**UNIVERSITA' DEGLI STUDI DI TORINO**

DIPARTIMENTO DI ONCOLOGIA

SCUOLA DI DOTTORATO IN SCIENZE

DELLA VITA E DELLA SALUTE

**DOTTORATO DI RICERCA IN MEDICINA MOLECOLARE**

**CICLO XXXIII**


# Semaphorin 6C controls pancreatic cancer cell viability and growth


**TESI PRESENTATA DA**

Damon Fard

**TUTOR**

Prof. Luca Tamagnone

Prof. Silvia Giordano

# CONTENTS

INTRODUCTION ........................................ 3

Semaphorins ........................................ 4

Semaphorin functions in health and diseases at a glance ..................... 5

*Semaphorins in neurological diseases*

*Semaphorin function in cardiovascular development and angiogenesis*

*Semaphorin function in bone homeostasis*

*Semaphorin function in the immune system and immunopathology*

*Semaphorin functions in cancer* ........................................ 11

Plexins ........................................ 14

Transmembrane semaphorins can trigger forward and reverse signaling ... 14

Neuropilins ........................................ 16

Sema6C: a poorly studied family member........................................ 16

AIM OF THE WORK ........................................ 18

RESULTS ........................................ 20

Sema6C knockdown in cancer cells hampers proliferation and elicits cellular senescence ........................................ 21

Sema6C-depleted cells increase the autophagic flux ................................. 31

Sema6C-induced phenotypic changes and cancer cell motility ................... 32

Sema6C sustains cancer cell viability under nutrient deprivation and metabolic stress ........................................ 37

Sema6C-dependent regulation of cancer cells is dependent on Focal Adhesion Kinase ........................................ 40

Sema6C-dependent regulation of cancer cells depends on its intracellular portion ........................................ 44

DISCUSSION ........................................ 51

MATERIALS AND METHODS ........................................ 57

BIBLIOGRAPHY ........................................ 64

# INTRODUCTION

## Semaphorins

Semaphorins are a large family of evolutionary conserved extracellular signaling molecules, which mediate cell-cell communication controlling a variety of cellular functions (Alto and Terman 2017). Semaphorins were primarily described as axon guidance cues, but were later implicated in the regulation of different biological processes, such as cardiovascular development, angiogenesis, bone homeostasis, and immune responses; in the tumor microenvironment, they modulate cancer cell growth, invasion and metastasis (Fard and Tamagnone 2021)

Hitherto, more than 20 semaphorin protein family members have been discovered, comprising secreted, transmembrane, and cell surface-attached proteins, subdivided into eight classes based on structural features and sequence similarity (Figure i).

Except for the first two classes that contain invertebrate semaphorins, 5 out of the 8 classes of semaphorins are expressed in vertebrates, while class V includes semaphorins encoded by viral genomes (Nakamura, Kalb, and Strittmatter 2000). While class 3 semaphorins are secreted as soluble molecules, all other vertebrate semaphorins exist primarily as membrane-bound forms, even though they can be sometimes shed in the extracellular space (Battistini and Tamagnone 2016). Semaphorin dimerization and receptor binding occurs through a *sema* domain, which is an N-terminus 500-amino-acid moiety with seven-blade beta-propeller structure.

Alongside the sema domain is located a cysteine-rich motif of 80 amino acids known as plexin-semaphorin-integrin domain (PSI), also referred to as MET-related sequence (MRS). Other features, specific for some semaphorin subclasses include: immunoglobulin-like domains, a basic C-terminus-domain in class 3, or thrombospondin repeats in class 5 (Capparuccia and Tamagnone 2009).

Class 3 semaphorins are secreted into the extracellular space and act in paracrine and autocrine manner. Classes 4, 5, and 6 include transmembrane semaphorins, which are capable of interacting with surface molecules on neighboring cells (*in trans* signaling) or via

shedding into the extracellular space. In both cases, this ("forward") signaling mode typically implicates specific receptors called Plexins.

Actually, transmembrane semaphorins also include a cytoplasmic domain, which allows for a two-way signaling: i.e. *forward* signaling that implicates semaphorins to act as ligands and plexins as receptors; and *reverse* signaling mode, when semaphorins act as receptors and the extracellular domain of the plexins functions as a ligand (Battistini and Tamagnone 2016). The forward and reverse signaling modes of semaphorins will be described in more detail in one of the following paragraphs.

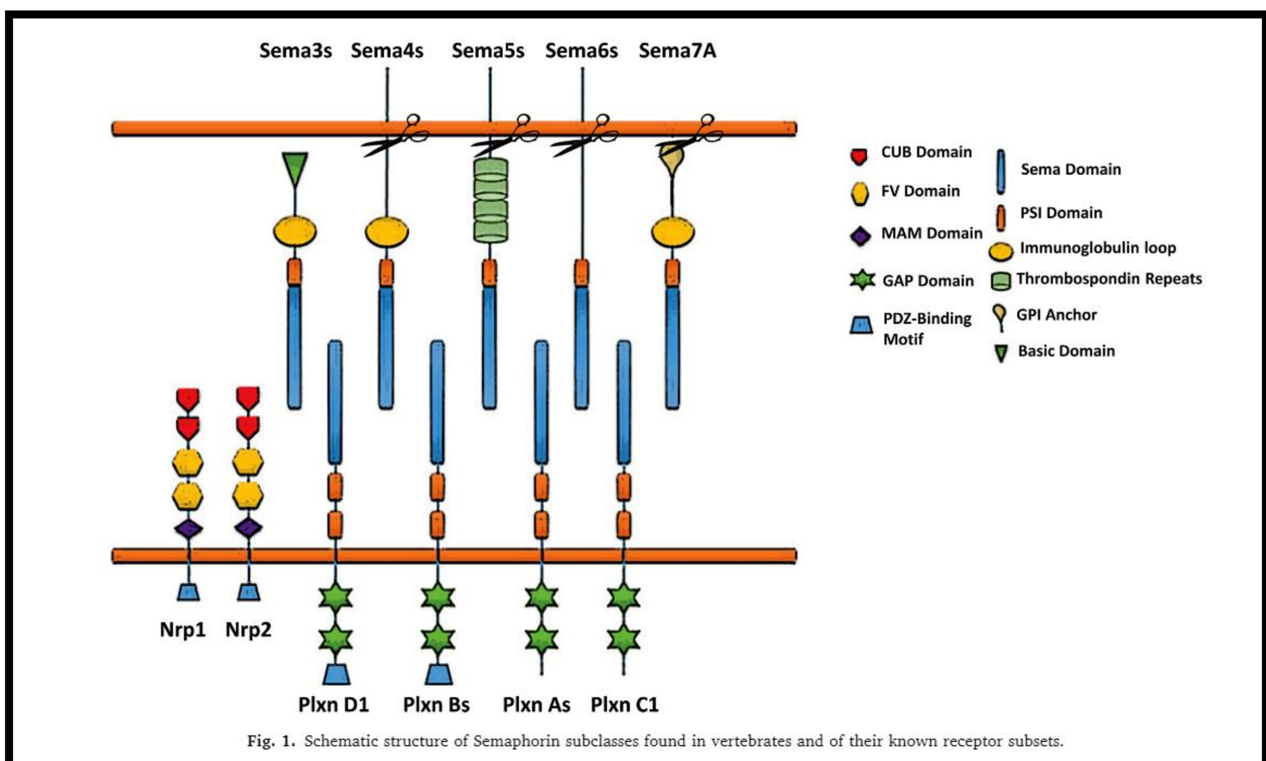



**Figure i.** Semaphorin and semaphorin-receptor structure (modified from *Fard et al, 2021*)

## Semaphorin functions in health and diseases at a glance

### *Semaphorins and neurological diseases*

In addition to their fundamental role in nervous system development (Carulli, de Winter, and Verhaagen 2021), multiple lines of evidence indicate that semaphorins can regulate neural

regeneration and the homeostasis of the adult nervous system (Koropouli and Kolodkin 2014) (Pasterkamp and Giger 2009). Therefore, not surprisingly, this protein family also plays an important role in diverse neurological disorders.

In the adult brain, they can function as limiting factors for neuronal regeneration after damage, and, in experimental mouse models, inappropriate semaphorin activity has been related to epileptic seizures and autistic behavior (Sahay et al. 2005; Li et al. 2019).

The regulatory role of Sema3A in hippocampal neurons has been studied in the early stages of Alzheimer's disease. This semaphorin seems to contribute to neuronal degeneration or delay the recovery of damaged neurons, by phosphorylating intracellular CRMP2 protein, which controls cytoskeletal stability (Good et al. 2004).

Furthermore, it is disputed the involvement of Sema3A in ALS, as some studies suggested its association with the typical muscle denervation, due to its role in distal motor axonopathy (Venkova et al. 2014).

In multiple sclerosis (MS) lesions, an abnormal expression of Sema3A hinders remyelination by repelling oligodendrocyte progenitor cells (OPC) that express PlexinA1/NRP1; while an opposite activity has been reported for Sema3F (Williams et al. 2007; Piaton et al. 2011). Additional semaphorins known to regulate immune responses (e.g., Sema3A, Sema4A, Sema4D, Sema6D, and Sema7A) have been putatively implicated in MS pathogenesis, based on experimental studies in mouse models (Okuno, Nakatsuji, and Kumanogoh 2011).

### *Semaphorin function in cardiovascular development and angiogenesis*

Sema3C/PlexinA2 signaling regulates the migration of neural crest cells involved in the septation of the heart outflow tract and in the formation of the aortic arch (Feiner et al. 2001; Goldmuntz and Emanuel 1997). Moreover, Sema3E/PlexinD1 signaling regulates cardiac ventricular compaction (Sandireddy et al. 2019).

Sema3D knock-down in zebrafish resulted in dysmorphic hearts with smaller atria and ventricles, although this activity has yet to be confirmed in vertebrates (Sato, Tsai, and Yost

2006; Fard and Tamagnone 2021). Sema3A's involvement in cardiac sympathetic innervation patterning, which regulates the heart rhythm, has been studied, and a missense mutation in SEMA3A has been linked to illnesses including sudden cardiac arrest and the Brugada syndrome (Ieda et al. 2007; Boczek et al. 2014; Nakano et al. 2013).

Notably, due to its ability to engage diverse receptor complexes, Sema6D has been shown to have a dual effect in chicks: as an enhancer of endocardial cell migration in the cardiac outflow tract, and as an inhibitor of migration in the ventricle region. Furthermore, Sema6D/PlexinA1 regulates myocardial layer differentiation and heart chamber development via forward and reverse signaling (Toyofuku, Zhang, Kumanogoh, Takegahara, Suto, et al. 2004).

The anti-angiogenic activity of secreted class 3 semaphorins (i.e. Sema3A, 3B, 3C, 3D, 3E, 3F, and 3G) is crucial for balancing stimulatory cues and preventing inappropriate vessel development (Valdembri et al. 2016). In contrast, other family members, such as Sema4D and Sema5A, have been recognized as pro-angiogenic factors, due to their regulatory activity in endothelial cells (Kim et al. 2011; Sadanandam et al. 2012; Conrotto et al. 2005). Additional semaphorins have been implicated to promote angiogenesis due to their regulation of tumor-associated macrophages (TAMs), which are a known source of pro-angiogenic factors in the tumor microenvironment (Figure ii).

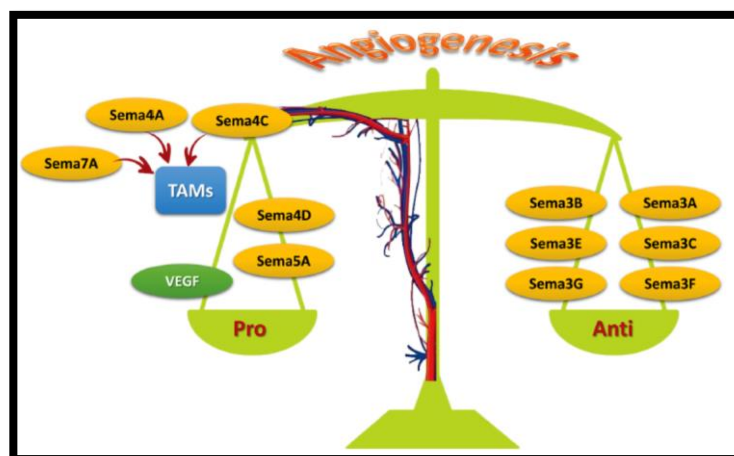



**Figure ii.** Semaphorin function in angiogenesis (modified from *Fard et al, 2021*)

### *Semaphorin function in bone homeostasis*

Semaphorins have been studied for their role in bone homeostasis and in associated diseases. For example, the Sema4D/PlexinB1 pathway is a key regulator of osteoblast/osteoclast function, and negatively controls bone formation. In fact, upon treatment with Sema4D-blocking antibodies, or genetic loss of Sema4D or PlexinB1, an increase in bone mass was observed in mice (Negishi-Koga et al. 2011; Dacquin et al. 2011) (Figure iii).

Sema3A, in contrast, has been linked with osteoclast/osteoblast activity as an inhibitor of bone resorption, leading to enhanced bone formation in mice (Kang and Kumanogoh 2013; Hayashi et al. 2012).

The regulatory function of Sema7A on osteoblasts and osteoclasts has been shown in vitro. In particular, it stimulates osteoblast migration by binding to the integrin 1 receptor and enhances osteoclast development by favoring precursor cell fusion (Delorme et al. 2005). Moreover, the SEMA7A gene polymorphisms are associated with decrease in bone mineral density and increased risk of fracture (Koh et al. 2006).

