUND
UNIVERSITY

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**Abstract**

Therapy is the often-used treatment for cancer patients, even those that undergo resection surgery. While milestones have been surpassed throughout the decades for a number of cancer types, there are those that comprise of characteristics limiting effectiveness of treatments. For these, such as some breast and pancreatic cancers, other pursuits must be explored to identify avenues for beneficial therapies.

In this study, we explore mechanisms in breast cancer cells that potentially lead to recurrence. Alterations in both COMP and the STRIPAK complex are able to further affect cellular processes leading to recurrence capability in different ways. Over-expression of COMP leads to activation of Notch which affects both Wnt/β-catenin and AKT pathways, already affected by COMP. The resulting effect is the rise in stem-like cells within the COMP-overexpressing population, able to propagate further even when in limited quantities. Similarly, depletion in the STRIPAK component STRIP1 affects activation of GCKIII kinases and cell cycle disruption through elevated expression of cyclin dependent kinase inhibitors p21 and p27, enhanced levels of which lead to a protective effect from therapeutic treatments and increased proliferation. Both of these altered proteins lead to the eventual ability of cancer cell recurrence.

The tumour microenvironment (TME) contains several other cell types apart from cancer cells which play a role not only in the regulation of the environment but in response to treatments. Cancer associated fibroblasts (CAFs) are vital in their role to affect the TME through manipulation of the structural components and through secreted factors. In attempting to understanding ways to gauge therapeutic response to treatment, a 3D coculture model was established for quick, high throughput analysis of treatment on CAF functionality and subsequent effect on invasion capability. As a component of the TME, a highly specific chondroitin sulfate was investigated as a likely drug target for the purposes of stromal targeting within breast and pancreatic cancers. Through high specificity, targeted treatment can overcome the unfortunate side effects to normal tissue.

In this compiled work, we elaborate on the effect of protein expression alterations and their resulting effect on recurrence capability of cells. We explore signalling alterations resulting in cancer stem cells as well as cell cycle arrest and cell fate determination. Other TME components are investigated for the purpose of anti-stromal therapy as a method to bypass the desmoplastic reaction within certain tumour types.



**Key words:** COMP, STRIPAK, Notch, p21, oncofetal chondroitin sulfate, therapy, tumour microenvironment

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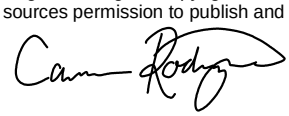
Signature  Date 2022-05-03

# Cancer and Therapy

## A look at stemness and the tumour microenvironment

Carmen Rodriguez-Cupello


LUND
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Cover: Immunofluorescence staining of oncofetal chondroitin sulfate detection by rVAR2 (yellow), epithelial cells by cytokeratin (green), αSMA+ fibroblasts (red), and the nucleus by DAPI (blue) in a sample of human pancreatic cancer by Carmen Rodriguez



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**CAF deposition of Oncofetal Chondroitin Sulfate onto ECM correlates with cancer progression**
**Rodriguez-Cupello C**, Gustavsson T, Madsen CD, Pietras K, Clausen TM, Salanti A - *Manuscript*

# List of Abbreviations

| | |
|---|---|
| αSMA | alpha smooth muscle actin |
| APC | adenomatous polyposis coli |
| ATM | ataxia telangiectasia mutated |
| ATR | AMT- and Rad3-related |
| Axin | axis of inhibitor 1 |
| CAF | cancer associated fibroblast |
| CCM3 | cerebral cavernous malformation 3 |
| CDK | cyclin dependent kinase |
| CDM | cell-derived matrix |
| CKI | cyclin dependent kinase inhibitor |
| COMP | cartilage oligomeric matrix protein |
| CS | chondroitin sulfate |
| CSC | cancer stem cell |
| CSGalNAcT-1 | chondroitin sulfate N-acetyl-galactosaminyltransferase 1 |
| CSL | CBF1/Suppressor of Hairless/Lag-1 |
| CSPG | chondroitin sulfate proteoglycan |
| CTC | circulating tumour cell |
| DNA | deoxyribonucleic acid |
| DS | dermatan sulfate |
| DSL | delta-serrate-lag |
| ECM | extracellular membrane |
| EMT | epithelial-mesenchymal transition |
| ER | estrogen receptor |

| | |
|---|---|
| FBS | fetal bovine serum |
| FOLFIRINOX | 5-fluorouracil, folinic acid, irinotecan, oxaliplatin |
| GAG | glycosaminoglycan |
| GCK | germinal center kinase |
| GOF | gain-of-function |
| HA | hyaluronan |
| HCC | hepatocellular carcinoma |
| Hep | heparin |
| HER2 | human epidermal growth factor receptor 2 |
| Hes | hairy and enhancer of split |
| HIF-1A | hypoxia inducible factor 1 alpha |
| HS | heparan sulfate |
| IHC | immunohistochemistry |
| IKKε | IκB kinase ε |
| IL6 | interleukin 6 |
| KO | knockout |
| KS | keratan sulfate |
| LOF | loss-of-function |
| MAPK | mitogen-activated protein kinase |
| MED | multiple epiphyseal dysplasia |
| MMP2 | metalloproteinase 2 |
| NECD | Notch extracellular domain |
| NICD | Notch intracellular domain |
| ofCS | oncofetal chondroitin sulfate |
| OS | overall survival |
| PanINs | pancreatic intraepithelial neoplasms |
| PCNA | proliferating cell nuclear antigen |
| PDAC | pancreatic ductal adenocarcinoma |
| PDGF | platelet-derived growth factor |

| | |
|---|---|
| PDK1 | phosphoinositide-dependent kinase-1 |
| PEGPH20 | pegvorhyaluronidase alfa |
| PfEMP1 | *Plasmodium falciparum* erythrocyte membrane protein-1 |
| PG | proteoglycan |
| PI3K | phosphatidylinositol-3-kinase |
| $PIP_2$ | phosphatidylinositol (4,5)-biphosphate |
| $PIP_3$ | phosphatidylinositol (3,4,5)-triphosphate |
| PLA | proximity ligation assay |
| PR | progesterone receptor |
| PSACH | pseudo achondroplasia |
| PTEN | phosphate and tensin homolog |
| RB | retinoblastoma protein |
| RTK | receptor tyrosine kinase |
| rVAR2 | recombinant VAR2CSA |
| scRNAseq | single cell ribonucleic acid sequencing |
| SHH | sonic hedgehog |
| SIKE | coiled-coil protein suppressor of IκB kinase-ε |
| SLMAP | sarcolemmal membrane-associated protein |
| STRIP1 | striatin interacting protein 1 |
| STRIPAK | striatin-interacting phosphatase and kinase |
| SV40 | simian virus 40 |
| T-ALL | T-cell acute lymphoblastic leukemia |
| TACE/ADAM17 | tumour necrosis factor-α-converting enzyme |
| TME | tumour microenvironment |
| TNBC | triple negative breast cancer |
| TSP | thrombospondin |

# Popular Science Summary

In many instances for cancer patients, chemotherapeutics are the only viable options for treatment. There are however, varying effects that it can have on the patients and type of cancer involved. Both the cancer cells and cancer associated fibroblasts (CAFs) can be affected by therapies and propagate the effects to their surroundings, however, depending on the function of each cell, the effect of therapy upon it can vary.

In this study, we investigate the effects that alterations of proteins can have on tumour signalling and cell fate when we examine the role of COMP and a STRIPAK complex component, STRIP1, in breast cancer. Enhanced COMP expression within breast cancer is shown to lead to a poorer prognosis in patients while altering pathways such as Notch, Wnt/ß-catenin, and AKT. Consequently, due to these alterations, cancer stem cells (CSCs) increase in the population, being able to evade immune and therapeutic responses and aid in the continued growth of the tumour. In a somewhat similar manner, when STRIP1 is depleted, there is hyperactivation of the GCKIII kinases, leading to cell cycle arrest. Upon treatment with therapy however, these STRIP1-depleted cells better survive the treatment and become more proliferative than wildtype treated cells. The varying effect of therapy on the heterogeneous population of cancer cells conveys the difficulty of identifying proper treatment for patients and the possible eventuality of recurrence.

CAFs similarly play a significant role in the tumour and in therapeutic response, and the identification of new ways to gauge response to therapy is vital. With the difficulty of understanding the functionality of CAFs in the typical 2D culture system, we redeveloped an established 3D coculture model for faster, more high-throughput needs. Within a matter of days, rather than weeks, CAF functionality can be analysed when comparing varying cell lines, cell manipulations, and/or drug treatments. Functionality of CAFs and their effects from therapy is only one aspect of whole role they provide in the tumour microenvironment (TME). CAFs often secrete and deposit material to create the structural extracellular matrix (ECM) which can affect signalling between numerous components. We have identified that CAFs secrete a highly specific chondroitin sulfate, not found in normal tissue outside the placenta. The deposition of this oncofetal chondroitin sulfate (ofCS) is found to increase as cancer progresses. Importantly, the use of a recombinant malarial protein, rVAR2, specifically binding to ofCS, is now providing the

prospect to deliver drugs to the specific tumour areas and decreasing unnecessary toxicity to normal cells.

In conclusion, this study describes several aspects of cancer and the tumour microenvironment. We shed light on the difficulty of treatment for cancer patients as their physiological uniqueness and the uniqueness of the tumours pose distinctive characteristics that can respond differently. We emphasize the continued pursuit of personalised therapies due to molecular signatures and even combination therapies to enhance the specified functions of currently used treatments.

# Populärvetenskaplig sammanfattning

För cancerpatienter är kemoterapi (cytostatikabehandling) ofta det enda möjliga alternativet för behandling. Effekterna av denna typ av behandling varierar dock hos patienterna samt beroende på vilken typ av cancer det gäller. Både cancerceller och cancerassocierade fibroblaster (CAF) kan påverkas av terapier och sprida effekterna till sin omgivning, men beroende på funktionen hos varje enskild cell kan effekten av behandlingen på cellen variera.

I denna avhandling har vi karaktäriserat effekterna som förändringar av proteinerna, COMP och STRIPAK-komplexkomponenten STRIP1, har på tumörsignalering och cellernas utvecklingsöde i bröstcancer. Förhöjt uttryck av COMP i bröstcancer leder till sämre prognos hos patienter samtidigt som det påverkar signaleringsvägar såsom Notch, Wnt/ß-catenin och AKT. Följaktligen, på grund av dessa förändringar, ökar andelen cancerstamceller (CSCs) i cellpopulationen, som undviker både immunsförsvar och behandlingen samt understödjer den fortsatta tillväxten av tumören. På ett nästan likartat sätt, när uttrycket av STRIP1 reduceras sker en hyperaktivering av GCKIII-kinaser, vilket leder till att cellcykeln stannar upp. Vid cytostatikabehandling påvisar emellertid dessa STRIP1-utarmade celler ökad överlevnad och tillväxt jämfört med vildtypsbehandlade celler. Den varierande behandlingseffekten på den heterogena populationen av cancerceller illustrerar svårigheten att identifiera lämplig behandling för patienter och den potentiella risken för återfall.

CAF har på ett liknande sätt en betydande roll i tumören och för det terapeutiska svaret i densamma, och behovet av att identifiera nya sätt att mäta behandligseffekten är stort. På grund av svårigheterna med att förstå funktionaliteten hos CAF i det typiska 2D-odlingssystemet, vidareutvecklade vi en etablerad 3D-samodlingsmodell för snabbare användning i större skala. Inom bara några dagar, snarare än veckor, kan funktionen hos CAF analyseras när man jämför olika cellinjer, cellmanipulationer och/eller läkemedelsbehandlingar. Funktionen hos CAF och deras effekter av behandling är bara en aspekt av alla de möjliga roller de spelar i tumörmikromiljön (TME). CAF utsöndrar och deponerar regelbundet material för att skapa den strukturella extracellulära matrixen (ECM), som kan påverka signalering mellan en rad olika komponenter. Vi har påvisat att CAF utsöndrar ett mycket specifikt kondroitinsulfat, som inte finns i normal vävnad utanför moderkakan. Avsättningen av onkofetalt kondroitinsulfat (ofCS) visade sig öka när cancern fortskrider. En betydelsefull aspekt är att detta möjliggör

användandet av ett rekombinant malariaprotein, rVAR2, som uteslutande binder till ofCS, för att på så sätt kunna leverera läkemedel till specifika tumöromården och därmed minska onödiga toxiska effekter på friska normala celler.

Sammanfattningsvis beskriver dessa studier ett flertal aspekter av tumörer och dess mikromiljö. Vi belyser svårigheten att behandla cancerpatienter då deras fysiologiska särdrag och tumörernas unika karaktärsdrag leder till en stor variation av behandlingens effekt. Vi framhåller den fortsatta strävan efter att finna skräddarsydda cancerbehandlingar inklusive kombinationsterpier på individnivå med hjälp av molekylära signaturer för att ytterligare förbättra egenskaperna hos för närvarande använda behandlingar.

# Abstract

Therapy is the often-used treatment for cancer patients, even those that undergo resection surgery. While milestones have been surpassed throughout the decades for a number of cancer types, there are those that comprise of characteristics limiting effectiveness of treatments. For these, such as some breast and pancreatic cancers, other pursuits must be explored to identify avenues for beneficial therapies.

In this study, we explore mechanisms in breast cancer cells that potentially lead to recurrence. Alterations in both COMP and the STRIPAK complex are able to further affect cellular processes leading to recurrence capability in different ways. Over-expression of COMP leads to activation of Notch which affects both Wnt/β-catenin and AKT pathways, already affected by COMP. The resulting effect is the rise in stem-like cells within the COMP-overexpressing population, able to propagate further even when in limited quantities. Similarly, depletion in the STRIPAK component STRIP1 affects activation of GCKIII kinases and cell cycle disruption through elevated expression of cyclin dependent kinase inhibitors p21 and p27, enhanced levels of which lead to a protective effect from therapeutic treatments and increased proliferation. Both of these altered proteins lead to the eventual ability of cancer cell recurrence.

The tumour microenvironment (TME) contains several other cell types apart from cancer cells which play a role not only in the regulation of the environment but in response to treatments. Cancer associated fibroblasts (CAFs) are vital in their role to affect the TME through manipulation of the structural components and through secreted factors. In attempting to understanding ways to gauge therapeutic response to treatment, a 3D coculture model was established for quick, high throughput analysis of treatment on CAF functionality and subsequent effect on invasion capability. As a component of the TME, a highly specific chondroitin sulfate was investigated as a likely drug target for the purposes of stromal targeting within breast and pancreatic cancers. Through high specificity, targeted treatment can overcome the unfortunate side effects to normal tissue.

In this compiled work, we elaborate on the effect of protein expression alterations and their resulting effect on recurrence capability of cells. We explore signalling alterations resulting in cancer stem cells as well as cell cycle arrest and cell fate determination. Other TME components are investigated for the purpose of anti-

stromal therapy as a method to bypass the desmoplastic reaction within certain tumour types.

# Background

Cancer was generally seen as a disease of old age, but over the decades has been more commonly known to affect millions of individuals regardless of age or gender. Cancer has a number of hallmarks it must obtain throughout tumour development that describe its complex nature[1]. The order in which these hallmarks are obtained is not vital to development and can therefore vary. Importantly, cells must be able to proliferate to a higher degree than they normally would; with this comes the need to resist growth suppressors and cell death signals. As cancer cells will continually replicate, they will require nutrients, either from nearby blood vessels or altering metabolic pathways when vessels are absent. Through multiple rounds of replication, there is a high probability of cells obtaining mutations. These changes allow the cells to evade the immune system and enhance survival. Eventually, cancer cells begin to invade into surrounding tissue and metastasize to other parts of the body[1].

Breast cancer is the most common type of cancer seen in women worldwide. Annually in the U.S., breast cancer accounts for 30% of diagnosed cancers in women, with 1 in 8 women likely to develop invasive breast carcinoma in their lifetime[2]. In Sweden during 2019, roughly 8,300 women were diagnosed with breast cancer[3]. Development of breast cancer can be, in some cases, resulting from a genetic predisposition, but despite this, it serves to allow for early detection and prevention[4, 5].

Breast cancers arise from either the lining of the epithelium surrounding the ducts or the lobules that provide the ducts with milk, known as invasive ductal carcinoma and lobular carcinoma, respectively[6]. Breast cancer can be further divided into several subtypes dependent upon expression of hormonal receptors, leading to varying treatments and survival for each. The markers for breast cancer are: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), while simultaneously considering the proliferative ability of the cells using Ki67[7, 8]. In terms of outcomes and prevalence, the luminal subtypes are the most common, accounting for approximately 65-75% of patients. Both luminal subtypes express ER and PR receptors while the Luminal B subtype can express HER2 and Luminal A does not. In terms of patient outcome, the luminal subtypes have the best outcome, Luminal A more so than Luminal B due to the high proliferation in the latter. The HER2 enriched subtype accounts for around 10% of patients and has a poor outcome. This subtype only expresses the HER2 receptor

and is highly proliferative. The triple negative (TNBC) or basal-like subtype, not expressing ER, PR, or HER2, accounts for around 15% of patients and generally has the worst outcome with high proliferation. Due to the variations between the subtypes, this would also mean that the therapy used differs, with Luminal subtypes receiving endocrine therapies, the HER2 enriched receiving a HER2 targeted therapy, and the triple negative receiving chemotherapy.

Pancreatic cancer is a less common type of cancer, with roughly 3% of diagnosed cancers estimated for this year in the U.S for men and women[2]. However, it accounts for 8% of cancer related deaths for both men and women. In Sweden, pancreatic cancer accounts for 3.4% of diagnosed cancers, roughly 2100 individuals, and 8.3% of cancer related deaths, roughly 2000 individuals[9].

Pancreatic cancer is known for its poor survival rate and lack of viable therapeutic options for most patients[10, 11]. Generally, there are no outward symptoms of the disease and therefore diagnosis happens at later stages than in other types of cancer, to the point where most have already started to metastasize. Only about 20% of patients are diagnosed with a resectable tumour. The initiation cells of pancreatic cancer defines which type it becomes, as the pancreas performs both endocrine and exocrine functions, these are the main types which are found: ductal tumours and neuroendocrine tumours[12]. Approximately 90% of pancreatic cancers are those formed from the cells that are responsible for its exocrine functions, ductal adenocarcinomas. While there may not be a great incidence of pancreatic cancer, it has a roughly 8% 5-year survival rate.

Pancreatic ductal adenocarcinomas (PDAC) have the characteristic of being highly stiff tumours, containing a large amount of extracellular matrix (ECM) composed of collagens and hyaluronan[11]. Due to the stiffness of the tissue, there is a collapse of vasculature, leading to hypoxic compartments within. However, even with the loss of vasculature and typical nutritional sources, the tumour thrives. The development of PDAC takes a very typical route that includes pancreatic intraepithelial neoplasms (PanINs). These early-stage precursors of PDAC have varying grades of complexity within them, known as PanIN I-III, PanIN III being carcinoma *in situ*[13, 14]. Once progressed through the PanIN stages, the tumour begins its invasion of local tissue. Often times, pancreatic cancer will metastasise, with typical regions being the liver, lungs, and/or peritoneum.[15]

Pancreatic cancers majoritively contain *KRAS* mutations which are considered the initiating and maintenance factor, with roughly 90% of patients containing this mutation. This allows for the initiating steps of PanINs and to some degree, the transformation to carcinoma in situ[16-19]. Roughly 50 – 80% of pancreatic cancers contain a secondary, inactivating mutation of either *TP53*, *SMAD4*, or *CDKN2A*. These mutations lead to increased aggressiveness of the tumours and help drive progression.

# Stemness Altering Pathways

Over the years, there have been several theories to explain the initiation of cancer in patients but two models stand out as those that best explain the process, the hierarchical model and the stochastic model. The hierarchical model describes how the population of cells within the tumour are considered to be a heterogenous mixture wherein roughly less than 1% of cells would have tumour-initiating capabilities[20, 21]. These cells would have the traditional characteristics known to stem cells: self-renewing capability, clonal tumour initiation capacity, enhanced evasion from the immune system, phenotypic plasticity, and resistance to both chemotherapy and apoptosis. In the hierarchical model, tumour initiation arises from the resulting immune evasion of a subset of cells leading to tumour formation. This also describes the ability of cancers to recur in patients after treatment[20, 21]. The stochastic model states that all tumour cells are homogenous in their equal characteristic ability to initiate, maintain, and promote tumour growth. Differences between the cells arise from the internal and external signals that the cells receive. Eventually, the differences in signalling allow certain cells to gain oncogenic mutations, initiating cancer[21]. Another model describes the ability of cells to be reprogrammed and revert back, or retro-differentiate, into a stem-like phenotype. This model would describe an intermingling of both the hierarchical and stochastic models, highlighting the importance of signalling factors within the tumour and the resulting effects it can have on survival.

There have been several methods developed in order to identify stem cells within different organs and tissue. For example, well-known markers of breast cancer stem cells are $CD44^{high}/CD24^{low}$, but this is not entirely viable for all breast cancer cell lines as, for example, the MDA-MB-231 cells naturally have a high CD44 expression. Other markers for stemness include: NANOG, OCT4, SOX2, CD133, ALDH1, CD13, CD90, and CD45[22]. The benefit of specific markers for stemness is for the possibility of targeting these specific cells as the initiators of cancer and mediators of recurrence[22].

## Notch Pathway

The Notch family of proteins, consisting of Notch 1, 2, 3, and 4, function as transmembrane receptors which are able to respond to five Delta-Serrate-Lag (DSL) type ligands (Delta-like 1, 3, 4, and Jagged 1, 2)[23, 24]. Notch proteins play a significant role in development of numerous tissues. In the breast, Notch is responsible for alveolar development, maintenance of luminal cell fate, and prevention of uncontrolled basal cell proliferation during pregnancy[23, 25]. In the pancreas, Notch is responsible for endocrine cell differentiation, maintenance of endocrine precursor cells, and control of epithelial branching[23, 26]. In other tissue,

Notch is able to regulate cell adhesion and proliferation, maintain stem cells, and affect immune cell development[27-29].

Activation of Notch requires two cells to interact with one another, their ligand and receptors working in trans. After having received a signal from its ligand, the intracellular domain of Notch localizes to the nucleus to form a transcriptional activator complex in order to affect genes of differentiation, proliferation, and apoptotic programs, thus allowing Notch and its ligands play a role in cell fate determination. The mechanism of action for Notch proteins is as follows: when bound to a ligand, tumour necrosis factor-α-converting enzyme (TACE/ADAM17) is able to extracellularly cleave the protein, allowing for the notch extracellular domain (NECD) to be endocytosed by the signalling cell and a final intracellular cleavage site to be exposed. For complete activation, gamma-secretase cleaves the membrane bound fragment, intracellularly, releasing the intracellular domain (NICD) which then localizes to the nucleus. In the nucleus, the NICD interacts with CBF1/Suppressor of Hairless/Lag-1 (CSL) to allow for DNA binding and transcriptional activation of various genes[23, 24, 30].

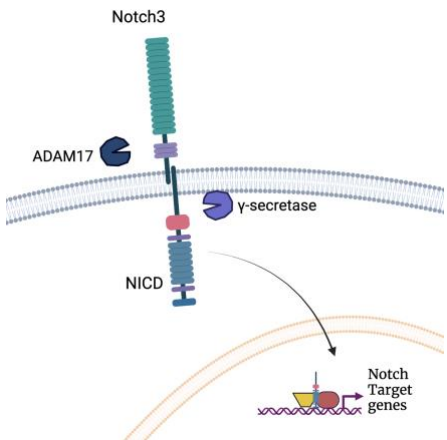


**Figure 1. Notch pathway.** Notch activation begins with interaction with a ligand. This interaction allows for ADAM17 cleavage of the extracellular domain (NECD), which will be endocytosed into the signalling cell with the activating ligand and degraded. Cleavage of the NECD exposes the final intracellular cleavage performed by γ-secretase. The unbound intracellular domain (NICD) then localises to the nucleus to act as a co-receptor for transcription of numerous genes.

The NICD can be regulated by modifications such as phosphorylation, ubiquitylation, hydroxylation and acetylation. These processes affect Notch by inhibiting its ability to transcriptionally induce other genes and decreasing its half-life[31, 32]. Notch itself can also regulate its own activity, being able to inhibit its cis receptors[23]. Notch target genes consist of genes from the hair and enhancer of split-

related (*HES*) family, *PIK3CA (*PI3K*), AKT, CCND1* (cyclin D1), *CDKN1A* (p21), and *CDKN1B* (p27)[33].

As mentioned, Notch proteins play a role in the developmental process and their mutation leads to an assortment of diseases[23]. In cancer, the Notch family proteins have already been discovered to play a significant role, affecting progression and tumorigenesis both as a tumour-promotor and as a tumour-suppressor[34, 35]. Notch 1 was found to be constitutively active in acute lymphoblastic leukemia (T-ALL) due to chromosomal translocation, causing the malignant phenotype[36]. Notch 1 and 4 have both been linked to the initiation of breast cancer in mouse and human cells *in vitro*[37, 38]. Notch 1, along with its receptor Jagged 1, has been linked to poor prognosis in a variety of cancers, including breast cancer[39]. Notch 3 has been shown to affect the development of ErbB2-negative (HER2) breast cancer tumours[40]. Constitutive activation of Notch, *in vitro,* has been shown to cause a 10-fold increase in the quantity of mammospheres formed, which similarly could be inhibited with a Notch 4 inhibiting antibody or with gamma-secretase[41]. In bladder cancer, Notch has been found to be inactivated in roughly 40% of patients, where activation was also shown to supress proliferation[42]. Notch 1 deletion in mouse models has additionally been shown to spontaneously develop basal cell carcinoma[43]. The interchanging role of Notch signalling within cancer exposes its significance in cells and the surrounding environment.

## PI3K/AKT Pathway

The PI3K/AKT pathway is known to affect a large variety of proteins, being able to affect proliferation, cell cycle, and cell-fate[44-48]. Three isoforms of AKT exist, though mainly AKT1 and AKT2 are prevalent within cells. AKT contains two phosphorylation sites that allow for its activation, Thr308 and Ser 473, both of which require phosphorylation for complete activation of AKT[45]. Activation of AKT can occur from signals due to nutrients, growth factors, or hormones from receptors such as: receptor tyrosine kinase (RTK), cytokine receptors, and integrins [49]. Once a signal is received, stimulation of phosphatidylinositol-3-kinase (PI3K) occurs. Once activated, PI3K phosphorylates phosphatidylinositol (4,5)-biphosphate ($PIP_2$) which becomes phosphatidylinositol (3,4,5)-triphosphate ($PIP_3$). $PIP_3$ then recruits AKT to the cell membrane where it can be negatively regulated by phosphoinositide-dependent kinase-1 (PDK1)[45, 48]. Phosphatase and tensin homolog (PTEN) is able to regulate PI3K's ability to phosphorylate $PIP_2$ along with PP2A phosphatase inhibition of AKT providing the ability for further regulation of AKT[50]. PI3K is often amplified and/or mutated in cancers and therefore, can play a role in alterations in AKT activation during cancer.