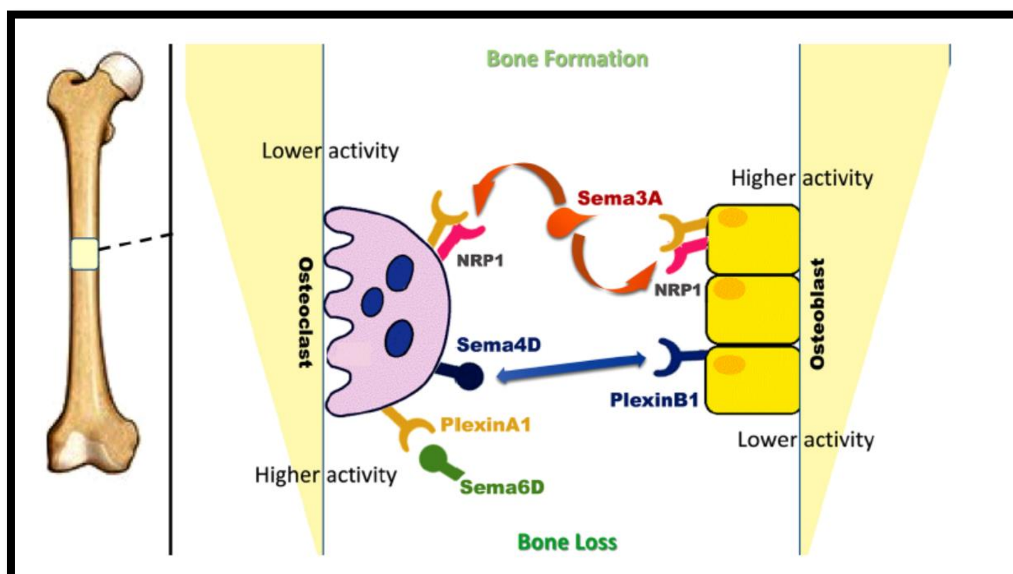



**Figure iii.** Semaphorin function in bone homeostasis (modified from *Fard et al, 2021*)

### *Semaphorin function in the immune system and immunopathology*

Semaphorins play multiple functions in the innate and adaptive immune system and in the pathogenesis of immune diseases. For example, inhibition of neutrophils activation and inflammatory response occurs upon engagement of Sema4D on the surface of the neutrophils with PlexinB2 exposed by endothelial cells (Nishide et al. 2017). On the other hand, inflammatory macrophages exposed to Sema3A are induced to produce and release more cytokines (Wen et al. 2010) (Figure iv).

In the frame of adaptive immune response, Sema3A regulates the migration of the dendritic cells (DC) in the lymphatic system. In particular, the interaction of Sema3A, released by lymphatic endothelial cells, with PlexinA1/NRP1 on dendritic cells, causes actomyosin contraction, which allows dendritic cells to pass across narrow vessel gaps (Takamatsu et al. 2010). In a different context, Sema3A has also been associated with negative control of immune responses, negatively controlling T-cell proliferation and fostering the differentiation of suppressor T cells (Catalano et al. 2006; Catalano 2010).

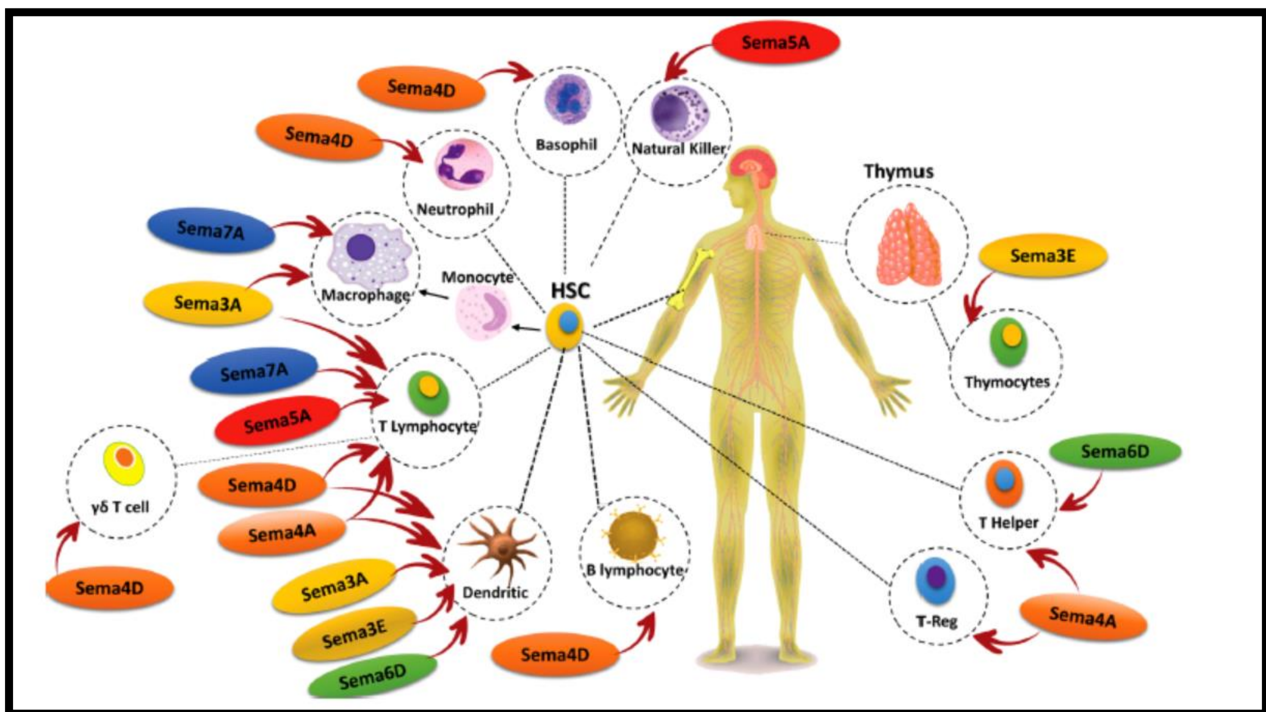



**Figure iv.** Semaphorin function in immune response cells (modified from *Fard et al, 2021*)

Sema3E functions as a regulator of the migration of CD4/CD8 double-positive T lymphocyte precursors in the thymus, during immune system development; in fact, these cells copiously express the receptor PlexinD1, which enables the maturing lymphocytes to migrate into the thymic medulla (Choi et al. 2008). Both Sema3A and Sema3E have been implicated to inhibit allergic responses, with a protective function in bronchial asthma (Adi et al, 2019)(Movassagh, Shan, Chakir, et al. 2017).

On the other hand, Sema4A expressed by DCs promotes Th1 activation and differentiation, by binding to the non-canonical receptor TIM-2 on T-cell surfaces (Kumanogoh, Marukawa, et al. 2002). Actually, differentiated Th1 cells also express Sema4A at a high levels, and Sema4A knock-out mice show defective Th1 differentiation, while Th2 response increases, sustaining allergic inflammation of the airways (Kumanogoh, Shikina, Suzuki, et al. 2005; Adi et al. 2019; Nkyimbeng-Takwi et al. 2012; Movassagh, Shan, Mohammed, et al. 2017).

Sema4B expressed by lymphocytes negatively regulates basophil function and inhibits the secretion of cytokine IL-4; moreover, Sema4B-deficient mice show elevated levels of serum IgE, compared to wild type (Nakagawa et al. 2011). Sema4C has a role in the differentiation of B lymphocytes, and especially in B cell polarization in response to Th2 cell regulation (Xue et al. 2016).

The analysis of Sema4D knock-out mice demonstrated that this semaphorin promotes B-cell proliferation and antibody production; this forward signaling mechanism is mediated by the non-canonical receptor molecule CD72, containing intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM), the function of which is blunted upon Sema4D binding (Kumanogoh, Shikina, Watanabe, et al. 2005). Intriguingly, CD72 is furthermore exposed by DCs, eliciting reverse signaling by Sema4D in T-cells and stimulating their maturation and immune function (Kumanogoh et al. 2000; Kumanogoh, Suzuki, et al. 2002). Consistently, Sema4D-deficient mice were found to be defective in forming antigen-specific T-cells, and more prone to develop autoimmune encephalitis compared to wild-type (Kumanogoh, Suzuki, et al. 2002). Interestingly, also reverse signaling from PlexinB2 to Sema4D in γδ T cells enhances their activation and function (Witherden et al. 2012).

Sema5A acts as an inducer of the proliferation and activation of T-cell and natural killer (NK) cells, and enhances the secretion of proinflammatory Th1/Th17 cytokines, shifting lymphocyte differentiation towards the inflammatory Th17/Tc17 phenotype (Gras et al. 2014). It has been shown, by in vitro and in vivo experiments, that CD4+ T-cells induce Sema6D expression upon TCR engagement, which plays an important role in the late phase of T-cell activation (O'Connor et al. 2008).

Sema7A act as an activator of T-cells in the frame of immunological synapses with DCs and macrophages, expressing β1 integrin receptors (Suzuki et al. 2007) Also Sema7A shed in soluble form by metalloproteases stimulates Th1/Th17 cytokine secretion and immune responses in an integrin β1-dependent mode (Xie and Wang 2017). Moreover, Sema7A was reported to promote pro-inflammatory signaling cascade between cells of the innate and adaptive immune system, participating in the pathogenesis of rheumatoid arthritis. Indeed, hindering Sema7A-β1-integrin signaling leads to a considerable disease attenuation in an experimental model of autoimmune arthritis (Xie and Wang 2017).

### *Semaphorin functions in Cancer*

Tumorigenesis and metastatic dissemination is a multistep process: the growth of a tumor mass and the local invasion of adjacent normal tissues by cancer cells may compromise vital functions, leading to 10% of cancer-related deaths; however, nearly 90% of the patients succumb to the consequence of cancer cell dissemination forming metastatic secondary tumors (Seyfried and Huysentruyt 2013). In recent years, diverse semaphorin signals have been consistently implicated in the regulation of tumor cell proliferation, angiogenesis, invasion and metastasis (Rizzolio et al. 2012; Neufeld et al. 2016) (Figure v).

Multiple class 3 Semaphorins have been associated with suppression of tumor growth and/or of tumor angiogenesis. For instance, Sema3B or Sema3F levels are lower in small cell lung carcinoma and endometrial cancer, compared to normal tissue counterparts (Tomizawa et al. 2001; Nguyen et al. 2011). Consistently, it was shown Sema3B-dependent inhibition of tumor cell viability (Tomizawa et al. 2001; Tse et al. 2002; Dong et al. 2019). Sema3F levels

are reduced in colorectal carcinoma ad breast cancer, which sustains tumor cell proliferation and invasion (Wu et al. 2011; Nasarre et al. 2005).

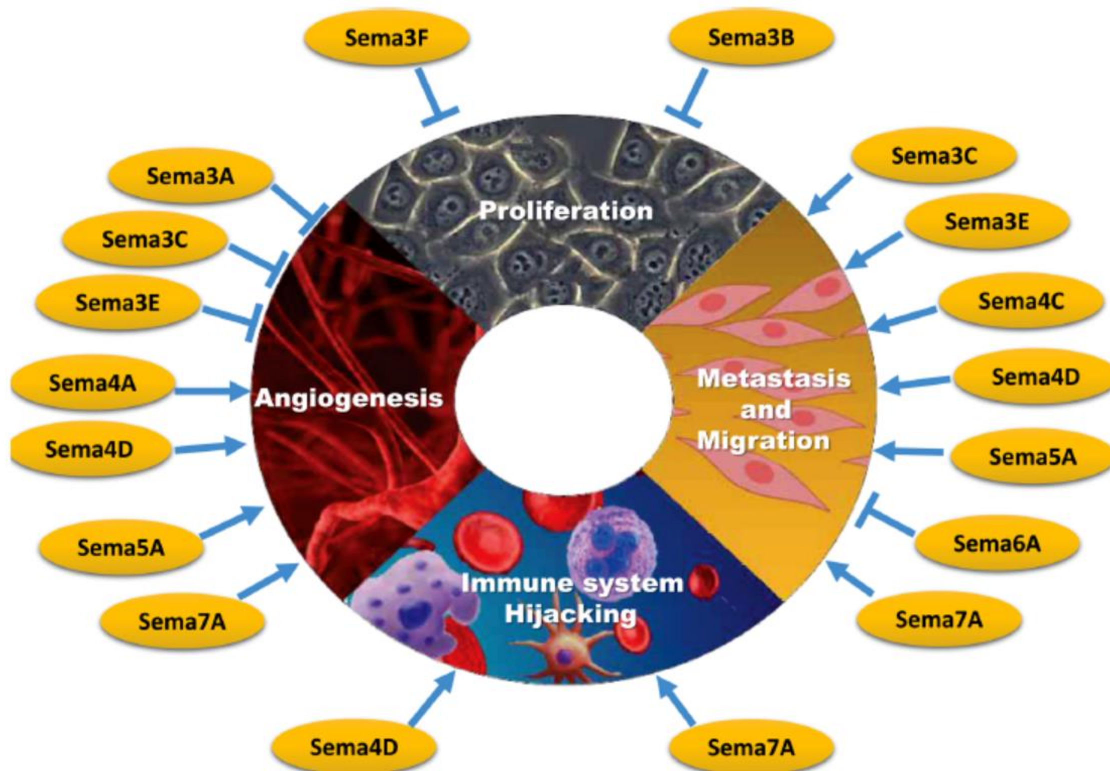



**Figure v.** Semaphorin function in cancer (modified from *Fard et al, 2021*)

A dual functional role of Sema3C has been reported: as tumor promoter in glioma, gastric, and prostate cancer (Vaitkienė et al. 2015; Peacock et al. 2018); as tumor suppressor due to its ability to inhibit pathological angiogenesis (Yang et al. 2015; Mumblat et al. 2015).

Similarly, Sema3E signaling through its receptor PlexinD1 negatively regulates angiogenesis in development and the tumor microenvironment (Tamagnone and Mazzone 2011; Casazza et al. 2012; Vivekanadhan and Mukhopadhyay 2019); however, other studies showed that Sema3E enhances cancer cell malignancy and metastasis via PlexinD1-associated oncogenic kinase ErbB2 signaling (Casazza et al. 2010). It was further reported that Sema3E sustain metastatic breast cancer cell survival by inhibiting a pro-apoptotic cascade promoted by PlexinD1 acting as a “dependence receptor” (Luchino et al. 2013).