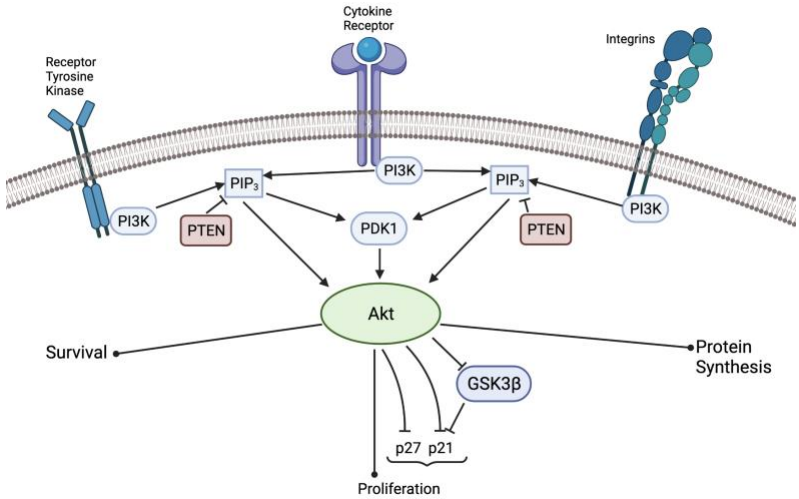


**Figure 2. AKT pathway.** Simplified overview of activation of AKT beginning with receptor activation though receptor tyrosine kinase, cytokine rectptors, integrins, and others. PI3K activation, due to receptor signal, leads to $PIP_3$ formation and recruitment of AKT to the cell membrane. Activation of AKT is performed by PDK1. AKT can affect survival, protein synthesis, proliferation, and other processes. Altering of proliferation can occur through inhibition of GSK3β or direct inhibition of CKIs p21 and p21. PTEN can inhibit $PIP_3$ function and regulate AKT.

In many cancers and other malignancies, AKT is generally found to be overactivated, likely being the cause of the malignancy[51]. This hyperactivation was first discovered in ovarian cancer where it was found in a subset of cell lines, eventually being linked to a more aggressive, undifferentiated form[52-54].

As a key player in signal transduction pathways, AKT is known to affect several processes. Overexpression and overactivation of AKT are able to enhance growth factor related signalling within cells. Cell proliferation can be altered by AKT through induction of cytoplasmic localisation of cyclin dependent kinase inhibitors (CKIs) p21 and p27 as well as through phosphorylation and inhibition of GSK3β to prevent cyclin D1 degradation[51, 55-57]. AKT can also inhibit/inactivate Bad and pro-caspase 9, proapoptotic factors, leading to enhanced cell survival and prevention of stress-induced apoptosis. AKT can affect angiogenesis, the replicative potential of cells, and invasion and metastasis[44]. Through its general overactivation in cancers, the belief that targeting AKT would be a viable target for therapy may not work for all cancer types. In hepatocellular carcinoma (HCC), multiple deletion of AKT isoforms in mice led to rapid mortality and loss of one isoform led to spontaneous development of HCC and increased metastasis[58]. With AKT's ability to interact with other vital pathways, its alteration is central to the resulting functions of cells.

## Wnt/β-catenin Pathway

Wnt is a conserved pathway that plays a role in development, differentiation, and self-renewal capabilities of stem cells. Wnt was discovered in *Drosophila melanogaster* where it affects the developmental patterning processes[59, 60]. The canonical Wnt pathway contains β-catenin, which responds to signals from the presence of Wnt. In the absence of a Wnt signal, β-catenin is phosphorylated in a complex with axis of inhibitor 1 (Axin), adenomatous polyposis coli (APC), and GSK3β, being able to regulate the cytosolic concentration of β-catenin through ubiquitination-dependent proteasomal degradation. In the presence of a Wnt signal, the receptor Frizzled releases Dishevelled, which inhibits GSK3β, thereby releasing β-catenin from the complex where it is then able to localize to the nucleus. Here, it interacts with other transcription factors, leading to the activation of target genes[30, 61-63].

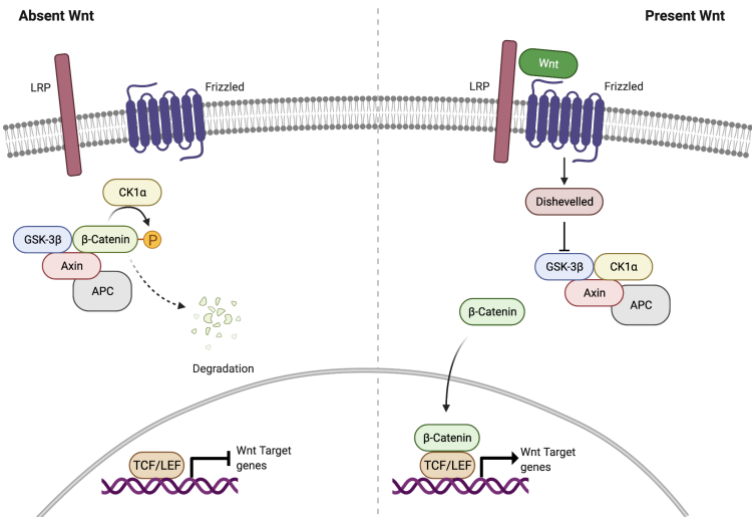


**Figure 3. Wnt pathway.** Activation of β-catenin requires a Wnt signal. In the absence of this signal, β-catenin is phosphorylated by GSK3β, leading to proteasomal degradation. In the presence of a Wnt signal, the phosphorylation of β-catenin is inhibited and it is then released from its complex, allowing for nuclear localisation and binding to transcriptional factors leading to activation of target genes.

Transcriptional targets of Wnt signalling are numerous and include: *CDKN2A*, *CD44*, *HES1*, *JAGGED1*, and *NOTCH2*[64]. In cancer, Wnt/β-catenin is found to be activated in roughly 50% of breast cancer patients and is correlated to poor survival in several other cancer types[65, 66]. In colorectal cancer it has been found to function as a tumour promotor[66]. Increased Wnt signals, with Notch dependence, have been shown to initiate an oncogenic transformation in breast epithelial cells[67]. In

hepatocellular carcinoma, Wnt/β-catenin and Notch3 were found to be inversely related, together being able to affect stemness[68]. The Notch ligand, Jagged1, is a downstream target of Wnt/β-catenin and is therefore subject to regulation of Notch, through Jagged1, by Wnt/β-catenin[69]. As a downstream target of AKT, GSK3β, and thereby the Wnt/β-catenin pathway, can be regulated through AKT. These intertwined pathways hold a significant role in cell fate determination.

## Stemness and Cancer

All cells of the body originate from a small population of cells, stem cells. These cells are able to renew themselves and propagate into all the cells found in the varying tissue and organs within the body[70]. During embryonic development, the pathways above play a vital role in shaping cells to their eventual outcomes. Specifically, Notch plays a role in the fate determination of cells through control of differentiation[71]. In animal studies, loss of Notch during embryogenesis lead to accelerated neuronal differentiation, deficiencies in hematopoietic stem cell generation, and abnormalities in vasculature formation and remodelling[72-74]. AKT plays a larger role in cell survival and promoting growth where it mediates anti-apoptotic signals[75]. Mouse models with knockouts (KO) of *AKT* isoforms display abnormal embryonic development[76]. Wnt is similarly responsible in cell fate through effects on cell differentiation. Enhanced Wnt signalling leads to osteoblast differentiation while decreased signalling leads to chondrocyte differentiation[77].

Cancer cells and stem cells share some similarities in that they have some self-renewal capabilities and clonal expansion, however, stem cells do so in a highly regulated system with normal cells as an outcome while cancer cell expansion results in abnormal DNA alterations[78]. However, populations of cancer stem cells have been discovered acute myeloid leukemia, breast cancer, and glioblastomas[79-81]. As described above, the Notch, AKT, and Wnt pathways are found to be dysregulated in many cancers and with their role in traditional stem cells, it is likely to conclude that they similarly play a role in cancer stem cells as well[82].

# Cell cycle

The cell cycle is quite obviously an important process in the cell allowing for proliferation and continual survival. It is a highly conserved and regulated process that is meant to ensure correct replication of genetic material for avoidance of aberrations[83-85]. The cell cycle is divided up into four phases, G1, S, G2, and M, each with required levels of cyclins and cyclin dependent kinases (CDKs); G0 designates a cell not within the cell cycle. Much of what is known about the cell cycle began through studying this regulatory process in yeast where only one CDK-like protein exists[86, 87]. In mammalian cells, there are numerous cyclins that oscillate throughout the cell cycle, interacting with CDKs to play important roles during each phase in order to continue progressing through the cycle. CDKs are serine/threonine kinases that are activated through phosphorylation at different points within the cycle; for example, cdk1 phosphorylation at the threonine 161 allows for kinase activity while phosphorylation at tyrosine 15 and threonine 14 inhibits kinase activity[88].

G1 is a gap phase where the cell prepares for synthesis of DNA. Here, the cell verifies the need for repair prior to continuing through the cycle. S phase denotes the point at which DNA synthesis occurs. G2 is another gap phase within the cycle. At this phase, the cell prepares for the process of division. M phase denotes mitosis, the process where replicated chromosomes are separated to eventually form two daughter cells. Importantly involved in the cell cycle are regulation points, which are found to occur during the gap phases of the cycle, G1 and G2. Key to these regulation points are CKIs. Two families of CKIs exist within cells: Cip/Kip family, consisting of p21, p27, and p57 (CDKN1C), and the INK4 family, consisting of p16 (CDKN2A), p15 (CDKN2B), p18 (CDKN2C), and p19 (CDKN2D). Both of the CKI families are able to affect the G1 checkpoint while only the Cip/Kip family can affect the G2 checkpoint.

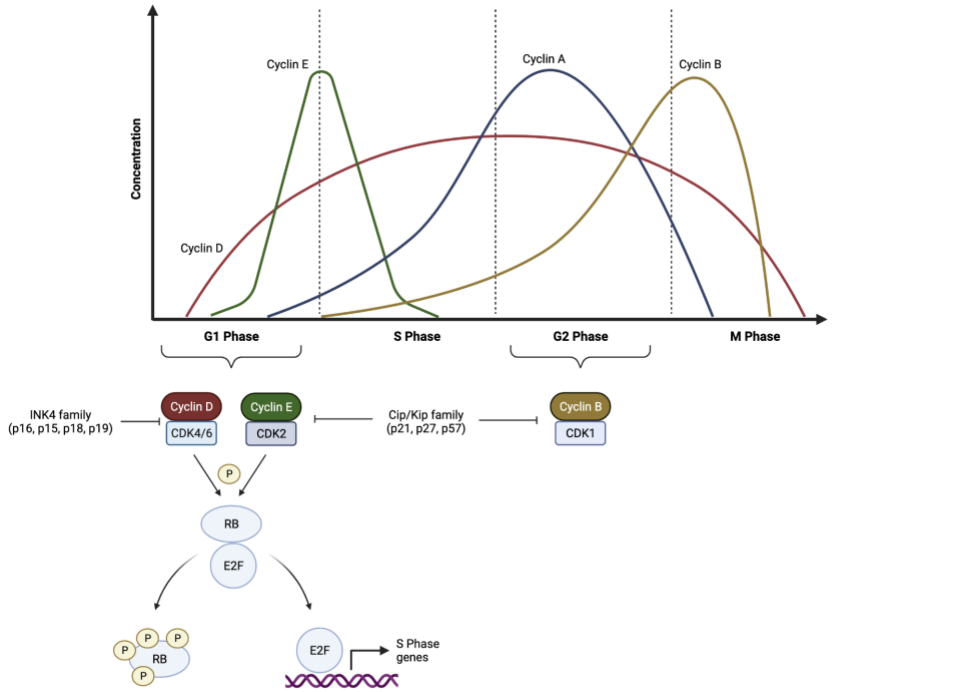


**Figure 4. The Cell Cycle.** The dynamic process of the cell cycle indicating the change in concentration of important cyclins during each phase of the cell cycle. Below indicates the two regulating points found during the gap phases and the further regulation based on the cyclin dependent kinase inhibitor families Cip/Kip and INK4.

Type D cyclins are induced when cells are first stimulated to enter the cell cycle from G0[89]. These cyclins then associate with and activate cdk4 and 6 for regulation in G1. Important in the G1 checkpoint is the retinoblastoma tumour suppressor protein (RB) and its interaction with E2F transcription factors. RB is able to bind to E2F and inhibit its transcriptional activation capability. Hyperphosphorylation of RB by cyclin/CDK complexes allows for the release of E2F and transcriptional activation of proteins involved in later stages of the cell cycle[90]. Notably, the CKI p21 can play a role in the regulation of E2F by inhibition of cyclin/CDK complexes that phosphorylate RB[91]. From E2F's activation cyclin E and cdk2 associate with one another to maintain the hyperphosphorylation of RB for transition from G1 to S phase[92].

The CKI p21 plays a major role in the cell cycle as part of the regulator family capable of arresting the cycle during both gap phases. The functional capability of p21 is dependent upon its nuclear localisation and can be altered through phosphorylation. There are 5 major sites of phosphorylation on p21: T57, S130, T145/S146, S153, S160[93]. The T57 site is phosphorylated by GSK3β, likely

responsible for ubiquitination and subsequent degradation of p21[94]. The T145/S146 site is phosphorylated by AKT, where T145 is preferred. When this point is phosphorylated, p21 loses its ability to bind to proliferating cell nuclear antigen (PCNA) and becomes localised to the cytoplasm. This prevents p21 from its cell arrest functions, allowing for uncontrolled cell proliferation[95]. From its compartmental localization p21 can also bind to other proteins apart from cyclins and CDKs[93].

Cyclin A accumulates at the G1/S transition and is maintained throughout S phase where it associates with both cdk 1 and 2. There are indications that cyclin A can interact with DNA, likely involved in regulating the replication of genetic material[96]. Loss of cyclin A2 and cdk2 have been shown to impair tumour cell proliferation in murine models of liver tumorigenesis[96]. Cyclin B/cdk1 activation drives the cell through its second gap phase, G2. However, there is no direct correlation of cyclin B/cdk1 with DNA for regulation of repair. In this phase, other proteins such as ataxia telangiectasia mutated (ATM) and AMT- and Rad3-related (ATR), are responsible for detection of DNA damage and inhibition of cell cycle progression[84, 97]. Drugs can also affect the cell cycle due to loss of required components for further phases. Nocodazole disrupts microtubule function, triggering the regulation point and arrest of the cell cycle[98]. Following mitosis, exit from the cell cycle is caused by the degradation of cyclins A and B, transitioning once again to G1.

In cancer, several of these cell cycle components are generally found to be altered. Cyclin D translocation has been linked to parathyroid adenomas and B-cell lymphomas[99, 100]. Alterations in cyclin D have also been shown in breast and colorectal cancers and in neuroblastomas[101-103]. While few alterations in p21 exist, it is likely affected in cancer due to its regulation by the tumour suppressor p53[104, 105]. As one of the most highly mutated genes in human cancers, p53, which was first discovered in complex with simian virus 40 (SV40) large T-antigen, was observed to be in abundance in several tumours but not in normal tissue[106, 107]. Wild-type p53 functions to determine cell fate by inhibiting cell cycle progression, inducing senescence, or promoting apoptosis[108-110]. Mutant forms of p53 can vary in their functions, being able to promote cancer as a ‘gain-of-function’ (GOF) mutation while others lose their tumour suppressing capability as a ‘loss-of-function’ (LOF) mutation[111, 112]. Generally, these mutations lead to a loss of control of p53, inhibiting its ability to arrest the cell cycle through p21, and the failure to repair DNA damage.

# Signalling

Often times in malignancies, there is an initiating mutation or alteration in cells that leads to a cascade of effects resulting in the modification of 'normal' characteristics and function of the cells. These effects on the cells, whether overexpression or depletions of proteins, can develop into a more aggressive form. Understanding these modifications can lead to comprehending the mechanisms involved and ways to combat them.

## Cartilage Oligomeric Matrix Protein

Cartilage Oligomeric Matrix Protein (COMP), also known as thrombospondin 5 (TSP5), is a pentameric molecule mainly found in cartilage and bone tissue[113-117]. COMP, likely discovered in 1984 but confirmed in 1992, was found to be a 'bouquet' of five identical arms containing four epidermal growth factor domains, eight thrombospondin type III domains, and a globular C-terminus, all held together through an N-terminus coiled-coil structure[118, 119]. As an extracellular component itself, COMP interacts with other ECM proteins, mainly several types of collagens, to aid in matrix assembly through interaction with one another or with the cell surface. COMP is known to also interact with the complement system, dealing with innate immunity, through the alternative pathway while inhibiting the classical and lectin pathways[117, 120].

Mutations of COMP are known to cause pseudo achondroplasia (PSACH), multiple epiphyseal dysplasia (MED), and other disorders[113, 121-124]. COMP has mainly been studied in skeletal conditions where it has been used as a diagnostic marker of osteoarthritis[125-127]. COMP is also believed to share characteristics of other TSPs[119]. TSP1 has been shown to affect cell adhesion by functioning as a molecular bridge, can promote macrophage recognition of apoptotic cells, and inhibit angiogenesis[128]. TSP1 and 2 have likewise been shown to Notch 3 and Jagged 1, where only TSP2 is able to enhance this interaction[129].

COMP does not have a significant amount of background investigations in relation to cancer. It has been shown to be overexpressed in HCC while generally low or not expressed in normal livers[130]. Previously, work with COMP was able to demonstrate that high levels were present in roughly 20% of breast cancer patients, regardless of subtype, through immunohistochemical (IHC) stainings of two patient cohorts[131]. As an ECM protein, COMP was expectedly found in the stroma in varying levels, but held no significance to patient survival. However, when looking at the levels in tumour cells, it was found that high levels of COMP correlated with a poor prognosis, leading to decreased survival and an increased chance of recurrence, indicating a role for COMP as a prognostic marker for breast cancer. COMP

overexpression was also linked to increased invasiveness of cells and resistance to apoptosis. Tumours formed from orthotopic implantation of COMP-overexpressing cells were found to have a number of enriched pathways including PI3K and Notch pathways[131]. COMP has also been shown to promote prostate cancer progression through increased invasion and alteration of calcium homeostasis[132].

## STRIPAK

The striatin-interacting phosphatase and kinase (STRIPAK) complex is a multi-component functional unit that possesses many roles in a variety of cells and organisms[133-136]. The STRIPAK complex has also been shown to play a role in diabetes, heart disease, cerebral cavernous malformations, and cancer. The components of the STRIPAK complex include: PP2A phosphatase, striatins, germinal center kinases (GCKIII) (MST3/STK24, YSK1/SOK1/STK25, and MST4/STK26), cerebral cavernous malformation 3 (CCM3), and the scaffold proteins FAM40A/striatin interacting protein 1 (STRIP1) & FAM40B/STRIP2. Other varying STRIPAK complexes exist, involving core proteins such as sarcolemmal membrane-associated protein (SLMAP), the coiled-coil protein suppressor of IκB kinase-ε (IKKε), known as SIKE, and Mob3/phocein[137, 138].

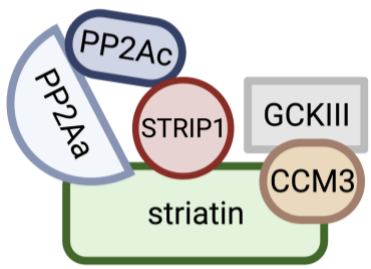


**Figure 5. STRIPAK complex.** STRIPAK complex consists of striatins, PP2A phosphatase, STRIP1/STRIP2, CCM3, and GCKIII family proteins (MST3, MST4, SOK1)

The PP2A phosphatase plays a major role, not only in the STRIPAK complex, but within the cell as a whole, being able to affect proliferation and differentiation. Being a key contributor to the cell, during the investigation of PP2A's binding partners, the STRIPAK complex was discovered[137]. PP2A consists of trimer structure, containing the catalytic subunit PP2Ac, the scaffold subunit, PP2A A, and the regulatory subunit or B subunit, of which, the B''' family are the striatins. Through tandem affinity purification, STRIP1 was found to directly interact with both the catalytic and scaffold subunits of PP2A. Of note, PP2A was not found to

bind to any of the GCKIII family of proteins, CCM3, or STRIP2 in HEK293 cells. Further work into interacting partners revealed that STRIP1 also interacted with members of the GCKIII family, CCM3, and other proteins. Similarly, CCM3 had already been shown to interact with the GCKIII family[137].

The GCKIII family of kinases are part of a much larger family known as Ste20-like kinases. Some other GCK members are known to affect mitogen-activated protein kinase (MAPK) signalling cascades[139]. Others are known to regulate cytoskeleton organisation, apoptosis, and the cell cycle.

Not much work has been performed regarding the various STRIPAK components and human diseases. Inhibition of STK25 has been shown to reduce lipid depositions and improve insulin sensitivity in mouse models of type 2 diabetes while overexpression enhanced lipid accumulation, impaired skeletal muscles, and decreased endurance of mice[140, 141]. MST4 overexpression has been correlated to increased proliferation and tumorigenesis in prostate cancer cell lines[142]. In yeast, *Saccharomyces cerevisiae*, a complex containing STRIP1/2 homolog (Far11) was able to affect the cell cycle leading go G1 arrest during pheromone response[143].

Previous work on the STRIPAK complex has described its effect on cancer cell migration through the activation of the GCKIII kinases which lead to increased aggressiveness[135]. The work describes the role of STRIPAK components in mechanotransduction of breast cancer cells. Loss of STRIP1 and 2, denoted as FAM40A and B respectively in the work, affect the morphology of the cells through changes in actomyosin contractility and interaction with plasma membrane linker proteins. STRIP1 was identified to be an upstream regulator of MST3 and 4, while CCM3 was found to affect MST 3 and 4 localisation but not biochemical activity. STRIP1 depleted cells showed decreased movement speed compared to cells depleted of other STRIPAK components, but had increased migration capability, therefore increasing malignancy. During this investigation, it was discovered, but not reported, that depletion of STRIP1 altered the proliferation of cells.

# Tumour Microenvironment

It has long been known that the environment in which tumour cells exist plays an important role in the characteristics and behaviours they portray[144-150]. The TME is composed of a large variety of cells, such as tumour cells, immune cells, fibroblasts, and structural components of the ECM. Even within these populations, cells can be identified with varying characteristics, expressing differing markers or perhaps expressing them at an altered level, which would eventually cause the cells to perform other functions. This creates heterogeneous niches within tumours. The TME is vital in the initiation process of cancer, altering the environment to allow for cancer cell survival and invasion into the local tissue. It collaborates with cancer cell signalling to provide for nutrition through blood vessel availability or removal of waste products of cells[147]. It is essential to understand the role of the TME and its components within cancer to identify the best methods to overcome the disease.

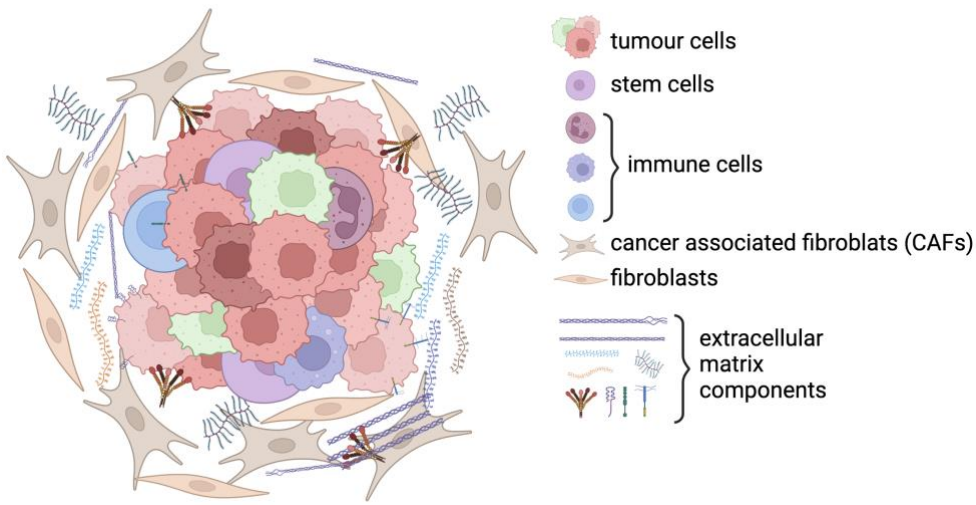


**Figure 6. Tumour Microenvironment.** Overview of components within the tumour microenvironment (TME) including several cell types (immune, tumour cells, fibroblasts) and extracellular matrix (ECM) components.

There have been many studies performed in the past to identify the role of TME components. Effects from the TME can begin as simply as the pressure found within the space. Normal tissue generally is a softer environment than a dense tumour and therefore, cell morphology can be altered which may modify cell function. One such example is found through YAP and TAZ, transcriptional activators identified to be vital for tumour initiation and progression[151]. YAP and TAZ are found to be

inhibited in normal tissue but become induced upon tissue stiffening, which leads to enhanced cancer cell proliferation. But not only do the cancer cells become affected by the stiffening of the surrounding tissue through YAP and TAZ, CAFs can similarly be altered by YAP and TAZ through mechanosensing[152]. In CAFs, YAP depletion alters functionality through reduced contractility and focal adhesions. This resulted in decreased remodelling ability of CAFs which decreased cancer cell invasion. Platelet-derived growth factors (PDGF) have also been shown to play a vital role in the TME[153]. In this study, PDGF-C within tumour cells was identified to enhance proliferation while simultaneously inducing recruitment of CAFs to the tumour. While these are only two of many examples, they exemplify the significance of signalling within the TME and between the inherent components.

## Cancer Associated Fibroblasts

As one of the major components of the TME, CAFs play a significant role in shaping the function of the tissue and surrounding cells[145]. CAFs originate from a variety of cell types including activation of resident fibroblasts, endothelial cells, pericytes, stellate cells, bone marrow-derived mesenchymal cells, or cancer cells themselves, resulting in subsets with varying functions[148, 154, 155]. CAFs play a fundamental role in the structural composition and organisation within the TME through deposition and remodelling of ECM components. These ECM components, such as collagens, proteoglycans, and glycoproteins, aid in signalling between cells and can affect the interstitial pressure within the tumour, leading to eventual blood vessel collapse and rise in hypoxic regions[156, 157]. The accumulation of CAFs within the TME is generally associated with a poor prognosis but CAFs are able to be both tumour promoting and tumour supressing[152, 158-160]. For example, CAFs, when in co-cultures with bladder cancer cells, secrete interleukin 6 (IL6) and induce the cancer cells into epithelial to mesenchymal transition (EMT) which leads to enhanced growth and migration[161]. CAF-dependent production of cross-linking enzymes and ECM remodelling leads to tissue stiffening, which in turn, enhances invasiveness, pro-survival and -proliferative signalling in the cancer cells[162-166]. For the tumour supressing aspects of CAFs, when using PDAC mouse models for depletion of α smooth muscle actin (αSMA), a marker of myofibroblasts, it was identified that these mice resulted in more invasive, undifferentiated, and necrotic tumours compared to their non-depleted counterparts[167] . This was believed to be due to the loss of collagen I and enhanced population of cancer stem cells from the tumours due to myofibroblast depletion. Similarly, when attempting to alter CAF signalling within tumours through depletion of sonic hedgehog (SHH) signalling, the resulting tumours were more aggressive, with increased proliferation and vascularity, leading to earlier initiation of tumours[168].

With the ascent of new techniques, like single cell RNA sequencing (scRNAseq), more insights into cells have come to light, and with this the uniqueness of CAFs

and the diversity of these cells[169-171]. Within each cancer type, CAFs can be clustered into several populations with characteristics of myofibroblasts, immune CAFs, antigen-presenting CAFs, or even further segmented within these general subsets[171, 172].