Accumulating evidence revealed the role of membrane-bound semaphorins in cancer. Sema4C expressed by endothelial cells of tumor-associated lymphatic has been found to promote cancer cell invasion (Wei et al. 2017). Moreover it has been shown that Sema4C controls breast cancer cell growth, hormonal dependency, and invasiveness (Gurrapu et al. 2018). Also Sema4C reverse signaling was found to promote cancer progression, by controlling gene expression and phenotype change through SMAD1/5 and ID1/3 transcriptional regulators ((Gurrapu et al. 2018; Gurrapu et al. 2019).

Sema4D has been associated with the stimulation of oncogenic tyrosine kinases, such as Met, ErbB2, Src, and PYK2 and it can regulate both tumor angiogenesis and cancer cell invasiveness (Conrotto et al. 2005; Giordano et al. 2002; Worzfeld et al. 2012). Interestingly, a reduction in cytotoxic lymphocyte activity and an increase in tumor rejection occurred through the treatment with an antibody against Sema4D (Evans et al. 2015), consistent with the relevant role of this semaphorin in immunoregulation.

Sema5A expression drives cancer cell invasion and metastasis in gastric and pancreatic carcinomas, while the signaling cascade needs more investigation (Sadanandam et al. 2012; Pan et al. 2013; Saxena et al. 2018).

Among the members of class 6, Sema6A has been reported to act as a suppressor for lung cancer cell migration, its expression is reduced in lung cancer and inversely associated with tumor recurrence and  (Chen et al. 2019). Sema6A reverse signaling was implicated to initiate apoptosis in lung and glioblastoma cells (Shen et al. 2018; J. Zhao et al. 2015). In contrast, BRAF-mutated melanoma cells were shown to be dependent on Sema6A signaling, and its silencing resulted in cell death (Loria et al. 2015).

Sema7A controls the metastatic behavior of melanoma and breast cancer cells, presumably through factors released by macrophages that abundantly infiltrate the tumor microenvironment in response to this signal (B. Ma et al. 2015; Garcia-Areas et al. 2014; Allegra et al. 2012).

## Plexins

Plexins are the main receptor of semaphorins (Tamagnone et al. 1999). The plexin family is characterized by single-pass transmembrane proteins originally discovered for their role as axon guidance receptors in the developing nervous system. However, they are currently known for their broader role in cell-cell communication in diverse tissues, including the immune system, and in the cancer context (Jiang et al. 2021).

Vertebrate plexins include 9 members, which according to structural homology are sub-grouped into four subfamilies: Plexin-A1/4, Plexin-B1/3, Plexin-C1, and Plexin-D1 (Worzfeld and Offermanns 2014) (see Figure 1).

Analogous to their ligands, plexins comprise a sema domain in their ectodomains, plus two or three times repetition of PSI domains; moreover, three immunoglobulin-like folds are shared by plexins and transcription factor domains (Ig-like, plexins, transcription factors domains, IPT).

The intracellular domain of plexins, which is crucial for triggering intracellular signaling pathways, is outstandingly conserved among family members (57-97% similarity) and across evolution (>50% similarity between invertebrates and humans)(Tamagnone et al. 1999). The intracellular moiety includes C1 and C2 domains, which carry GTPase Activating Protein (GAP) activity on monomeric G-Proteins, such as R-Ras (Oinuma et al. 2004) and Rap-1(Ueda et al. 2016); moreover, it contains a “linker” domain which interacts with GTP-bound monomeric GTPases of the Rho family (Hota and Buck 2012). A C-terminal motif shared by plexins of the B-subfamily associates with PDZ domains of effector and adaptor proteins (Swiercz et al. 2002).

## Transmembrane semaphorins can trigger forward and reverse signaling

“Forward” signaling is the classical mode of functional activity of semaphorins through the binding to a receptor ectodomain. The signaling cascade is mostly triggered through the cytoplasmic GAP domain of plexins, and inhibits the activity of R-Ras, M-Ras, and Rap1, a

mechanism that can explain the observed negative regulation of integrin function. Furthermore, it has been demonstrated that some plexins can trans-activate other pathways upon semaphorin binding, such as plexin-associated receptor tyrosine kinases and RhoA-dependent kinases (Cagnoni and Tamagnone 2014; Gurrapu et al. 2018).

Other aspects of semaphorin function are mediated by "reverse" signaling. In fact, transmembrane semaphorins can act not only as ligands, but also as receptors themselves, eliciting a signaling cascade through their own cytoplasmic domains (Figure vi).

In the intracellular part of these semaphorins, are found domains mediating protein-protein interactions. For instance, class-4 semaphorins contain potential PDZ-domain interaction motifs (Burkhardt et al. 2005); or class-6 semas contain large proline-rich motifs interacting with Src homology 3 (SH3) domains. In particular, it has been demonstrated that Sema6B associates with Src kinase (Eckhardt et al. 1997), and Sema6D interacts with c-Abl (Eckhardt et al. 1997).

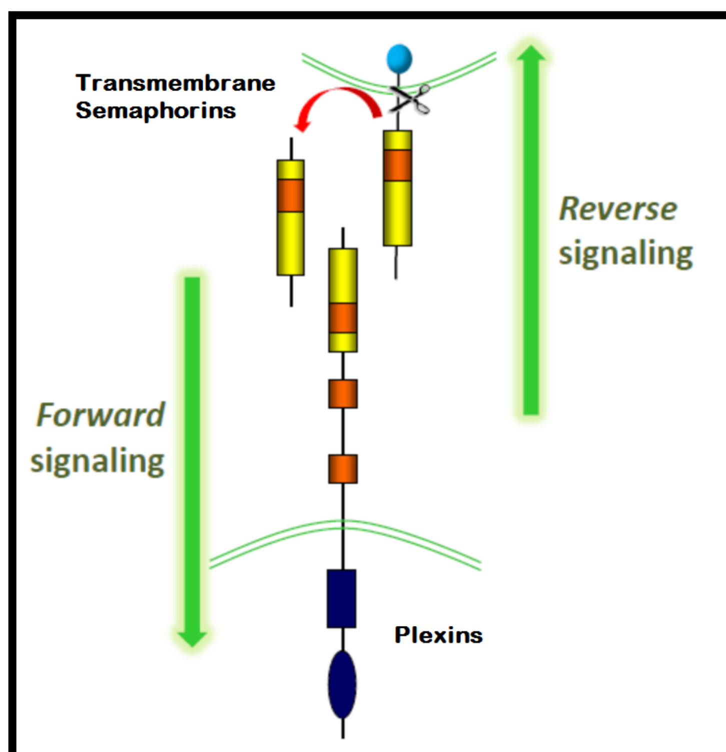



**Figure vi.** Semaphorin forward and reverse signaling.

## Neuropilins

Class-3 semaphorins, apart from Sema3E which binds to Plexin-D1, are unable to interact with plexins directly; thus, co-receptor molecules are required to form a ligand-receptor complex, called Neuropilins (Nrp) (see Figure 1). In humans, there are two types of neuropilin (neuropilin-1 and neuropilin-2), with approximately 44% structural homology at the amino acid level (Takagi et al. 1991; Neufeld and Kessler 2008). The extracellular domain of neuropilins contains two complement-like (CUB) domains (called a1/a2) that are responsible for binding the Sema domain of class 3 semaphorins, two FV/FVIII coagulation factor-like domains (b1/b2), and a meprin-like MAM domain (c domain). The intracellular C-tail of neuropilins is too short to support independent signal transduction, but it comprises a PDZ binding motif implicated in receptor localization at the cell membrane (Cai and Reed 1999; Gao et al. 2000).

## Sema6C: a poorly studied family member

Semaphorin 6C (Sema6C) is a poorly studied family member, initially known as semaphorin Y (Kikuchi et al. 1999). It is a transmembrane molecule, considered a distal member in class-6, as it has a longer and partly divergent cytoplasmic domain, and its sema domain is phylogenetically distant from other paralogues. Sema6C C-terminal sequence is highly hydrophobic, but lacks the typical C-terminal residues (A, V, L, I) representing the consensus motif for binding PDZ-containing proteins (Sheng and Sala 2001). One study has indicated Plexin-A1 as bona fide Sema6C receptor (Yoshida et al. 2006); however, the signaling cascades elicited by this semaphorin, in either forward or reverse mode, are almost totally unknown and await elucidation.

Similar to other semaphorins, Sema6C was found to act as axon repelling and growth cone collapsing factor for DRG neurons. Intriguingly, Sema6C not only is expressed in neurons, but

also in the embryonic targets: the myotome and dermatome; thus, it has been posited to regulate the navigation of extending axons, their halting in target areas, and synapse formation during development (Kikuchi et al. 1999). Interestingly, Sema6C expression in adult tissues is mostly localized to the brain and skeletal muscle (Qu et al. 2002). Although it is reduced after muscle denervation, Sema6C expression stay high at the neuromuscular junction, suggesting that it plays a role in synapse stability and axon sprouting inhibition in skeletal muscles (Svensson, Libelius, and Tågerud 2008). Moreover, a splice variant of Sema6C (Sema6C1), extensively expressed in the mouse brain, was shown to elicit GSK-3 activity, in analogy to Sema3A and Sema3F, triggering growth cone collapse of developing entorhinal and hippocampal axons (Eickholt, Walsh, and Doherty 2002; Burgaya et al. 2006).

Other studies linked to Sema6C signaling with the regulation of early primordial follicle dormancy/activation. In particular, between day 5 and day 7 after birth, when primordial follicle activation starts, the expression level of Sema6C protein in the ovaries drops dramatically. Based on this study, Sema6C may control the maintenance of primordial follicle quiescence, and its downregulation may hasten the start of their differentiation (Zhou et al. 2018).

# AIM OF THE WORK

Pancreatic cancer is a highly aggressive disease with a fatality rate that is nearly equal to its incidence. Moreover, approximately 80% of pancreatic cancers are detected at an advanced stage, when the efficacy of current therapeutic approaches is extremely low.

The identification of new molecular targets and functional liabilities in pancreatic cancer cells is thus particularly relevant, in perspective to develop novel therapeutic approaches.

Semaphorins are extracellular signals, initially discovered as guidance cues in embryonic development, which recently emerged as significant regulators of tumor growth and metastasis, and potential cancer biomarkers. Moreover, preclinical research has revealed some semaphorin family members as promising molecular targets for cancer therapy.

In this thesis work, I investigated Semaphorin 6C, a poorly studied transmembrane semaphorin, expressed in human tumors and statistically correlated with patient prognosis.

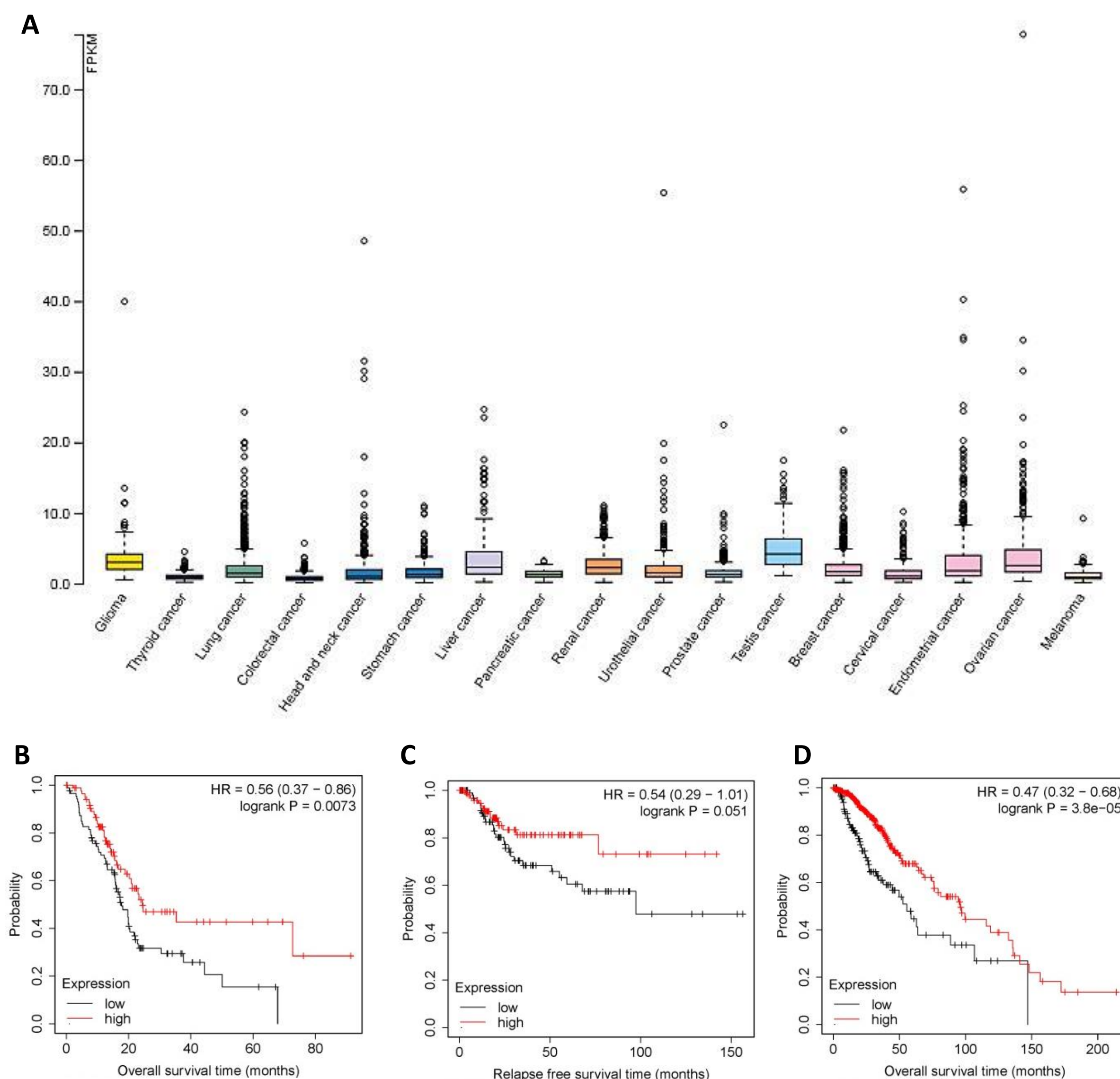
Very little is known in literature about this molecule, and I especially aimed at understanding its function and signaling mechanisms in cancer cells with a special focus on pancreatic cancer.

To this end, I planned to generate gene knock-down and overexpression models to investigate the functionale role of Sema6C in cancer cells. Moreover, I took advantage of Sema6C full length and truncated mutant to investigate the presently unknown signaling pathways mediated by this transmembrane semaphorin in cancer cells.

# RESULTS

## Sema6C knockdown in cancer cells hampers proliferation and elicits cellular senescence

TCGA dataset analysis revealed that Sema6C is widely represented in human tumors, at low levels on average, but with a significant subset of high expressors (**Fig. 1A**). In consideration of a potential role in cancer progression, we initially surveyed Sema6C distribution in diverse tumor types and its association with patient survival. Kaplan-Meier analysis of TCGA data indicated that high Sema6C levels were associated with better prognosis in pancreatic adenocarcinomas, lung squamous (but not adeno) carcinomas, and low grade gliomas (but not glioblastomas); while they coupled with poor patient survival in several tumor types, including colon, stomach, and prostatic adenocarcinomas, ovarian and endometrial carcinomas, and sarcomas (**Fig. 1B**). These findings could indicate a differential relevance of Sema6C expression in diverse cancer cells, or reflect variable microenvironmental parameters of undetermined functional impact on tumor progression.



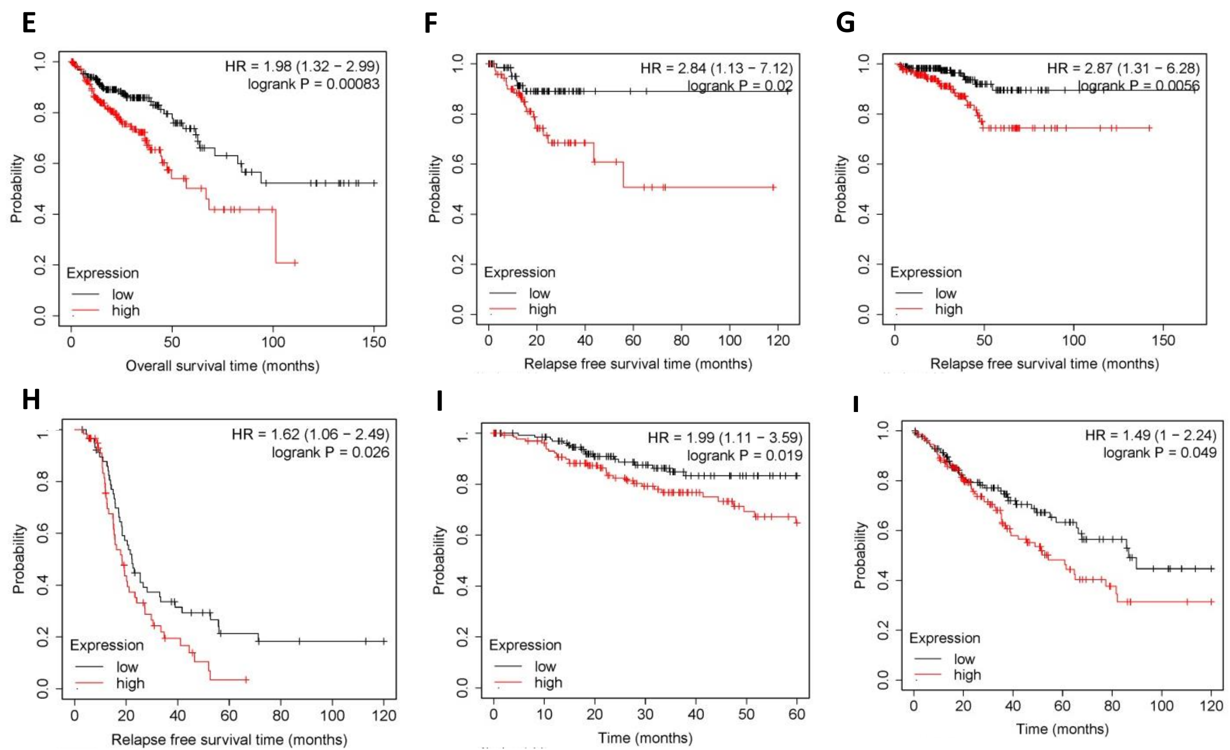



**Figure 1. A)** Sema6C expression in human tumors (TCGA data; https://www.proteinatlas.org/). **B-L)** Kaplan-Meier plots (TCGA data; http://kmplot.com/private/) revealing the correlation between Sema6C expression and patient survival in: pancreatic adenocarcinomas (B), lung squamous carcinomas(C), low-grade gliomas (D), colon adenocarcinomas (E), gastric cancers (F), prostatic adenocarcinomas (G), ovarian cancers (H), endometrial carcinomas (I), and sarcomas (L).

In order to address these questions, and investigate the functional role and molecular signaling mechanism of Sema6C in cancer cells, we assayed the impact of gene knock-down in different tumor cell types. Sema6C knock-down was achieved by the lentiviral-mediated transfer of cDNA constructs driving the transcription of short hairpin RNA (shRNA) sequences targeting *SEMA6C* mRNA. Gene silencing efficiency was validated by quantitative reverse transcriptase-PCR (qRT-PCR) analysis, revealing a 90% reduction of mRNA levels in presence of Sema6C-targeted shRNAs (**Fig. 2**).

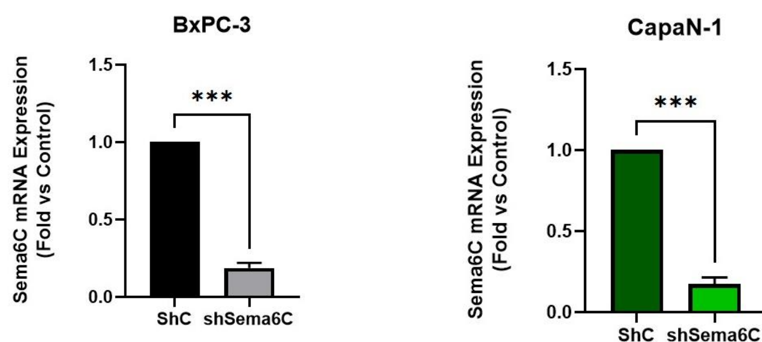
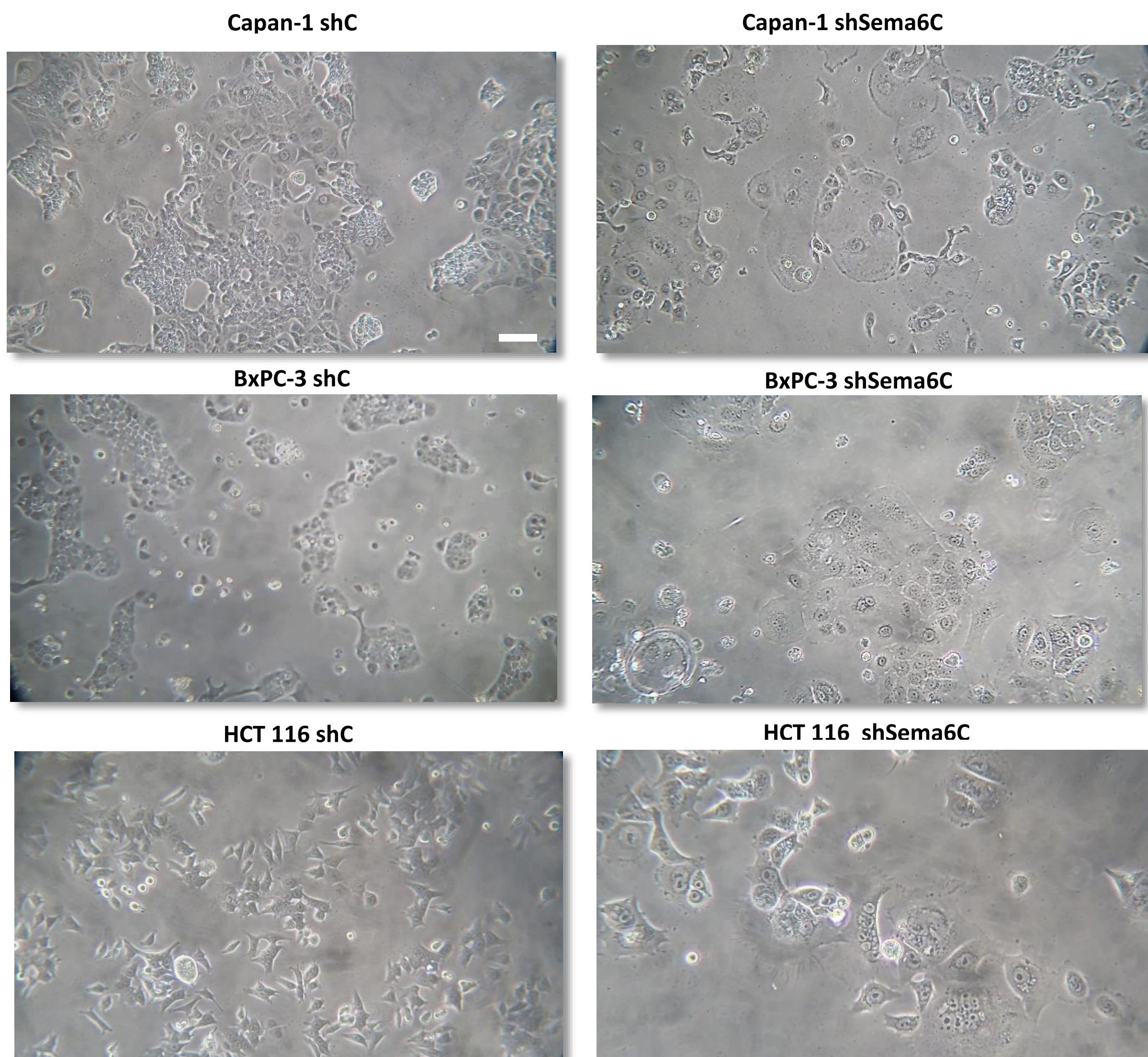



**Figure 2.** qPCR assessment of Sema6C silencing in BxPC-3 and Capan-1 cells

Interestingly, a few days after transferring shRNAs, we observed dramatic changes in the phenotype of Sema6C-depleted cells, compared to control cells (transduced with an empty vector). In particular, in pancreatic cancer cells (such as BxPC-3, Capan-1), in colon cancer cells (HCT116), in ovarian cancer cells (HEY), and in breast carcinoma cells (MCF7), we observed the onset of a similar phenotype, characterized by growth inhibition and flattened morphology (**Fig. 3**). These results suggested that Sema6C function is required for cell viability, and this feature is conserved among different cancer cell types. Actually, most cancer cells shortly died after the achievement of Sema6C silencing, and it was not possible to establish stable cell lines; however, some Sema6C-depleted PDAC cells could be maintained in culture for a few weeks, and their phenotype was further analyzed.



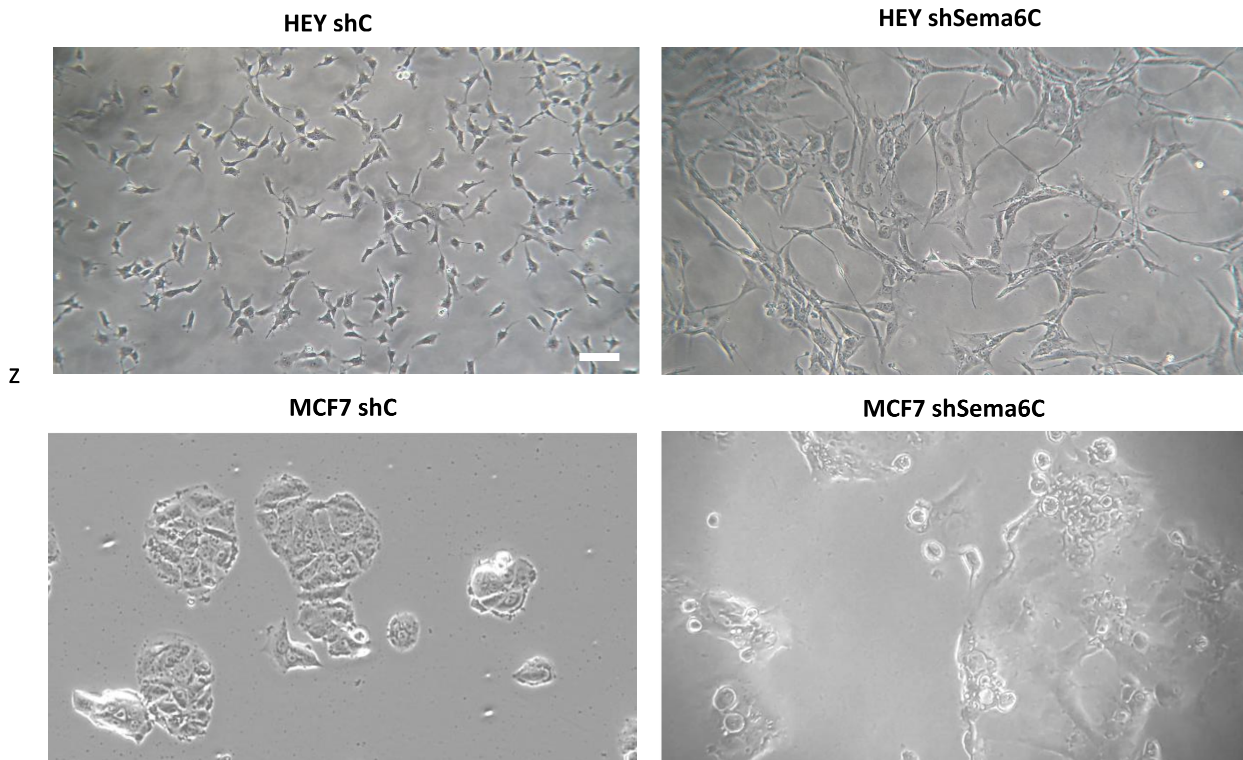



**Figure 3.** Pancreatic cancer cells (BxPC-3, Capan-1), colon cancer cells (HCT116), ovarian cancer cells (HEY), and breast carcinoma cells (MCF7) were transduced with lentiviral particles carrying shRNAs against Sema6C; this resulted in overt phenotypic changes of Sema6C silenced cells, compared to controls. Scale bars=50 µm.