*Methods for Examining CAFs*

CAFs obtain much of their role in the TME through interactions with the ECM and tumour cells[173]. Due to this, traditional methods of studying cells, as are used with cancer cells, would not be entirely suitable when looking at the range of functionality of CAFs. There is a greater movement towards studying cells in a more typical environment that they would be subject to in patients, meaning a more three-dimensional environment and generally with at least two cell types[174]. Cell derived matrices (CDMs), hydrogels, microfluidic devices, and other methods have been used to study the interaction of cells and ECM to replicate a more natural environment[175].

CDMs produced through CAF deposition, under the presence of ascorbic acid, provide a scaffold to mimic the ECM and study the varying structure[176]. After ECM deposition is complete, generally taking 1-3 weeks, the CAFs can be removed from the developed matrix and, if desired, cancer cells can be subsequently added on the CDM surface to monitor their interactions and cellular behaviour.

Other types of co-culture systems with CAFs, cancer cells, and ECM have been used to study their interplay. Organoid cultures are often used to study stem cell properties. These cultures start out as cell aggregates that are then subjected to a matrix and growth factors to enhance its progression[177, 178]. Direct co-culture methods with both CAFs and cancer cells have also been used to identify how stromal cells effect cancer cell drug response[179]. The direct interaction allows for secretion of growth factors from both cell types to interact and affect the other. Similarly, indirect cultures are used to identify the effects of only secreted factors on a different cell type. Hydrogels are generally collagen rich gels with cells embedded within. CAFs used in combination with these hydrogels, prior to polymerisation, can be used to study contraction ability. This method takes roughly two weeks to allow for fibroblast contraction[180].

It is important to consider which of the many models is the best for specific investigation that will be performed. CDMs would be best for ECM remodelling studies while hydrogels can identify contractility effects as well as invasion ability of cells[175]. However, with all these methods, it allows for an enhanced understanding of cancer cells, CAFs, the ECM, and their interaction with one another to affect progression.

## Proteoglycans

Proteoglycans (PGs) are complex, post-translationally modified macromolecules found in the glycocalyx, a network of molecules that act as a barrier between cells and the TME[181]. Proteoglycans, and the other components of the glycocalyx, glycolipids and glycoproteins, play a role in the signalling between cells and the TME through receptor-ligand interactions and affecting the ability of cells to migrate. Within tumour stroma, there is an abundance of PGs, suggesting a role in tumour promotion[182, 183]. At the cell surface, some PGs can act as co-receptors for activation of several other pathways such as Wnt.[184-186]

Glycosaminoglycans (GAGs) are side chains found on PGs, mainly comprised of glucuronic or iduronic acid and N-acetylglucosamine[187]. GAGs are considered modifications to their PG cores and therefore express a high level of heterogeneity in their composition, length, and importantly, sulfation patterns. There are six types of GAGs that have been identified: heparan sulfate (HS), dermatan sulfate (DS), keratan sulfate (KS), chondroitin sulfate (CS), heparin (Hep), and hyaluronic acid (HA), all of which are sulphated except for HA[188]. GAGs are synthesized in the Golgi, where they are modified by *O*-sulfotransferases, such as chondroitin sulfate N-acetyl-galactosaminyltransferase 1 (CSGalNAcT-1) for CS.

HS is the most abundantly studied GAG and is present in all cell types and tissues, where it acts as a regulatory molecule both in normal and pathological conditions. Proteoglycans containing HS are generally localised to the cell surface or within the basement membrane. Here, they are able to promote tumour growth, invasion, and metastasis, likely through interactions with secreted factors within the TME[189-192]. CS can be classified by their sulfation patterns on their *N*-acetylgalactosamine and glucuronic acid components, leading to five types: CS-A with 4-*O*-sulfated residues, CS-C with 6-*O*-sulfated, CS-D with both 2-*O*- and 6-*O*-sulfated, and CS-E with 4,6-*O*-disulfated. CS-B, now known as DS, varies from the other CS due to the presence of iduronic acid rather than glucuronic acid and either 4-*O*-sulfated or 6-*O*-sulfated residues. These sulfations and, in particular, the pattern they are found in, enable GAGs and their PG cores to specific interactions[193].

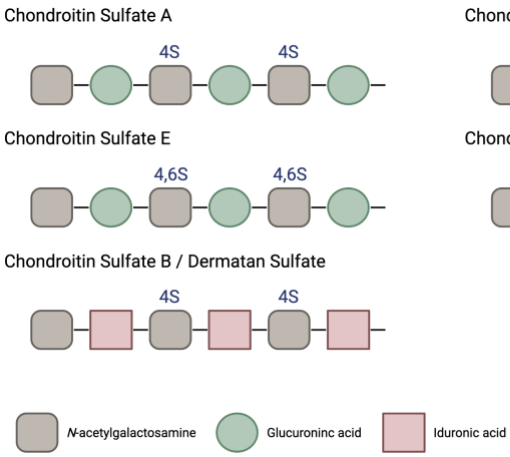


**Figure 7. Chrondroitin Sulfate subtypes.** Depiction of chondroitin sulfate subtypes with indicated sulfation pattern. Adapted from Soares de Costa *et al*. 2017.

Regulation of expression of CS proteoglycans (CSPGs) has been correlated to both normal and pathological conditions[194]. CS chains have been shown to maintain secreted factors within the ECM for sustained release, allowing for promotion of cell signalling[195, 196]. CSPG4 is a known marker of pericytes and some tumour cells. Already in glioblastoma and melanoma, therapeutic targeting of CSPG4 is being used to inhibit proliferation and angiogenesis[197-199]. Similarly, targeting of CSPG4 was attempted in soft-tissue sarcomas. Here, it was identified that targeting and depletion of CSPG4 was dependent upon the developmental stage of the tumour. When targeting was performed prior to tumour development, the resulting tumours were larger. However, when targeting CSPG4 after tumour initiation, no changes in growth factor signalling were observed[200]. Conversely in pancreatic cancer, CSPG4 does not appear to have an effect on malignancy but was found to be a marker of hypoxia[201].

As the structure of PGs is not encoded genetically within cells, the GAG modifications on them elicit heterogeneity within the TME. This ability can be altered through the enzymes that allow for the addition of these chains and the *N*-acetylgalactosamine and glucuronic acid monomers. Loss of these enzymes, such as glycosyltransferases used to create the backbone of the GAG chain, results in post-natal lethality and chondrodysplasia in mice[202]. Targeting of these enzymes for therapeutic purposes therefore is not a viable option as they have also been shown to play an important role in the brain during axon regeneration[203].

*Plasmodium falciparum*

Malaria or *Plasmodium falciparum* is an infection that can severely affect pregnant mothers and lead to neonatal mortality[204, 205]. *P. falciparum* displays a cell surface

ligand that is used for adhesion to placental tissue, *P. falciparum* erythrocyte membrane protein-1 (PfEMP1). Roughly 60 *var* genes encode these PfEMP1 proteins, one specific being VAR2CSA, which allows for binding to CS-A on the placental syncytiotrophoblast, an epithelial layer that separates the mother and fetal blood[206-208]. While CS-A chains can be found throughout the body, VAR2CSA binding is only found in placental tissue, indicating a variation in this type of CS-A[209, 210]. This placental CS-A was associated with high proliferation and the ability to invade, characteristics common to cancer[211].

Using a recombinant form of VAR2CSA, it was discovered that many cancers cell lines and cancer patient tissue contained this placental CS-A while having no reaction to any other normal tissue within the body, coining the term oncofetal chondroitin sulfate (ofCS)[212]. In a number of cell lines, recombinant VAR2CSA (rVAR2) bound to ofCS was found to inhibit cell adhesion through decreased phosphorylation of Src and Erk and decrease cell invasiveness[213]. Due to its high specificity, exploitation of ofCS is believed to be a viable therapeutic target for cancer patients[214-217]. It has already been identified that ofCS expression remained in high quantities after cisplatin-based neoadjuvant therapy in bladder cancer patients. This would allow a secondary targeting option for those patients that develop resistance to therapy[217]. ofCS detection through rVAR2 use has been shown to isolate circulating tumour cells (CTCs) in an EpCAM-independent manner, allowing for a greater, more varied set of CTCs captured, enhancing a non-invasive manner in which to detect, diagnose, and monitor patients[215]. This method has already been used in the detection and isolation of CTCs in glioma patients[218]. This previous work highlights the importance of ofCS in cancer and the benefits of exploring its presence and function in other types of cancer.

# Therapy in cancer

As the decades have passed, incidence of cancer has increased with new and improved methods of detection[219]. While this may seem detrimental, our understanding of cancer has similarly swelled, leading to enhanced therapies. Due to this, mortality rates overall have decreased for cancer patients. Although great strides have been made, there is still more work to be done.

## Breast Cancer

When a patient is believed to have breast cancer, physical examinations are performed wherein a biological sample is taken and imaging can be performed. From this, clinicians and pathologists determine the origin cells, the stage, and presence of molecular receptors[220-222]. As previously mentioned, breast cancer originates from the lobular cells or the epithelial lining of the ducts. The staging of breast cancer defines the size of the tumour, involvement of regional lymph nodes, and distant metastasis, leading to a TNM classification. Stage I consists of tumours up to 2 cm in size with no lymph node involvement. Stages II and III describe varying combinations of primary tumour size, either between 2 to 5 cm or larger than 5 cm, and lymph node involvement. Once the tumour has become distantly metastatic, it is considered stage IV[223]. As mentioned previously, molecular receptors for breast cancer include ER, PR, and HER2, as well as proliferation capability (Ki67). All this data together allows for clinicians to determine the best course of action for patients.

### *Clinically used Treatments*

Treatment for breast cancer is dependent upon the pathological results. Options for patients include resection of the tumour, hormonal therapy, chemotherapy, immunotherapy, or radiotherapy. Patients presenting with stage I, II, or III cancers generally undergo neoadjuvant therapy in order to reduce tumour volume, allowing for roughly 80% of patients to be operable[224]. Stage IV cancer is considered incurable and patients are only provided with chemotherapy and palliative care[225]. Patients that are hormone receptor positive, ER and PR, and HER2 negative receive endocrine therapy, such as tamoxifen. In some cases, chemotherapy is used for these patients where adriamycin, cyclophosphamide, paclitaxel, and docetaxel are generally used in varying combinations. For HER2 positive patients, a HER2-targeted therapeutic is used, such as trastuzumab and pertuzumab, in combination with the above-mentioned chemotherapies. For TNBC patients, chemotherapy, radiotherapy, and immunotherapy are the treatment options for patients with non-resectable tumours[226].

*Recurrence Rates*

Recurrence of cancer is an unfortunate phenomenon that often plagues patients. Generally, after having undergone treatments, these new tumours become resistant to any further treatment. While in the past, recurrence could have been attributed to lack of confirmation of negative margins when resecting tumours, more recently has been attributed to molecular subtypes and types of treatments administered after resection[225]. Overall, roughly 10% - 20% of patients develop local recurrence even after resection of stage I and II breast cancers. Recurrence rates are highest for patients with TNBCs, between 25 – 40%, and lowest for ER+/PR+/HER2- cancers[227-230]. Similarly, and expectantly, patients that had lymph node-positive breast cancer also had a higher rate of recurrence compared with node-negative patients[231, 232].

*Researched Treatments*

While currently used therapies have shown great strides in treatment of breast cancer, there are still side effects to these treatments, recurrence of cancer, and possible resistance to treatment upon recurrence. Because of this, investigations into more enhanced treatments must continue. Much of this work begins as *in vitro* investigations of singular cell lines, can become more advanced with three-dimensional organotypic cultures, generally moves to *in vivo* mouse and cynomolgus monkey models, and finally develops into a proper clinical trial which involves patients. It is important, particularly for TNBC, with chemotherapy and radiotherapy are the only treatment options and having many side effects, to discover better treatment options with less harsh effects on patients. One study performed looked into sequential use of an inhibitor of CDKs and doxorubicin as a method to enhance the effect of the chemotherapeutic drug[233]. The group describes the use of roscovitine, a pan-CDK inhibitor, to cause arrest of a larger population of cells at the G2/M phase. Use of doxorubicin, which inhibits the topoisomerase II enzyme involved in DNA damage response, following cell cycle arrest led to more DNA damage which leads to eventual cell death by apoptosis. When the combination was used in a breast cancer xenograft model, the combination similarly reduced tumour burden and enhanced survival[233]. Concentrations and durations of treatments can play a role in tumour response. Coupled with alterations of protein expression levels, chemotherapies can lead to undesired outcomes. In one study, p21 was identified as a marker of cell fate determination during drug treatments[234]. The level of p21 expression was said to determine whether cells would become senescent or proliferative after treatment and allotted recovery time. If levels of p21 were at the extremes, too high or too low, the cells would become senescent as is expected. However, at a median p21 level during drug treatment, named the ‘goldilocks’ zone, cells would become proliferative, indicating resistance to the drug[234]. Another study has shown that use of a drug, UNBS5162 increased apoptosis of cells *in vitro* and decreased AKT activation, which led to decreased proliferation, migration, and invasion of breast cancer cells[235]. As a major pathway in cell fate

determination, the PI3K/AKT pathway is often considered a target of therapies. One study has shown that in TNBC in particular, PI3K inhibitors enhanced wnt signalling through GSK3β, thereby allowing cells to require resistance and having minimal effect on tumour growth. This effect was not seen on HER2 enriched tumours, where tumour volumes were decreased with use of the PI3K inhibitor GDC-0941[236]. These studies all show the progress that is being made in the field of therapeutics, the difficulties that arise, and ways that investigators are overcoming the issues. Only time will allow for further developments in the field, leading to enhanced patient survival.

## Pancreatic Cancer

As mentioned previously, pancreatic cancer suffers from being generally diagnosed at later stages when patients have already progressed to a locally or distantly metastatic form[11]. While most patients do not develop symptoms, it can be that some of the symptoms which patients present with are an indicator of where in the pancreas the tumour is located, either the head/neck which is located near the stomach and small intestine or the body/tail which is located near the spleen[237]. The location also indicates what structures would become affected when the tumour begins to spread. When the tumour has already spread to the surrounding tissue, it becomes more difficult to treat and ensure that no recurrence develops over time. Some general risk factors for developing pancreatic cancer include obesity, type 2 diabetes, and tobacco use, though these are factors for other types of cancers as well[11, 237]. Only roughly 5-10% of pancreatic cancers can be attributed to genetic factors where some of the mutations involved include those in *STK11*, *BRCA1*, *BRCA2*, and *CDKN2A*[10].