Growth curve analysis demonstrated that in fact Sema6C-silenced cells slowed down or even stopped proliferation (**Fig. 4A**). We also serendipitously observed the recurring presence of multi-nucleated cells upon Sema6C silencing, a finding potentially consistent with cytokinesis blockade, further indicative of cell cycle progression inhibition (**Fig. 4B**). Moreover, not only they appeared overtly larger in size and flattened on the plastic dish, but cytofluorimetric analysis demonstrated that the actual volume of Sema6C-depleted cells was also significantly increased (manyfold larger than controls), possibly consistent with a metabolic change (**Fig. 4B-C**). Notably, cell shape on plastic was more circular, compared to controls, and lacked polarized protruding processes (**Fig. 4D**).

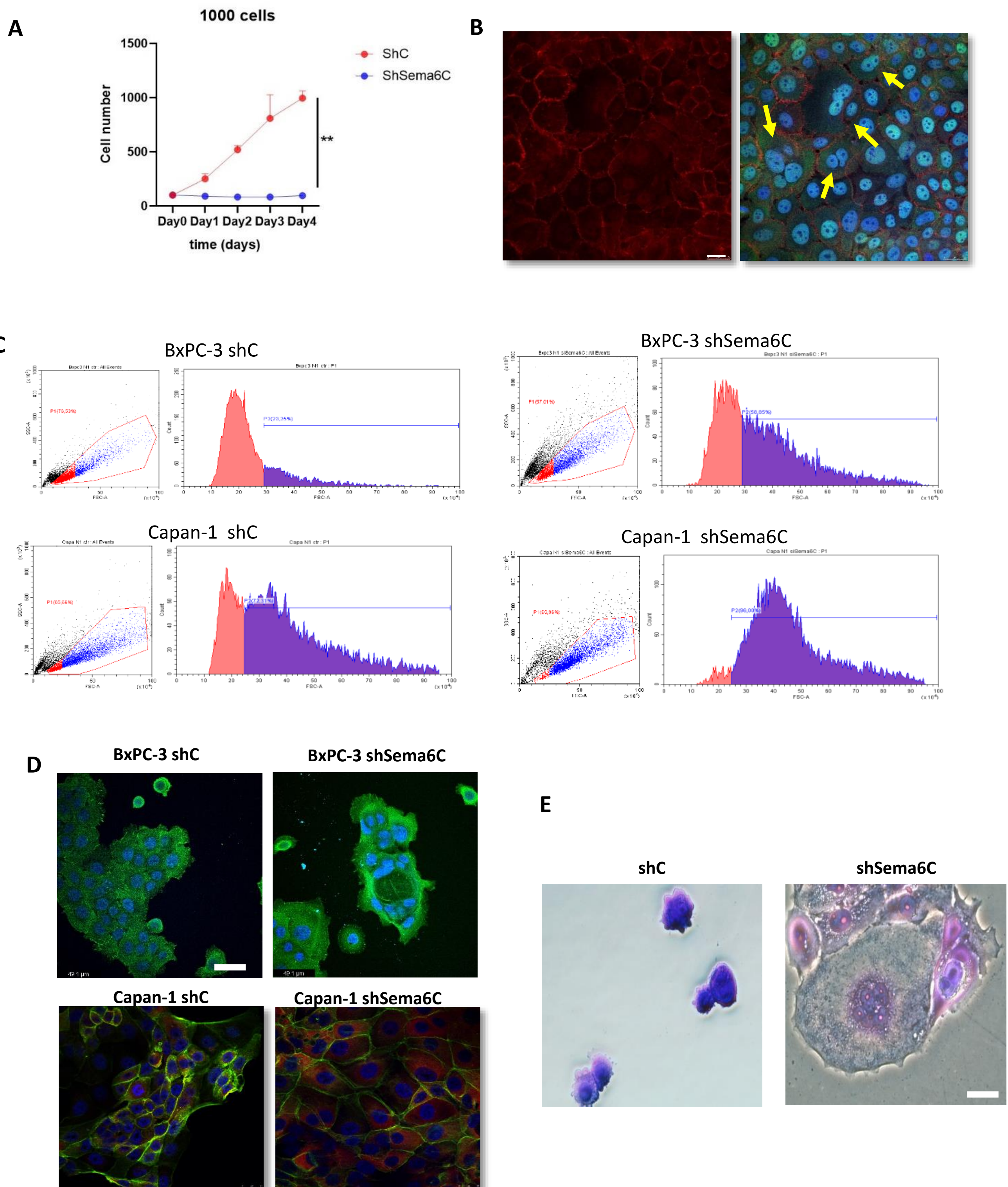



**Figure 4. A)** BxPC-3 cells, either control or Sema6C-silenced, were seeded in equal number in multiple wells, and their number was counted in 5 consecutive days. **B)** Sema6C-depleted BxPC-3 cells were immunostained for E-Cadherin (red) to reveal cell margins, and with DAPI (blue) to show the nuclei. Scale bar= 40 µm. **C)** BxPC-3 and Capan-1 cells, either control or Sema6C-silenced, were analyzed by flow cytometry to assess cell size (based on light scattering). **D)** The same BxPC-3 cells as above were stained with fluorescent Vinculin (in green) and DAPI (blue) and CapaN-1 cells stained with phalloidin (F-actin) (in green) and DAPI (blue). Scale bar= 30 µm **E)** Crystal violet staining of BxPC-3 control and Sema6C- silenced cells, Scale bar= 20 µm.

The specificity of gene silencing effects was confirmed by assaying another independent shRNA sequence targeting Sema6C, which resulted in the same cell phenotype (**Fig. 5A-B**). Moreover, we performed functional rescue experiments by re-expressing Sema6C in cancer cells, few days after depletion of the endogenous protein, at a stage in which cell proliferation is clearly inhibited but the cells have not yet become senescent. This was sufficient to restore cancer cell growth, as well as the parental phenotype (**Fig. 5C**).

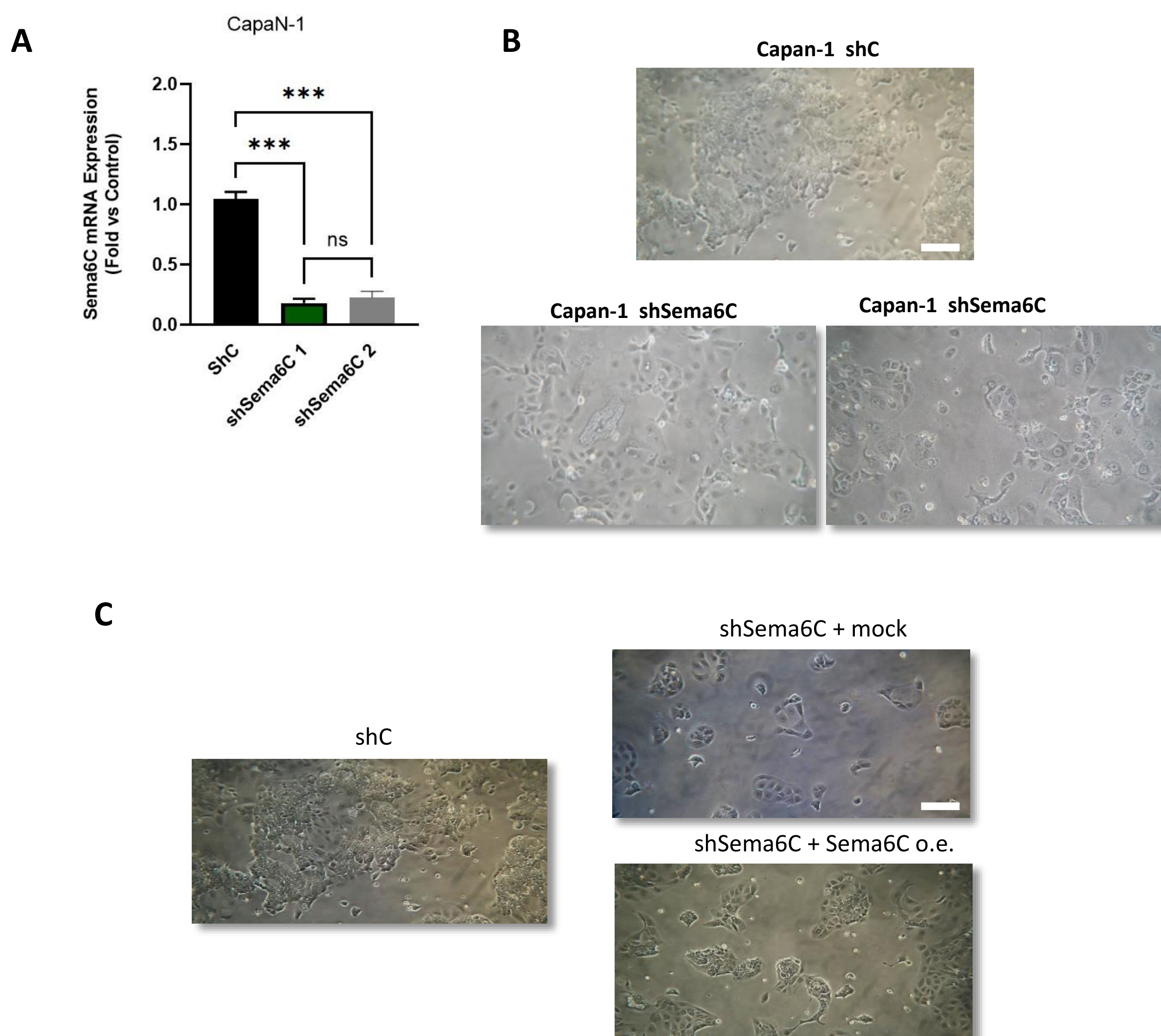



**Figure 5. A)** qPCR assessment of Sema6C silencing in Capan-1 cells with two different shRNA constructs (shSema6C-1 and shSema6C-2). **B)** Inverted phase contrast micrographs of Capan-1 cells, either control or subjected to Sema6C silencing with the two different shRNA constructs reported in the previous panel. Scale bar=100 µm. **C)** One week after transduction, Sema6C-silenced cells were further transduced with a Sema6C-overexpressing construct in order to restore its levels in cancer cells, which led to reversion of the parental phenotype after 48 hours. Scale bar=100 µm.

De novo production of cyclin D and its association with existing CDKs (cyclin-dependent kinases) is required for cells entering the proliferation cycle. However, the upregulation of cyclin D1 levels is insufficient to form an active cyclinD–CDK4/6 complex and initiate the cell cycle, since the suppression of CDK inhibitors (CDKIs) is also required for G1/S phase transition. Major CDKI molecules include p21 and p27, and the effector protein p53.

Thus, cell cycle progression across the G1/S phase transition is precisely controlled in normal cells; in contrast, defective regulatory machinery is observed in tumor cells, and the activity of CDKIs is usually suppressed, in order to promote uncontrolled proliferation. We therefore checked the CDKIs and cell cycle arrest-associated markers p21 and p27 in our models, and interestingly found a significant increase in the expression of these two eminent regulators in Sema6C-silenced cells compared to control cells (**Fig. 6**). TP53 cell cycle suppressor protein was also induced, further indicating growth inhibition and cell cycle arrest.