### *Clinically used Treatments*

Treatment for pancreatic cancer is dependent upon the size of the tumour and the stage of metastatic spread[11, 237]. For the 20% of patients with resectable tumours, neoadjuvant therapy is given at times, this includes those patients that are borderline resectable. After resection, additional chemotherapy is provided, a combination of gemcitabine and FOLFIRINOX (5-fluorouracil, folinic acid, irinotecan, and oxaliplatin). Treatment for locally advanced and metastatic patients is chemotherapy and, if necessary, radiotherapy. In some instances, for locally advanced patients, resection can become a viable option after chemotherapy.

### *Recurrence Rates*

Pancreatic cancer patients have a general 5-year survival rate of roughly 7-8%[11]. As previously mentioned, only 20% of patients are able to undergo resection of their tumours, increasing 5-year survival to 10-15%[238, 239]. This would indicate an 85% recurrence rate in these patients.

*Researched Treatments*

As majority of patients are only provided with chemotherapy as treatment and the survival rate remains extremely low, new avenues need to be explored when combating pancreatic cancer. As a driver for pancreatic cancer, the alteration of *KRAS* is often believed to be a viable target. Along with several other genes, multi-gene targeting was tested in mouse models, resulting in decreased tumour growth after siRNA silencing of *KRAS, XIAP, BCLXL, FLIP, MCL1L,* and *SURVIVIN*[240]. Targeting *KRAS* has also been shown to lead to compensatory mechanisms for tumour survival[241]. Using an inducible model of pancreatic cancer, investigators confirmed the dependence of the tumour upon mutant *Kras* by removal and subsequent decrease of tumour burden. However, when mice regained doxycycline, used to initiate oncogenic *Kras*, roughly 70% of mice presented with recurrent tumours. These tumours presented differently from the primary, having more mesenchymal-like morphology and increased Yap1, leading to increased aggressiveness of tumours. Even with loss of oncogenic *Kras,* Yap1 enhanced tumours were able to continue growth[241]. It is important to consider all the underlying mechanisms involved as cells have compensatory pathways in order to maintain homeostasis in normal conditions. As pancreatic tumours are stiff from high ECM content, resulting in loss of vasculature and increased hypoxia, tumour cells are required to find new ways to obtain necessary nutrients to maintain survival. One of these mechanisms is autophagy, degrading cellular components to be recycled and used as nutrients. To combat this, inhibition of autophagic processes is considered as a combination treatment. One study describes the combination of ERK and autophagy inhibitors as a viable treatment option for reducing autophagic dependency of PDAC cells. While both loss of mutant *Kras* and ERK increased autophagy in cells, loss of ERK was shown to cause cells to become dependent. Therefore, when combining both ERK inhibition and an autophagic inhibitor, such as hydroxychloroquine, tumour volumes were greatly reduced[242]. With the use of several chemotherapeutics, resistance to treatment rises within patients, likely resistance to gemcitabine. Studies have shown that key enzymes required for gemcitabine import and phosphorylation can affect sensitivity to the drug[243, 244]. Other factors such as epithelial to mesenchymal transition and the TME have been linked to gemcitabine resistance as well[245]. Several ongoing clinical trials are aimed at combination therapies targeted towards DNA damage response, immunotherapy, tumour cell specific therapy, and the TME, listed in review[237].

## Stromal Therapy

Traditionally, therapy has been mainly aimed at targeting of the tumour cells. However, within the last couple of decades, therapies targeting other components have risen, such as immune checkpoint therapies which target the immune cells[147]. In pancreatic cancer, stromal targeting has increased in popularity as an avenue to

explore further due to the highly desmoplastic reaction. The increased tissue stiffness, increased interstitial fluid pressure, and decreased vasculature all affect the ability of drugs to perfuse into the tissue and circulate within the tumour[246-248]. Therefore, finding mechanisms to reduce this would be the path forward. The following described will focus upon targeting the structural ECM and CAFs.

As an attempt to disrupt signalling in the TME, one study explored SHH pathway inhibition[249]. Using mouse models, treatment with the inhibitor IPI-926 with or without use of gemcitabine decreased the desmoplastic reaction within PDAC tumours, with reduced proliferation in αSMA+ fibroblasts. This was accompanied by increased numbers of CD31+ endothelial cells and increased vessel density within the tumours. As previously mentioned, CAFs and cancer cells affect one another through signalling pathways, allowing for changes to the TME to promote or suppress tumours. As an important mutagenic driver of not only PDAC but many other cancer types, p53 mutants are highly studied. Here, p53 status of cancer cells was able to affect CAF functionality, being able to 're-educate' CAFs to perform desired outcomes[250]. Hydrogels created with CAFs derived from tissue containing varying p53 mutational status were incubated in conditioned media from their original cultures, the other CAFs, or cancer cells with varying p53 status were analysed for remodelling. Gels created from the p53 LOF CAFs were greatly affected by conditioned media from the p53 GOF CAFs and cancer cells, indicating importance of secreted factors for signalling. Of the components found secreted in the conditioned media, HSPG2 or perlecan was found to be elevated and led to improved invasion. With a combination of HSPG2 KO and chemotherapy, tumour growth was delayed and survival increased in mouse models[250]. Another investigation of altered signalling is explored through the use of necuparanib, a heparin mimetic which can interact with several heparin-binding growth factors, chemokines, and other molecules[251]. In this study, single doses and combinations of necuparanib and gemcitabine decreased spheroid size in 3D cultures as well as extended survival and decreased metastasis in mouse models.

When investigating new treatments for malignancies, an option is to repurpose clinically used drugs for other malignancies. As several treatments are already clinically relevant, drug concentrations and durations of treatments are already calculated. Tamoxifen is an estrogen receptor modulator used to treat breast cancer patients but its role in affecting other types of cancers is being explored. One study identifies a role of tamoxifen in reduction of hypoxia within PDAC leading to an increase in vascularisation[252]. Use of tamoxifen on mouse models of PDAC led to downregulation of proteins involved in ECM organisation, cell adhesion, and wound healing and an upregulation of genes in vessel morphogenesis. Looking into more detail, it was discovered that hypoxia inducible factor 1 alpha (HIF-1A), a regulator of the cell's response to hypoxia, was reduced upon treatment. When using hydrogels, it was identified that tamoxifen altered fibrillar collagen diameter and length, collagen synthesis, and matrix remodelling through further alterations of

fibronectin and matrix metalloproteinase 2 (MMP-2). With all these effects, it appears that tamoxifen reduces the stiffness of the TME, allowing for vascularisation of tumours, which could lead to enhanced perfusion of treatments to the cancer cells[252]. As reduction and alterations of structural ECM components, such as collagens and fibronectin, has shown promise for reduction of stiffness within PDAC tumours, this avenue is a highly sought-after goal. The richness of the PDAC ECM comes from a combination of several collagens, glycoproteins, and proteoglycans, of which, hyaluronan is highly prevalent[253]. HA is degraded by hyaluronidases; therefore, a recombinant human form was created, named pegvorhyaluronidase alfa (PEGPH20). In a previous study using xenograft mouse models of PDAC, PEGPH20 was shown to enhance tumour uptake, inhibit tumour growth, and decrease metastasis when used in combination with gemcitabine[254]. Phase I clinical trials indicated a dose tolerance for patients that was able to decrease HA serum levels, allowing for alterations in tumour perfusion similar to the mouse models[255]. Two phase Ib/II trials performed with PEGPH20 focused on its combination with currently relevant treatments, gemcitabine and FOLFIRINOX. In combination with gemcitabine, overall survival (OS) was increased, however the incidence of thromboembolic events was roughly 30%[256]. Unfortunately, the combination with FOLFIRINOX proved detrimental to patients, reducing their OS as toxicity was increased by the combination[257]. In a phase II study combining PEGPH20 and nab-paclitavel/gemcitabine in untreated metastatic patients, patients with HA-high tumours resulted in the greatest improvements with an increased OS, from 8.5 months to 11.5 months[258]. Finally, in a phase III, the continuation of the phase II, specialised with high-HA only metastatic PDAC patients, while response rate was improved, OS and progression free survival did not improve[259]. Consequently, PEGPH20 was discontinued as it failed to meet the primary endpoints. Despite this unfortunate outcome, there are still clear benefits towards targeting of the ECM within PDAC, however further study is required before a treatment can become clinically available.

# Present Investigation

Cancer can be an extremely detrimental disease with years of involvement. While there have been great strides over the past decades in the field, there is still much to be learned and tackled in order to progress in all areas. Of the areas that needs more understanding, stromal-rich cancers, such as breast and pancreatic cancers, are common enough to affect thousands of individuals but also have a difficulty treating. For breast cancer, this is particularly true for the TNBC subtype, where no hormonal receptors are expressed. With the luminal and HER2 enriched subtypes, these receptors (PR, ER, and HER2) are the basis of some therapies. While there do exist some therapies that are being used for TNBC patients, 25 – 40% of patients develop recurrent tumours. Pancreatic cancers suffer from late diagnoses in patients, where tumours are generally found to be metastatic. These tumours do not respond well to currently used therapies due to decreased penetration from the desmoplastic reaction. Importantly, the role of the ECM and CAFs are important to identify viable ways to overcome a desmoplastic reaction. The currently aim of investigations now is to target the stromal environment first with subsequent use of traditional therapies. In this way, the ECM can be reverted back to its normal state and therapies are able to become effective for patients.

## Paper 1

The most accepted belief for the reason for recurrence of breast cancer is due to cancer stem cells. These are cancer cell that carry specific characteristics in order to allow them to survive best and proliferate. There have been several theories in the past about how these stem cells arise in the system, but the theory is that these cells already exist in the tumour. The tumour itself is a heterogenous mixture of different cell types which can actually be divided further into smaller niche environments, each with their own combination of cell types in varying proportions, trying to provide a particular function. However, it does appear that even normal tumour cells can gain some of the characteristics of stem cells and be the cause of recurrence.

In **paper 1**, we encountered a unique set of circumstances that eventually led to the increase in cancer stem cell-like cells within cell populations both *in vitro* and *in vivo*. We look into breast cancer with an overexpression of COMP, an extracellular matrix protein that had been previously found to be upregulated in roughly 20% of

all breast cancers[131]. The aim of **paper** 1 was to understand the mechanisms involved during overexpression of COMP which led to increased invasiveness, resistance to apoptosis, and poor survival in patients, with relation to the increased expression of Notch pathway components. When these cells are implanted into immunocompromised mice at low quantities, they are still able to produce tumours while their non-overexpressing counterparts do not, indicating the ability of the cells to survive under stress conditions. Several other methods such as side population analysis and CSC marker expression were also used to confirm the higher presence of cancer stem cells in COMP-overexpressing cells.

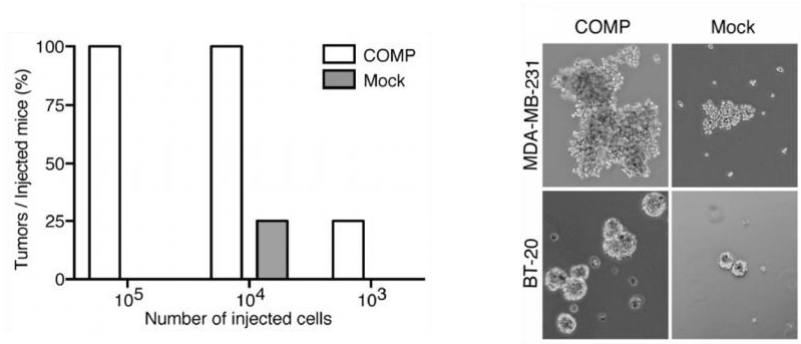


**Figure 8. COMP overexpresison enhances CSC population.** COMP-overexpressing cells, serially-diluted and injected into immunocomprimised mice, produced tumours when Mock control cells rarely produced. COMP-overexpressing cells similarly produced larger mammospheres, showing enhanced survival capability.

The overexpression of COMP resulted in an alteration in the Notch pathway, specifically, the upregulation and activation of Notch 3. After identification of the specific receptor, Jagged1, involved in Notch's activation, the role of COMP in this interaction was investigated. As COMP's traditional role in the ECM is as a scaffold to bring together components such as collagens, we hypothesised a similar role in aiding the interaction of Notch3 and Jagged1. To that end, we investigated the binding capability of all three proteins, COMP, Notch3, and Jagged1, to one another with a number of methods: proximity ligation assay (PLA), sandwich ELISAs, and co-immunoprecipitations. Most importantly was the addition of recombinant COMP into control cells as confirmation of COMP's direct role and not only the sustained alterations resulting from COMP overexpression. All of these methods confirmed the proximity and binding of the three proteins to one another.

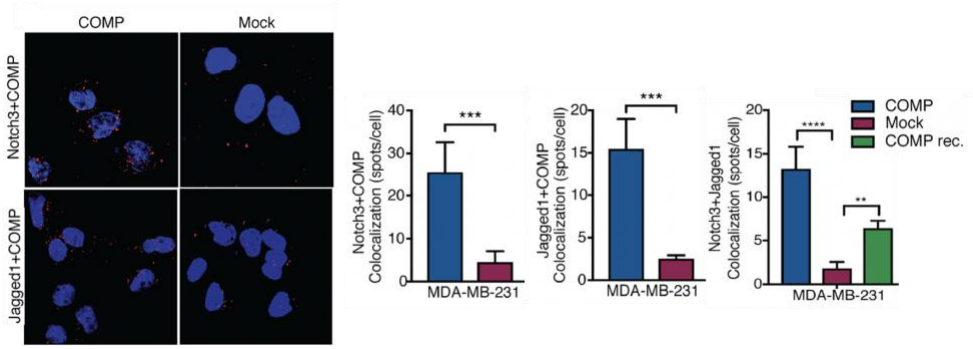


**Figure 9. COMP enhances Notch3 and Jagged1 interaction.** Proximity ligation assays indicating the proximity and interaction of Notch/COMP, Jagged1/COMP, and Notch3/Jagged1.