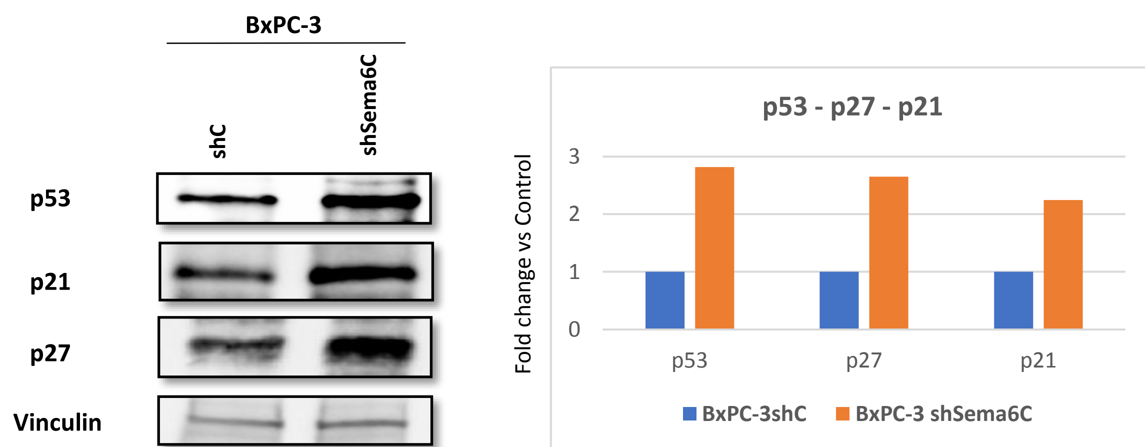


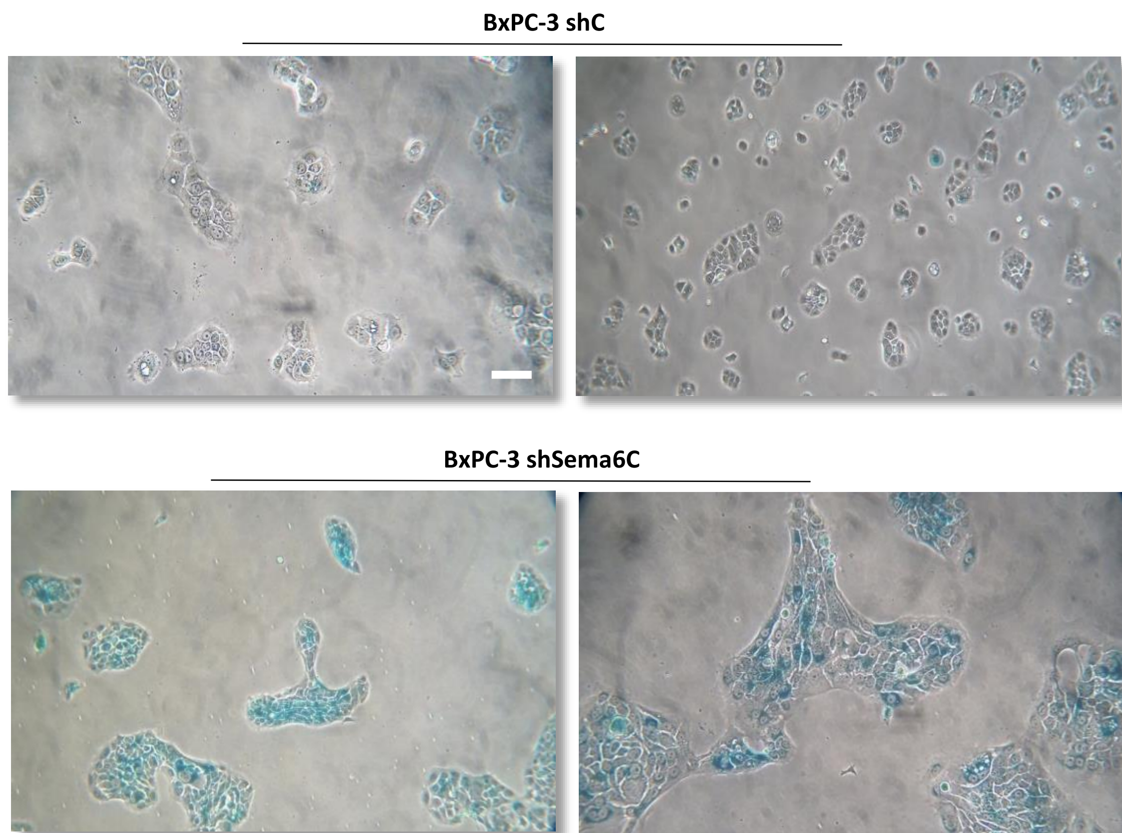

**Figure 6.** Western blotting to analyse p27, p21 and p53 expression level in BxpC-3 control and Sema6C-silenced cells.

The phenotype consistently observed upon Sema6C knock-down in diverse cancer cells was suggestive of the progressive establishment of cellular senescence. In fact, senescence is caused by persistent cell cycle inhibition induced by various stressors, and it is believed to prevent old or damaged cells from replicating, without directly leading to apoptosis (Herranz and Gil 2018; Fridlyanskaya, Alekseenko, and Nikolsky 2015; Dimri et al. 1995; Vogelstein,

Lane, and Levine 2000). Notably, the transcriptional and metabolic profile of senescent cells is substantially altered. Indeed, we investigated the putative senescent phenotype of Sema6C-depleted cancer cells by analyzing some of its typical hallmarks, such as increased beta-galactosidase activity and induction of cell cycle inhibitor genes.

We stained the cells to reveal the senescence-associated marker β-galactosidase, which is linked to the enhanced lysosomal number and activity, typical of senescent cells. A significant increase in β-galactosidase activity was observed in Sema6C knock-down cells compared to control cells, as revealed by the chromogenic conversion of X-gal substrate (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in the flattened cells that failed to complete cell division (**Fig. 7**).

**A**



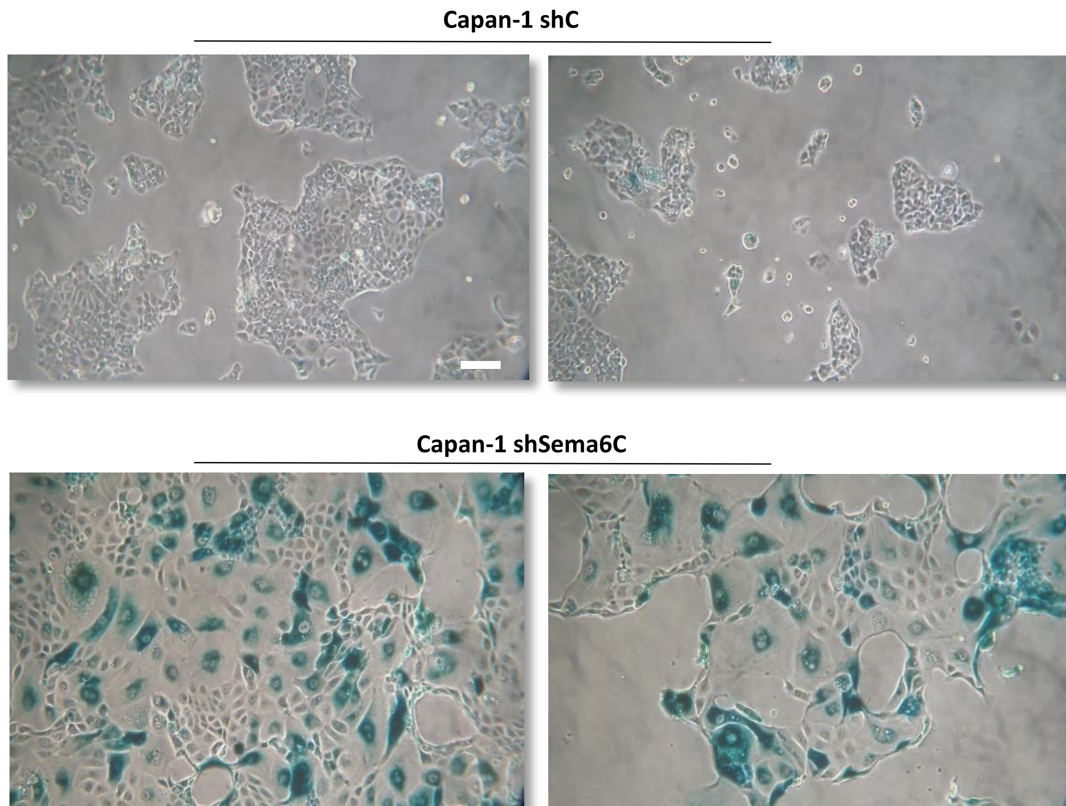
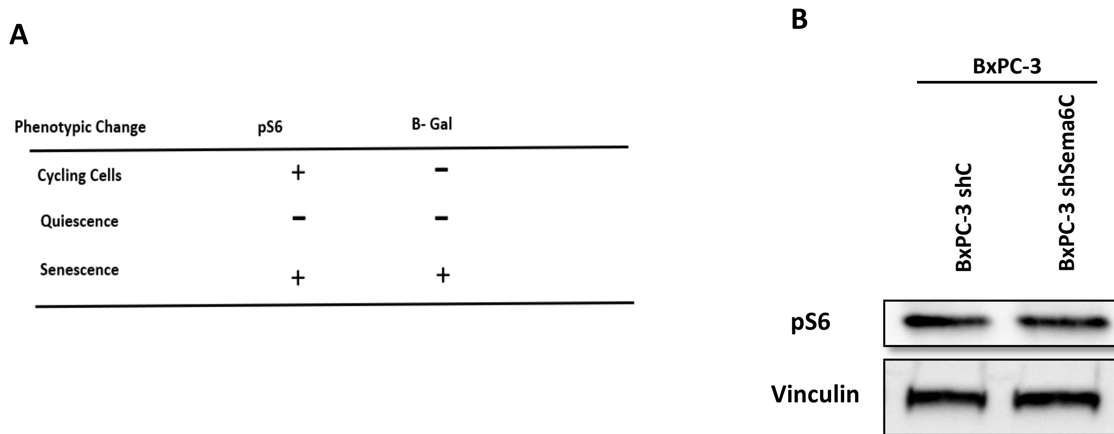



**Figure 7.** Beta galactosidase chromogenic assay in BxPC-3 (A) or Capan-1 (B) either control of Sema6C-silenced. Two representative images are shown for each condition. Scale bars=50 µm.

The inhibition of G1/S phase transition is obviously a shared feature of senescent and quiescent cells. However, the latter (considered in a so-called G0 phase) is commonly characterized by a slow metabolism, and does not grow in size during interphase; in contrast, senescent cells are typically associated with an enlarged phenotype. This observation is strikingly reflected by the activation state of the major metabolic regulator S6, a ribosomal protein phosphorylated in response to mTOR activation, and found to discriminate senescent from quiescent cells. Not surprisingly, in senescent cells, rpS6 phosphorylation is similar to that of cycling cells, while in quiescent cells it is very low (Alessio et al. 2021; Blagosklonny 2012).

Thus, the combined assessment of CDKI levels, S6 phosphorylation, and beta-galactosidase activity can provide an informative matrix to discriminate cycling, quiescent and senescent

cells. Remarkably, we found that in Sema6C-depleted senescent cells, characterized by p21/p27 and beta-galactosidase induction, phosphoS6 levels were comparable with control cells, further confirming their peculiar metabolic state (summarized in Table 1).




| Phenotypic Change | pS6 | B- Gal |
|---|---|---|
| Cycling Cells | + | - |
| Quiescence | - | - |
| Senescence | + | + |

**Table 1. A**) Molecular algorithm, based on pS6 and Beta galactosidase levels, to identify cell condition and functional phenotype. **B**) Western blotting for phosphoS6 level in BxPC-3 control and silenced cells

We then asked about the upstream mechanism responsible for cell cycle inhibition in Sema6C-depleted cells. One of the major players in late G1, driving cell cycle progression, is active (phosphorylated) ERK kinase, which enhances CDKs activity and inhibits CDKIs. (Chambard et al. 2007; Kerkhoff and Rapp 1997; Rivard et al. 1999; Delmas et al. 2001; Kortylewski et al. 2001; Foster et al. 2003; Bhatt et al. 2005).

The MEK/ERK pathway is actually implicated in both G1/S and G2/M transitions and its inactivation can cause G1 and G2 cell cycle arrest and senescence by inducing p21 and p27 CDKIs (Villanueva et al. 2007). Thus, we investigated whether depletion of Sema6C could affect phospho-ERK levels, in light of the potential relevance of this kinase in controlling cell cycle progression and cell senescence in our cancer cell models. Indeed, we observed a significant decrease in phospho-ERK upon Sema6C knock-down in BxPC3 (**Fig. 9A**). Similar results were obtained in colon cancer cells HCT116 subjected to Sema6C silencing (**Fig. 9B**), confirming Sema6C-dependent regulation of ERK activity in diverse cancer cells.

In sum, our data suggest that Sema6C loss is leading to persistent growth inhibition, indicated by increased p53, p27 and p21 cell cycle inhibitors, and by the decrease in pERK levels; this is eventually leading to cell senescence, revealed by accumulation of beta-galactosidase.

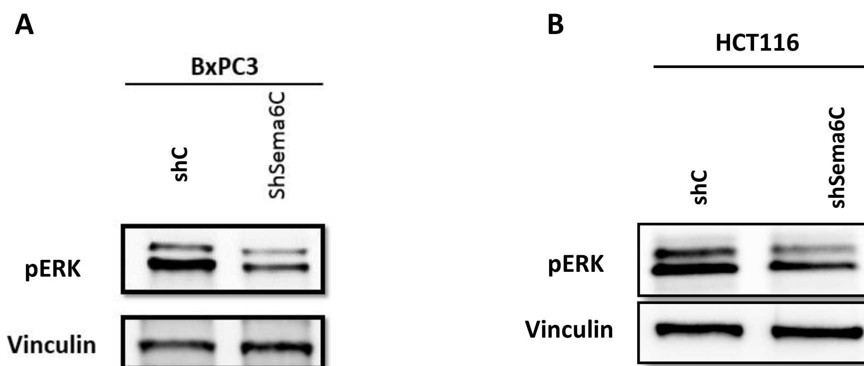



**Figure 9.** Western Blotting for pERK in BxPC-3 (A) and HCT116 cells (B), either control (shC) or Sema6C-silenced (shSema6C).

## Sema6C-depleted cells increase the autophagic flux

It was shown that persistent quiescence may lead to cell senescence (Fujimaki et al. 2019). Notably, as cells get deeper in quiescence and senescence, lysosomal gene expression and mass rise, as lysosomal function and lysosome destruction fall (Fujimaki et al. 2019).

We therefore wished to assess whether autophagy was triggered in Sema6C-silenced cells, in association with growth inhibition and senescent phenotype. The induction of autophagy was monitored using various approaches according to "Guidelines for detecting and monitoring autophagy" (Klionsky et al. 2021).

First of all, autophagosome (APs) formation was tracked by LC3 immunostaining. As shown in **(Fig. 10),** in Sema6C-depleted cells the LC3 immunofluorescent signal was higher than in CTRL cells. The quantitative analysis of densitometric values of LC3 immunostaining in the different conditions showed an increase of LC3 signal in Sema6C-depleted cells compared to ctrl cells (CTRL=1 vs shSema6C=3.25; $p<0.001$ **), suggestive of an increase in the AP formation in Sema6C-depleted cells.