Through investigating the interactions of Notch target genes and even those that affect its receptor, Jagged1, we identified Notch and COMP's ability to affect other signalling pathways like Wnt/β-catenin and PI3K/AKT. Specifically, COMP-overexpressing cells contain more β-catenin within the cell as a whole and in the nucleus, confirmed with luciferase activation assay. Nuclear β-catenin and luciferase activation indicate further transcriptional activation of β-catenin target genes. When inhibiting Notch through γ-secretase inhibitor, DAPT, the activation of β-catenin was enhanced, indicating a role of Notch in β-catenin degradation. Similarly, when looking at the AKT pathway, we identified an altered state in COMP-overexpressing cells. In this case, AKT activation was decreased when compared to Mock cells with Notch seemingly inhibiting the activation.

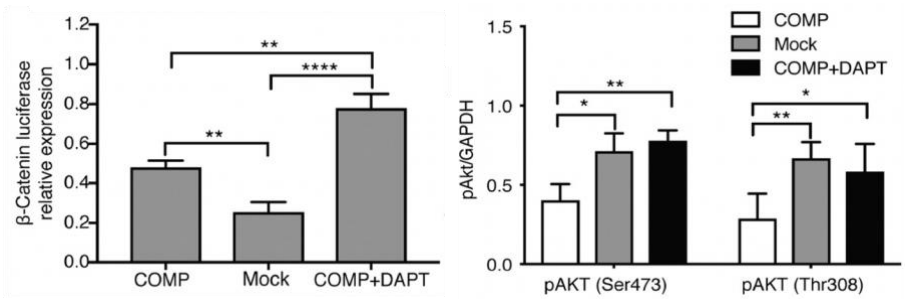


**Figure 10. Notch Activation and its Effect on Signalling Pathways.** COMP-overexpressing cells alter β-catenin activation, with direct effect by Notch on its activation, and alter AKT pathway, by decreasing its activation similarly effected by Notch.

All together, these pathways play a vital role in proliferation, the cell cycle, and a plethora of other pathways, allowing for great effects on the function of cells. We have shown already that these COMP-overexpressing cells implanted into mice showed enhance tumour initiating ability at decreased cell populations. To complete our study, we probed into the altered state of Notch3 in tumours produced in transgenic mice with varying expression of COMP. In these spontaneously formed tumours, we confirmed the activation of Notch3 in $COMP^{+/+}$ mice compared to $COMP^{+/-}$ and $COMP^{-/-}$ mice.

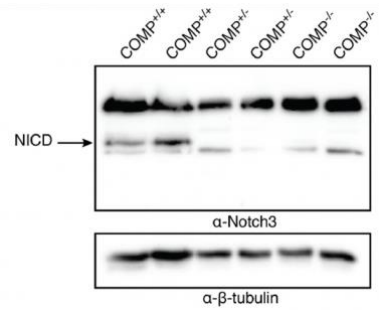


**Figure 11. Notch Activation in MMTV-PyMT breast cancer model with varying COMP expression.** Tumours from the MMTV-PyMT mouse model with varying expression of COMP were dissociated and probed for Notch3 expression.

In the end, the overexpression of COMP cause activation Notch3 which led to further activation and alterations in several key pathways within the cell that led to cells obtaining stem-like characteristics. This is an important finding in understanding the heterogeneity of cancer, the various cell modifications that can be found even within the same type of cancer, and the daunting task in attempting to identify the best approach to overcome it.

# Paper 2

**Paper 2** brings another aspect into the varying ways that cells can undergo change but eventually leading to a similar outcome as in **paper 1**. In this case, we take a look at the STRIPAK complex and, more specifically, the scaffold protein STRIP1/FAM40A, in breast cancer. We investigate the alterations caused by loss of STRIP1 and overactivation of GCK kinases, which had originally been shown to cause a lower proliferation than the loss of other STRIPAK components. The aim here was to identify the cause of the decreased proliferation with loss of STRIP1.

We identified that this proliferation loss is related to G0/G1 arrest in STRIP1 depleted cells.

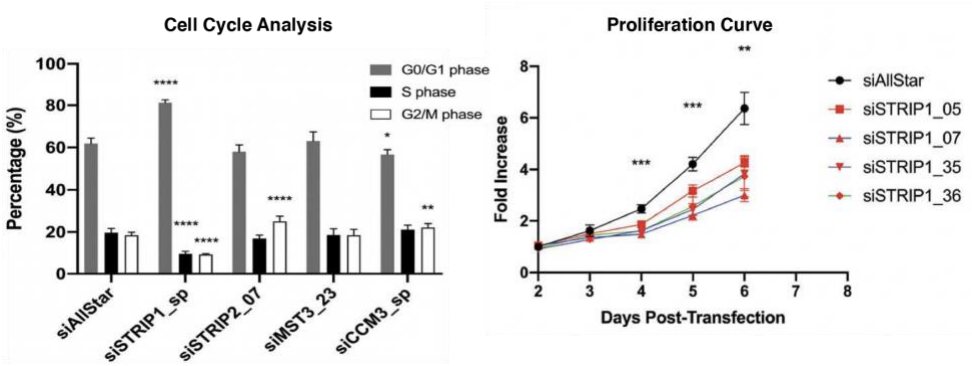


**Figure 12. STRIP1 depletion alters prolfieration through cell cycle arrest.** Flow cytometry analysis of cell cycle phases indicates increased population of cells within G0/G1 in STRIP1 depleted cells compared with cell depletion of all other STRIPAK components and control cells. Depletion of STRIP1 also decreases proliferation of cells compared to control cells.

This change of STRIP1 depletion was discovered to be due to the upregulation of the cell cycle inhibitors p21 and p27 but not associated with increased DNA damage in the cells, identified by decreased γH2Ax. It was surprising when looking at an individual cell analysis, through immunofluorescence, that there was such a striking difference between a basal level of p21 and its overexpression, which is found in greater population in STRIP1-depleted cells. The difference is another confirmation towards the heterogeneity of cell populations within tumours.

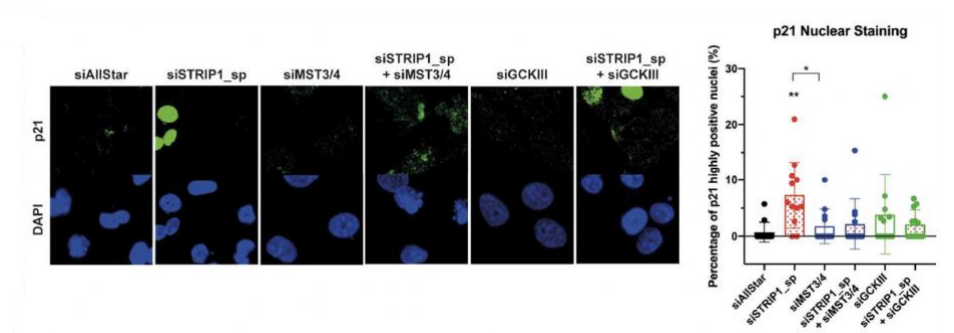


**Figure 13. STRIP1 depletion leads to nuclear overexpression of p21 in a subset of cells.** Heterogenous expression of p21 was found to exist in all cell condition, with STRIP1 depleted cells containing a greater population. Overexpression of p21 was distinctly found to be nuclear only.

In an attempt to enhance the overall levels of p21 to confirm the larger p21 overexpression in STRIP1-depleted cells, we found a curious effect when the cells were subjected to therapy. From previous work, it had been identified that the level of p21 during the phase in which cells were subjected to therapeutics was able to determine whether cells would become senescent or proliferative[234]. In this work, it was shown that highly elevated and decreased levels of p21 both led cells to senescent fates after an allowed recovery time while median levels, labelled as 'the goldilocks zone', resulted in proliferative cells. In replicating the study found, it was seen that a low dosage of therapeutic drugs did not have the same effect on STRIP1-depleted cells and control cells. While these cells had been less proliferative than control cells before the use of therapy, they became more proliferative than the control cells after therapy and an allowed recovery period. In the end, we confirmed that the elevated p21 and p27 levels were responsible for the protective effect towards therapy as depletion of both STRIP1 and p21 or STRIP1 and p27 rescued the increased proliferation to that of control cells.

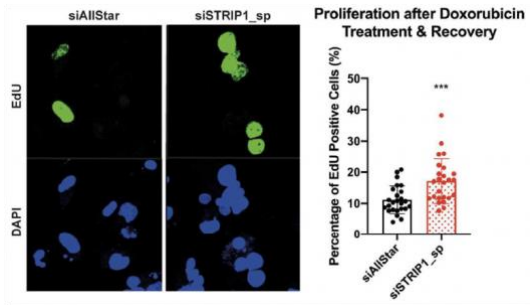


**Figure 14. STRIP1 depletion leads to increased proliferation following drug treatment and recovery.** Cells were subjected to low dose therapeutics for 24 hours and allowed 96 hours of recovery time before proliferation analysis. STRIP1 depleted cells were found to be more proliferative than control cells, both subjected to treatment and recovery.

In this paper, we were able to demonstrate once again how an alteration of protein expression can affect the cell. Loss of STRIP1 and subsequent hyperactivation of the GCKIII kinases lead to an altered cell cycle and increased p21 levels. These levels then provide the cell with the ability to protect themselves, providing another opportunity for recurrence to occur when therapy does not affect all of the cell population.

# Paper 3

It is not only the cancer cells that are important in the development of cancer. Therefore, the efficiency of therapies on the tumour would be dependent upon its effects to all the cells types found within. CAFs have always been a large component of the tumour environment, but the focus has always been on the tumour cells as the important factors. With work performed in the past years, it is clear that there is communication between cell types and given the heterogeneity of niches within a single tumour itself, it is likely that communication between CAFs, cancer cells, and any other cells, varies enough to allow for some niches to develop altered expression patterns leading to phenomena like increased stem like cells.

It has been postulated that the cause of the low efficacy of therapy in pancreatic cancer is due to the desmoplastic reaction within the tumour, causing a great amount of stiffness of the tissue and preventing penetration of drugs. Due to this, the belief for improving therapy is now to target the densely packed stroma in an attempt to decrease the amount of ECM and the pressure within the tumour, therefore allowing the current therapies that presently exist to be used afterwards and with an increased chance at having desired effects.

**Paper 3** describes a far simpler and quicker method to be able to study effects on CAF functionality and even the effects its remodelling capability would have on cancer cell invasion. In short, rat tail collagen is extracted and prepared for further use. Multi-well plates are coated with BSA in order to aid the formed gels to contract uniformly without adherence to the well surface. The desired CAFs are prepared, counted, and then combined in a mixture of the previously prepared rat tail collagen, collagen neutralising buffer, and fetal bovine serum (FBS). It should be noted here that the cells may, prior to the addition to the mixture, have been subjected to either transient or stable modification, and the mixture could likewise contain basement membrane components, as Matrigel. The new solution is added to each well of the plate and allowed to polymerise. Once the gels polymerise, therapeutic drugs can be added to the media. ECM remodelling occurs over a 72-hour time period and analysis of the contraction is calculated as:

$$(\%)Contraction = 100 \text{ x } \frac{(\text{well area} - \text{gel area})}{(\text{well area})}$$

For further investigation into the invasion capability of cells through the remodelled gel, the gels are placed on a mesh scaffold with an air-liquid interface and added cancer cells should be allowed up to 72 hours to invade into the gel. Analysis of invasion is calculated as:

$$Invasive\ Index = \frac{\text{Number of cells detected inside section}}{\text{Total number of cells seeded}}$$

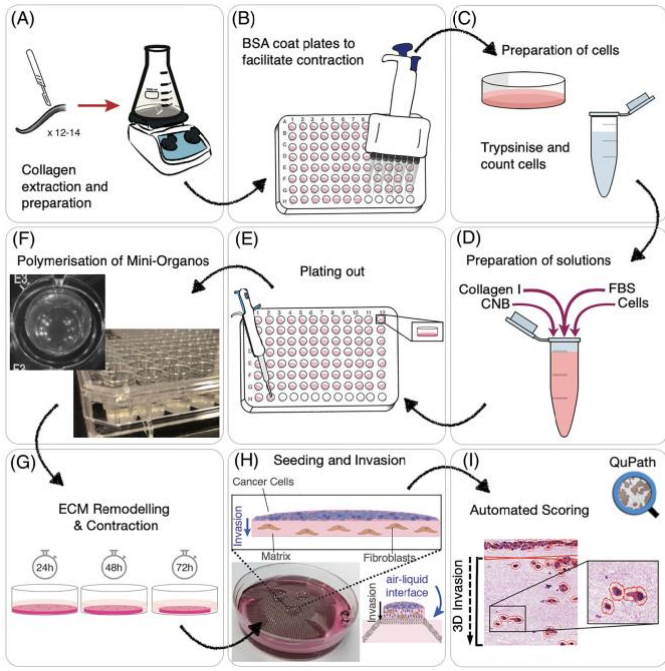


**Figure 15. Step-by-step approach to the mini-organo technique.** Schematic guide to the mini-organo procedure. Extraction and preparation of collagen preceeds preparation of multi-well plates used for creation of gels. The gel solution is created with the desired cells, the extracted collagen, and other factors. The evenly plated out solution is allowed to polymerise before being left to become remodelled by the included cells. Further invasion analysis can be performed using a scaffold and providing a nutrient attractant using an air-liquid interface.