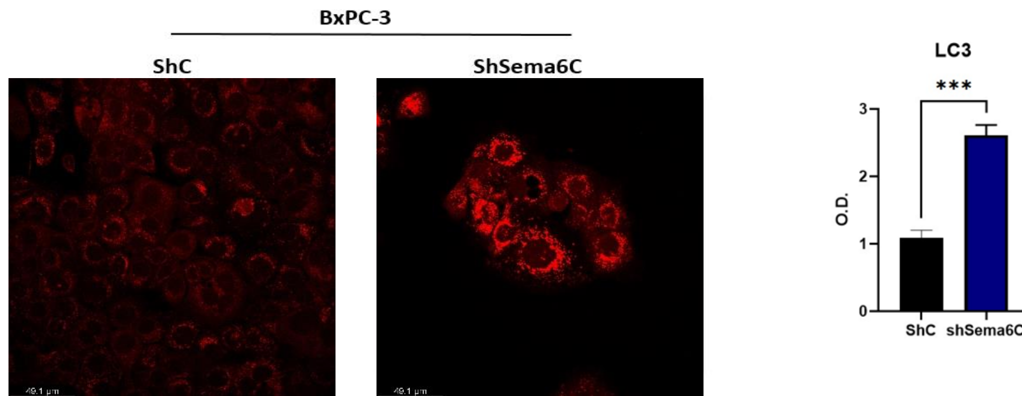



**Figure 10.** Immunofluorescence staining for LC3 (in red) in control and Sema6C-silenced BxPC-3 cells, Scale bars=50 µm.

Further, autophagic activity in Sema6C-depleted cells was monitored by Western blotting, assessing changes in the level of the autophagosomal protein LC3-II and the autophagy substrate p62/SQSTM1. The abundance of these two proteins is a clear index of the autophagy flux, as its course is indicated by degradation of p62, an ubiquitin cargo binding protein that brings different substrates to the phagosomes; and by the conversion of cytosolic LC3-I into the LC3-II lighter isoform. Intriguingly, in Sema6C-depleted cells, the relative levels of LC3-II were higher than in ctrl cells, while p62 was strikingly reduced (**Fig. 11**), indicating that Sema6C suppression considerably enhanced autophagosome formation.

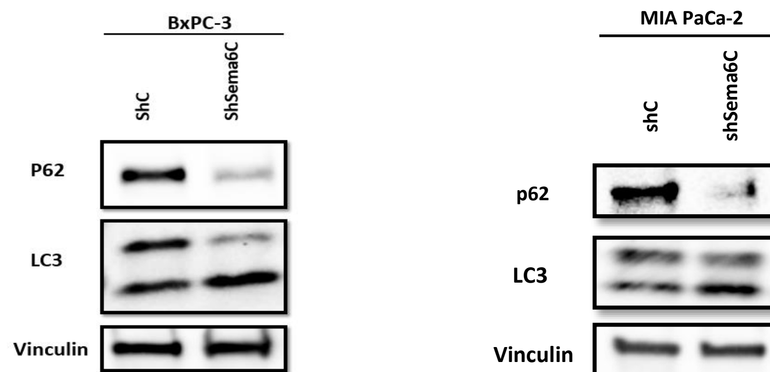
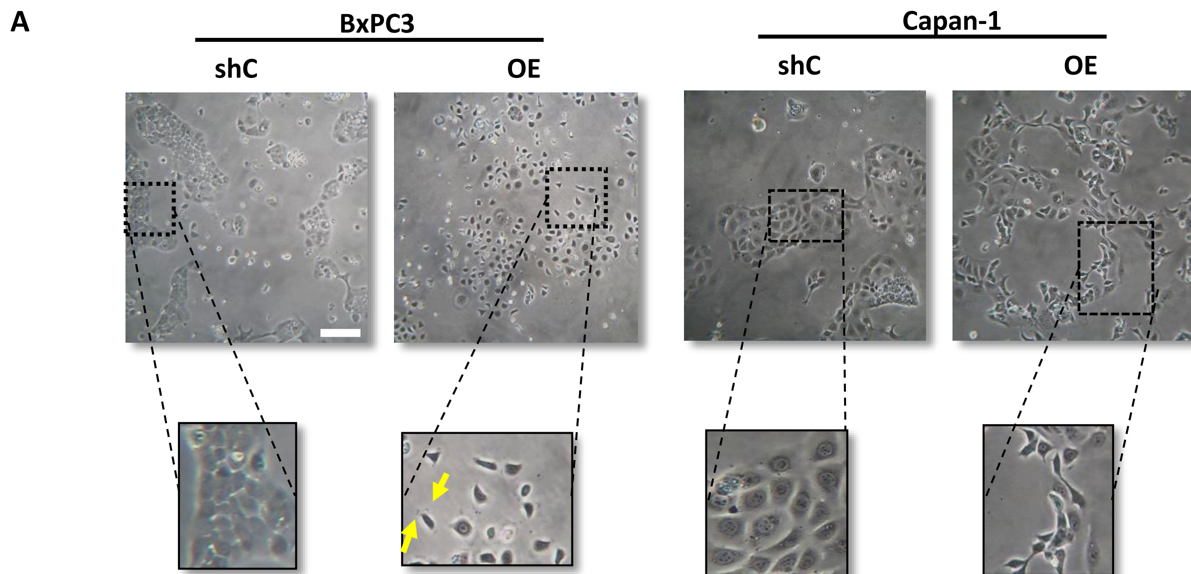


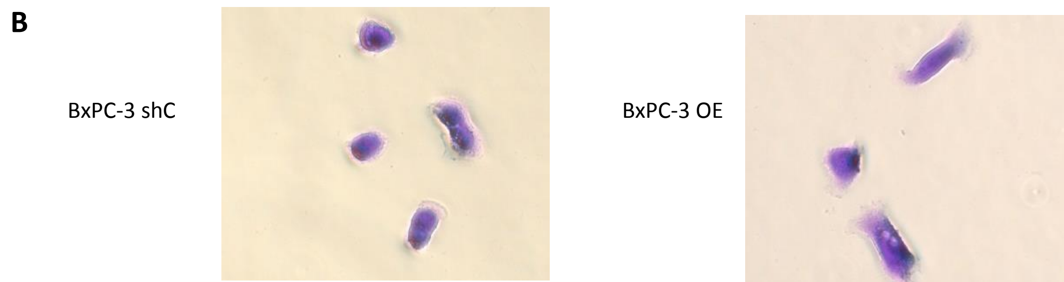

**Figure 11.** Western blotting for p62 and LC3-II expression level, in BxPC-3 and MIA Paca-2 cells, either control or Sema6C-silenced.

## Sema6C-induced phenotypic changes and cancer cell motility

The knock-down of endogenous Sema6C expression revealed its basal function in support of cancer cell viability and growth. In order to elucidate the molecular mechanisms elicited downstream to Sema6C, we enhanced its expression in two different PDAC cancer cells, by

transduction of a cDNA construct. At first, it was evident that cells acquired a complementary phenotype to that observed upon gene silencing: as shown in **Figure 12**, they appeared smaller in size, less connected to each other, and characterized by loss of circularity and by the presence of polarized protruding processes.



**D**

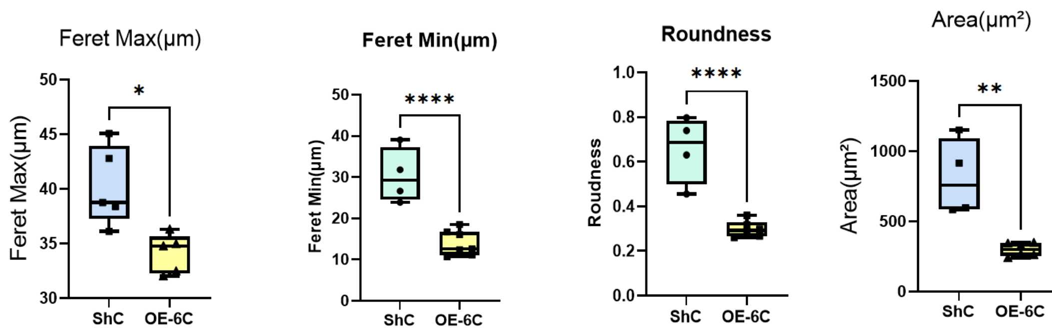



**Figure 12. A)** Inverted phase contrast micrographs of BxPC-3 and Capan-1 control (shC) or Sema6C overexpressing (Sema6C-OE) cells, Scale bar=100 µm. **B)** Control and Sema6C overexpressing cells stained with crystal violet **C)** The three-dimensional profile of BxPC-3 control and Sema6C-OE cells, calculated by Neurolucida, on the base of crystal violate staining. **D)** The graphs show cellular parameters calculated by Neurolucida in the cells above.

Suspecting that this phenotype could reflect an epithelial-mesenchymal transition (EMT), we initially checked EMT markers and transcriptional regulators, but we didn't observe any significant changes (**Fig. 13**). We also questioned if Sema6C, likewise another semaphorin, Sema4C, might activate the TGFβ/BMP signaling pathway, but we found no remarkable differences in SMADs phosphorylation (not shown).

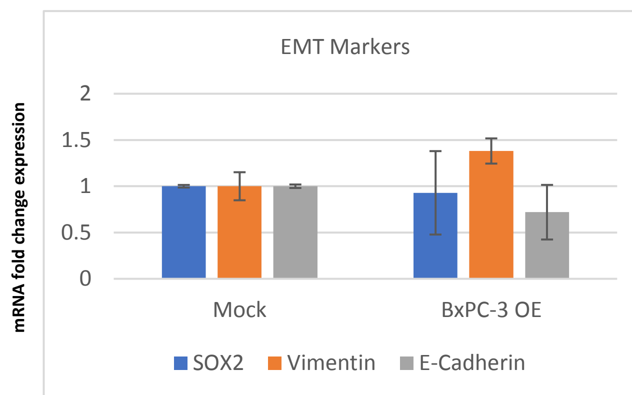



**Figure 13.** qPCR assessment of EMT markers expression, in control and Sema6C-OE BxPC-3 cells.

When analyzing the proliferation rate of Sema6C-overexpressing cells, we found a modest increase (**Fig. 14A**). Consistently, we found that the expression of the cell cycle inhibitors p21 and p27 was lower in Sema6C overexpressing cells, complementary to what was observed upon gene silencing (**Fig. 14B**). In contrast, pERK levels were markedly increased by Sema6C in cancer cells (**Fig. 14C**). Notably, it has been reported that elevated ERK activity can lead to activation of p90-ribosomal S6 kinase, resulting in hyper phosphorylation of ribosomal

protein S6 (rpS6)(Sawicka et al. 2016). In keeping with that, Sema6C overexpression in BxPC-3 cells was actually associated with remarkably increased phosphorylation of rpS6 (**Fig. 14D**).

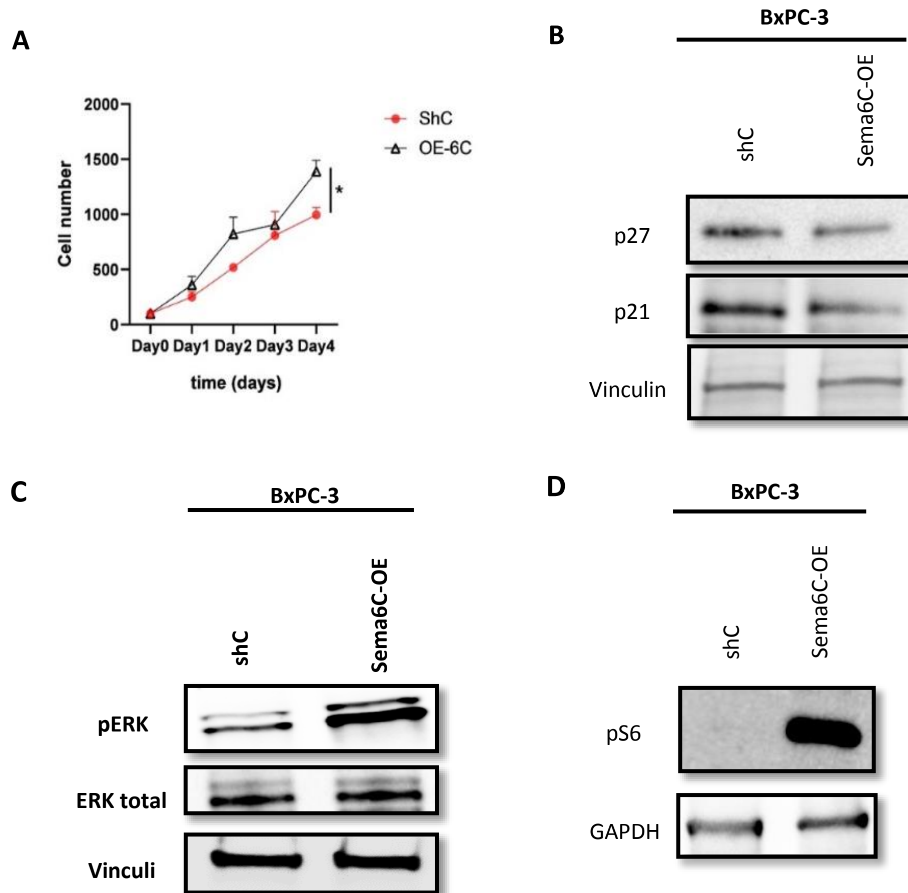



Figure 14. A) Cell counting assay was performed and Cell nuclei were counted in 5 consecutive days B) Western blotting for the cell cycle inhibitors proteins p21 and p27, upon Sema6C overexpressing in BxPC-3 cells. C) Protein immunoblotting revealed pERK level increased upon Sema6C overexpression. D) Hyper phosphorylation of ribosomal protein S6 verified by western blotting of Sema6C overexpressing cells.