Extraction of rat tail collagen described in this method could similarly be purchased commercially with identical results, therefore reducing the time and resources even further for this method. The move towards smaller gels for contraction and remodelling benefits the decreased use of materials such as unique cell types, collagen, and drugs used. Of course, optimization is required as for all new methods with varying cell lines, but this similarly can be done in mass due to the miniaturized format of the method. Calculation of contraction ability is extremely simple and imaging the plate does not require complex equipment. Continuing on with the invasion analysis is similarly a shorter time span due to the size of the remodelled

gel. Therefore, allowing for quicker experiments and screening of any manipulations placed upon cells.

This method was validated using cell lines derived from KPC mouse model variants, previously described[260], with mutant GOF p53 and LOF p53, which have varying degrees of aggressiveness. The inhibitors used similarly confirm the validity as these alter the mechanistic ability of CAFs to remodel their environment. The reproducibility of the model was confirmed by different laboratories using different collagen concentrations and cell quantities. All these provide proof of the stability of this enhanced method, allowing for reliable results.

# Paper 4

Finally, we look into a highly specific protein modification that could be used as a drug target for therapy. Oncofetal chondroitin sulfate was found to be present only in the placenta in normal, non-malignant tissue. It was eventually found to also be present in cancer cells and tumour circulating cells. Due to its high specificity, it was immediately believed to have a higher purpose as a drug target. Therefore, in **paper 4**, we investigated ofCS in cells, with the aim of finding its presence in pancreatic cancer cells and tissue and a role it might play. During the investigation however, we took advantage of a small panel of mouse pancreatic cells, of which included cancer cells and CAFs. Confirmation of presence of ofCS was identified in cancer cells, but was surprisingly found in CAFs, identifying varying expression and localisation of ofCS within the cells. Not only was this a novel discovery of ofCS in CAFs, but it was also noted that CAFs secreted factors containing the ofCS modification. We further explored the effect of hypoxia and the p53 GOF mutation R172H, detecting increased ofCS expression under both stressors.

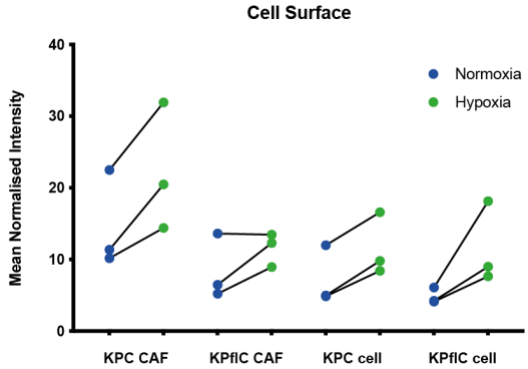


**Figure 16. ofCS expression is affected by hypoxia and p53 status.** Flow cytometry analysis of cells derived from two KPC mouse models. Cells were plated and grown in either normoxia (23% $O_2$) or hypoxia (1% $O_2$) for 72 hours. Cells were then probed with a recombinant VAR2 (rVAR2) for ofCS detection. Hypoxia, p53 mutational status, and cell type all appear to affect ofCS expression.

Understanding the desire to exploit the specificity of ofCS for therapeutic purposes, we were interested in understanding its initiation into cancer tissue. Taking two models of spontaneous cancer formation, the MMTV-PyMT model for breast cancer and the iKras*p53* model for pancreatic cancer, we took several time points during progression and identified ofCS already in the early stages of development. The abundance of ofCS within the tissue grew along with the tumour, indicating the possibility of early detection and targeting.

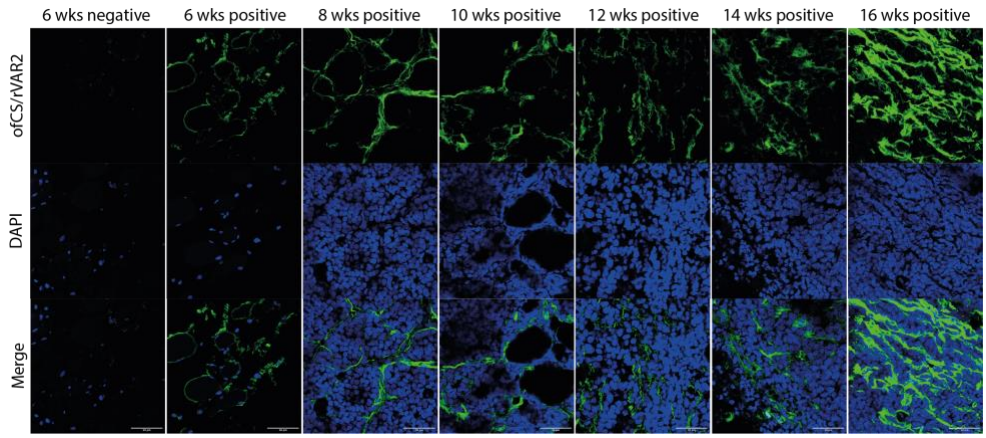


**Figure 17. ofCS expression initiates at early stages and increases during progression.** Tissue from MMTV-PyMT mouse model was probed with rVAR2 for ofCS detection at varying stages of development. ofCS is detected at the early stages of 6-8 weeks and appears to increase in abundance and intensity during progression. ofCS expression does not appear to exist in the epithelial cells.

Finally, we investigated ofCS's expression within human tissue and its dependence upon CAFs. Using commercially purchased TMAs, we confirmed the colocalization of ofCS with αSMA+ activated fibroblasts, confirming the CAF-dependence for deposition of ofCS onto the ECM. An attempt was made to correlate stage and grade of breast and pancreatic tumours to ofCS expression in human tissue, but this was not identified with the current samples.

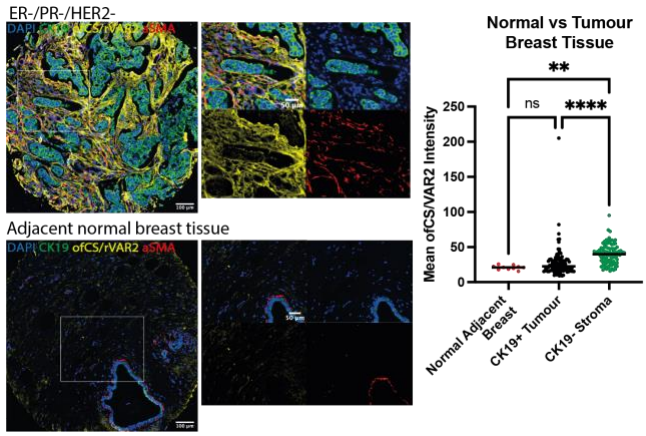


**Figure 18. ofCS expression is correlated to αSMA⁺ stromal presence.** Immunofluorescence image of two patient samples from a commercial tumour microarray (TMA) indicating a sample from a breast cancer patient and normal adjacent breast tissue, showing elithelial cells by cytokeratin (green), ofCS by rVAR2 detection (yellow), αSMA-positive fibroblasts (red), and nuclear DAPI (blue). ofCS detection appears to be absent from epithelial areas and present in the stroma.

This data shows the viability of pursuing further investigation into ofCS and its use as a drug target for anti-stromal therapy in breast and pancreatic cancers. The high specificity of ofCS would also allow for decrease in toxicity to patients.

# Conclusions

In conclusion, we demonstrate that cancer is a complex spectrum with many variables involved for its initiation, maintenance, and progression. It is important to understand all the possible nuances in order to comprehend the processes and find ways to combat them. Alterations of expression of COMP and STRIP1 affect cells enough to allow for resistance to therapy, likely for COMP overexpression but confirmed for STRIP1 depletion. Several of the affected pathways can be altered by signalling between cancer cells and CAFs, conditioning the CAFs to promote

tumorigenesis. As the components in the ECM acting as intermediates in this signalling, proteoglycans and other ECM components are vital tumour progression, all of which play a role in therapeutic response.

# Future Research

The role of aberrations of proteins and their subsequent effect on cells is crucial to understand when considering treatment options for patients. Both COMP and STRIP1 have been shown to have the ability to lead to recurrence in breast cancer. COMP has already been shown to be function as a prognostic marker in breast cancer through serum detection[261]. In this study, it was shown that metastatic patients contained twice as much COMP in their sera. However, not all cancers react in the same way. To understand if COMP could be used as a diagnostic or prognostic marker in other types of cancers, the expression needs to be examined in more patient samples.

Similarly, when looking at the role of the STRIPAK complex in pancreatic cancer, we found variances when compared to breast cancer. The nature of this specific type of cancer being rather important as it is not as clearly understood as breast cancer. In this case, using murine cells, we found that a loss of FAM40B/STRIP2 rather than STRIP1, was able to over-activate the GCK kinases.

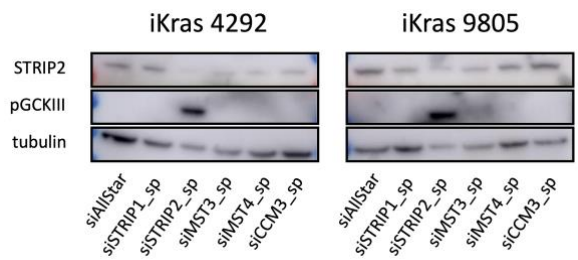


**Figure 19. Loss of STRIP2 induces activation of GCKIII kinases.** Western blot analysis of murine cell lines iKras 4292 and iKras 9805, derived from the indicible iKras*p53* model with Kras G12D and p53 R172H mutations, indicating only loss of STRIP2 leading to activation of GCKIII family of kinases.

While in the breast cancer cell line MDA-MB-231 we could see morphological variations when depleting the STRIPAK complex components, we cannot identify such a drastic variation with the murine lines iKras 4292 and iKras 9805. Of note, these lines are derived from an inducible model for pancreatic cancer that require doxycycline for sustained expression of the Kras mutation G12D. Depletion of

STRIP2 similarly affected the proliferation of the cells, being decreased in both cell lines. However, with simultaneous depletion of the kinases, a rescue of the decreased proliferation was not possible in the 9805 line.

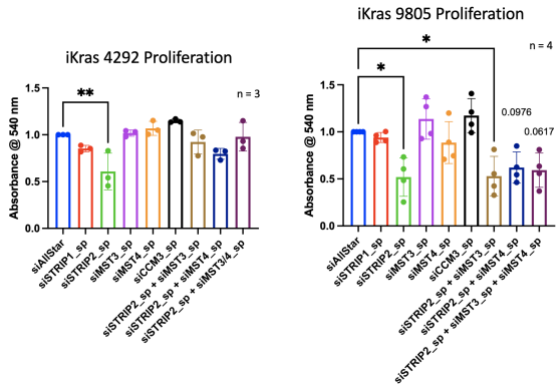


**Figure 20. STRIP2 depletion leads to decreased proliferation.** When depleting both murine cell lines with STRIPAK components, only loss of STRIP2 lead to a decrease in the proliferation. Simultaneous depletion of STRIP2 and the kinases was able to rescue the decreased prolideration in only one cell line.

Similar to the other investigations, looking into the localisation and expression of p21 in these cells revealed an increased population of high nuclear p21 expressing cells in a STRIP2 depleted population.

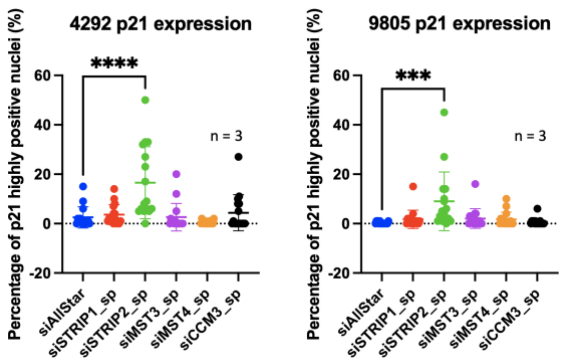


**Figure 21. STRIP2 depletion enhances nuclear p21.** Cells depleted with STRIP2 showed an increased percentage of cells with high nuclear p21 expression when compared to control and depletion with other STRIPAK components.

A quick look into a human pancreatic cell line revealed that the basal level of activated GCKIII kinases was detectable whereas this was never the case with either the MDA-MB-231 breast cancer cell or either of the murine PDAC cells. In this case, only STRIP1 depletion could affect the overactivation of the GCK kinases and p21 levels, indicating this change between the scaffold protein required for activation could be species specific.

Expansion of the investigation continued with a further look at how loss of STRIP2 could also affect the AKT or Wnt pathways. The hypothesis here is based on the limited population of high p21 expressing cells, roughly 10% in paper 2, and the resistance to therapy that is seen, likely due to a transformation to a stem-like population as was seen with COMP overexpressing cells in paper 1. While the experiments for this were not completed in full, we can already identify a trend of altered levels of both components of these pathways, with hyperactivation of AKT and depletion of β-catenin with loss of STRIP2. While there is evidence that AKT can affect p21 levels, its nuclear localisation rules out the possibility of this interaction. A further look into the mechanisms involved with STRIP1/2 depletion and subsequent activation of GCKIII kinases and the alterations if being to cells could reveal a novel role for the STRIPAK complex. With more clinical evidence of GCKIII activation on survival, both before and after treatment, this could assist doctors in navigating a treatment plan for patients that present with this unique feature.

Understanding how the TME changes over time would lead to enhanced knowledge of what components play a tumour promoting versus tumour suppressing role. Advances in scRNAseq have allowed for distinguishing the heterogeneity of tumours, however, most studies are temporally stagnant, only one time point is used. It is the changes during progression that are likely important and can be used as a targeting options. Therefore, a push towards identification of temporal changes and eventually spatial alterations in three-dimensional studies would allow for an improved comprehension of the cancer.

Most importantly for this work is the involvement in therapy, particularly the use of ofCS as a drug target. The current work with our collaborators has expanded greatly. The imaging and animal models used in **paper 4** have provided our collaborators a viable model for testing developed antibodies for efficiency testing. Thus far, the first attempts at antibody drug conjugate testing have yielded promising results.

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