These data suggested that Sema6C elicited an EMT-independent phenotypic change and a signaling cascade supporting cell proliferation, associated with increased ERK and pS6 activity. However, since the spindle-shaped phenotype induced by Sema6C-overexpression, including the presence of polarized pseudopodia, was reminiscent of highly motile cells, we decided to investigate potential changes in the migratory behavior elicited by Sema6C. Actually, by wound-healing assays, we discovered that BxPC3 cells overexpressing Sema6C had a greater capacity to move into the wound compared to control cells **(Fig. 15A)**, Consistently, Transwell assays, analyzing individual cell migration, confirmed a higher migratory attitude of Sema6C-overexpressing cells compared to controls. **(Fig. 15B)**

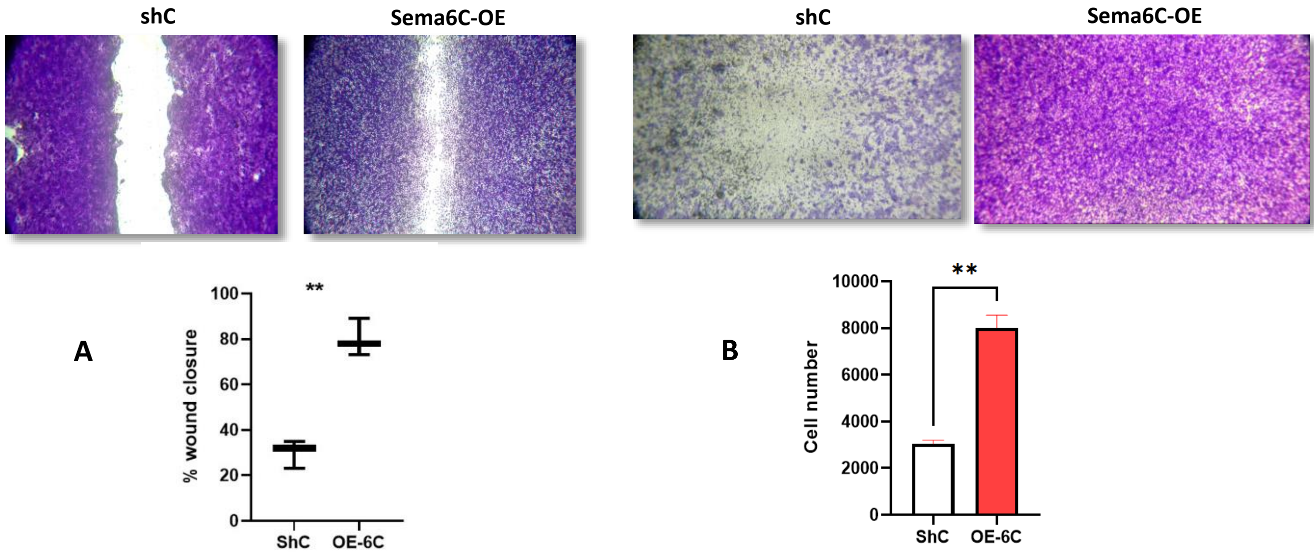



**Figure 15.** A) Wound healing assay with BxPC3 cells, either control or Sema6C overexpressing; wound closure was analyzed after 24 hours, comparing to $T_0$. B) Transwell Boyden chamber assays with control and Sema6C overexpressing cells; cell numbers were counted by ImageJ.

We than decided to monitor Sema6C-induced changes of cell motility in real time. Cell movement parameters of a series of individual cells were measured by time-lapse video microscopy in terms of covered distance and speed. Interestingly, the results were in alignment with the findings shown above, revealing that a longer distance was traversed on average by Sema6C-overexpressing cells, which were also moving considerably faster than control cells (**Fig. 16**).

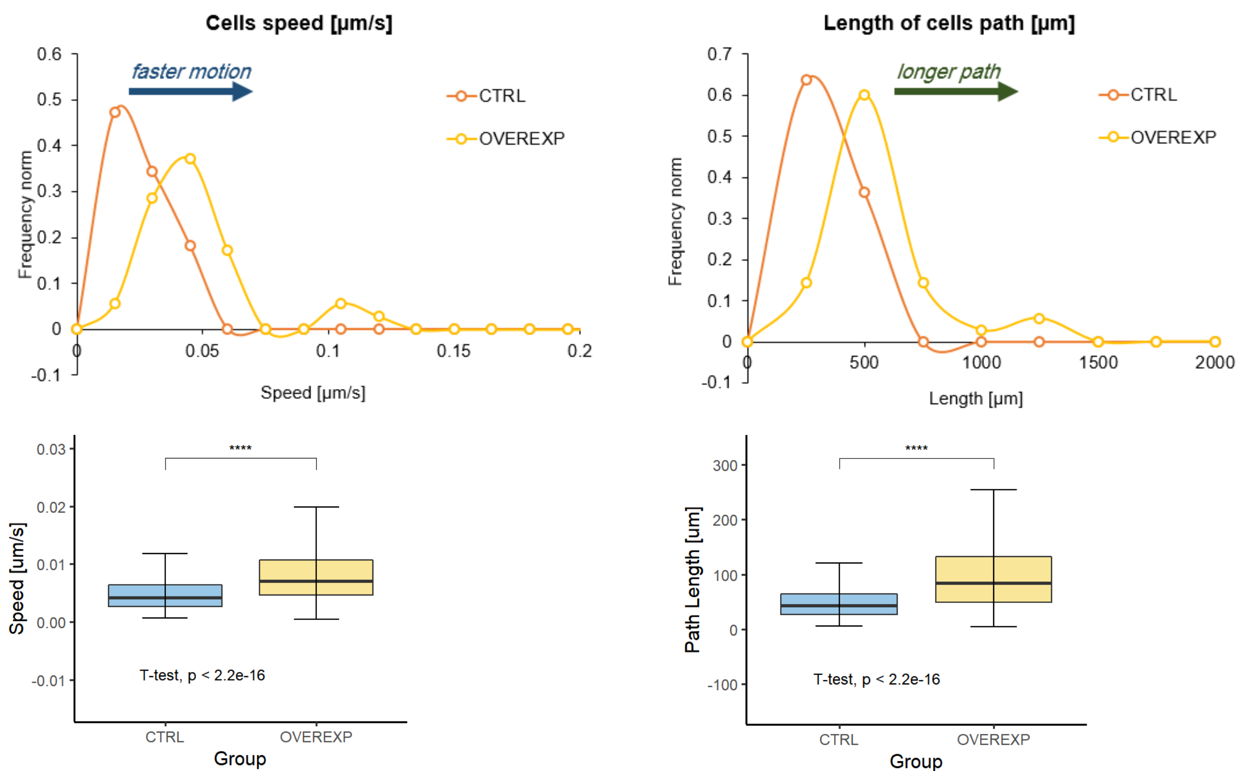
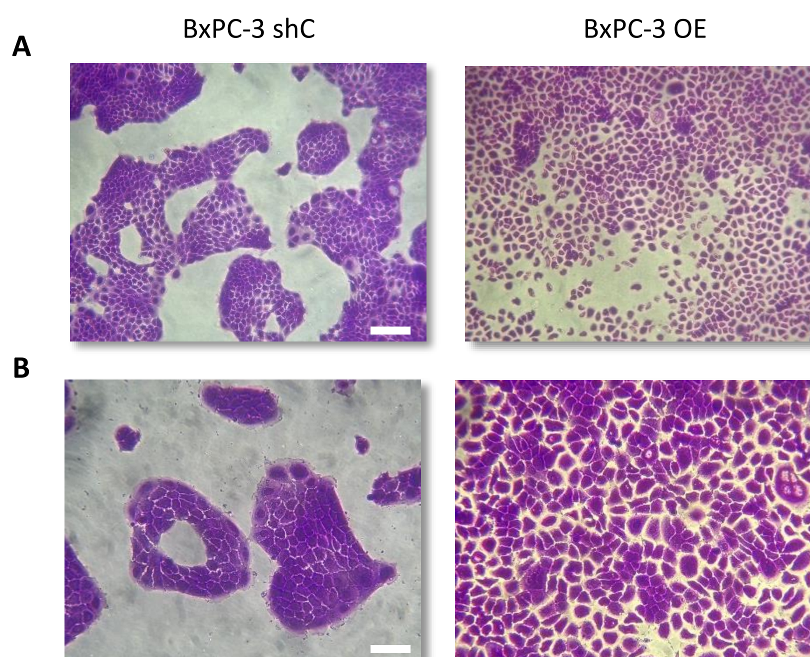


**Figure 16.** Time-lapse video microscopy, assessing the spontaneous motility of BxPC3 cells, either control or Sema6C-overexpressing.

## Sema6C sustains cancer cell viability under nutrient deprivation and metabolic stress

As mentioned above, Sema6C-overexpressing cells showed barely significant increased proliferation rate. However, likely due to their peculiar phenotype, these cells did not grow in clusters and did not fill the culture dish surface at confluency, as controls did. Instead, they seemed to leave small empty spaces between neighbors, detectable at a close look, even when the dish was fully covered (**Fig. 17A-B**).

Moreover, we serendipitously observed that Sema6C-overexpressing cells acquired the capacity to survive metabolic stress caused by serum and nutrients deprivation, far better than control cells. In particular, we challenged the cells by keeping them in serum-free medium for several days without refreshing the culture. Notably, in these conditions, Sema6C-depleted cells revealed their frailty, undergoing cell death after 4 days, whereas control cells lived for up to 12 days (**Fig. 17C**). On the other hand, Sema6C overexpressing cells survived metabolic stress by nutrient deprivation up to 20 days. Notably, long-term survival of these cells was not associated with senescence; in fact, after refreshing the culture with serum-completed medium on the last day, Sema6C-overexpressing cells resumed proliferation and attained confluency (**Fig. 17C**).



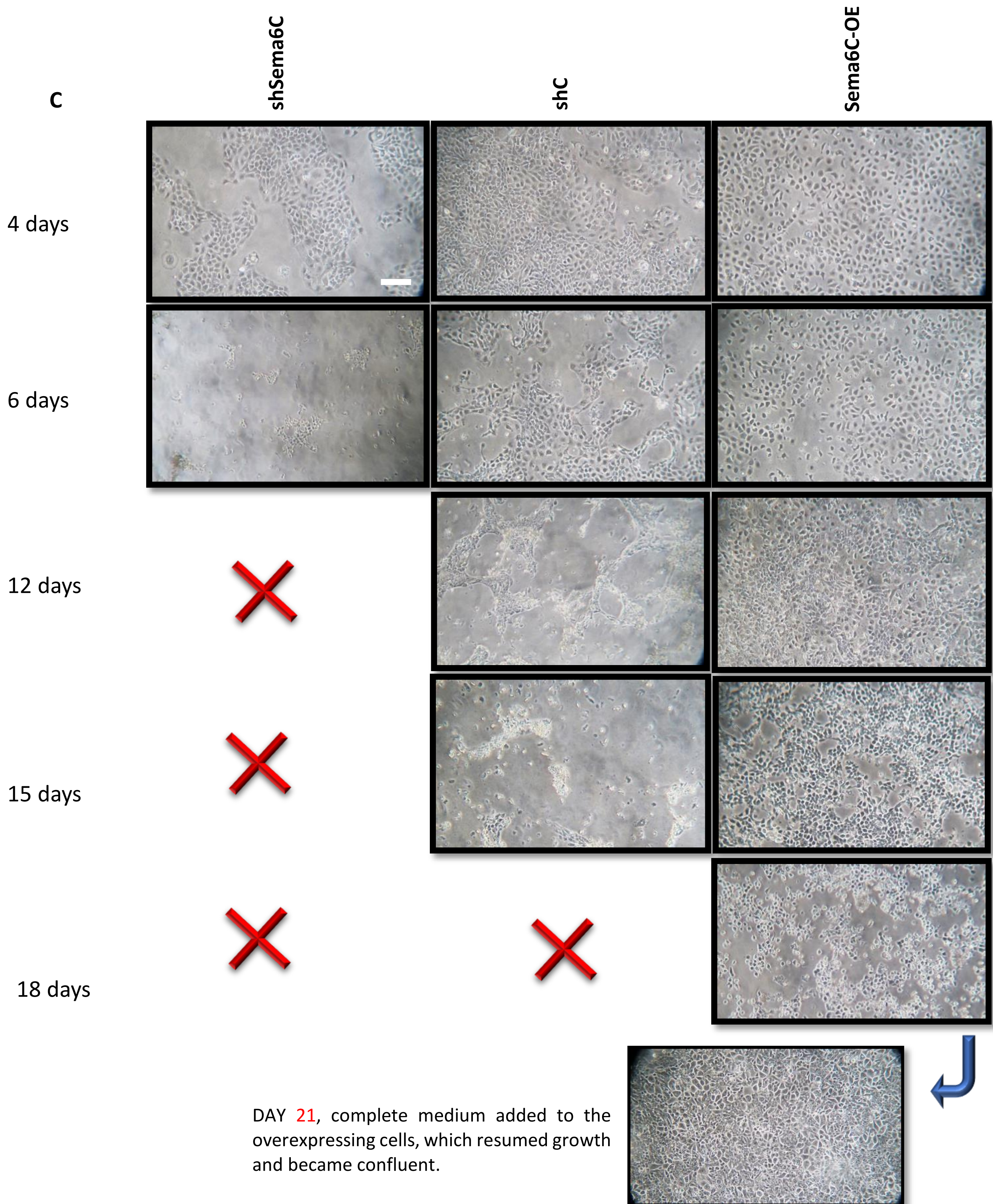



**Figure 17.** (A) Inverted phase contrast micrographs of BxPC-3 control or Sema6C-overexpressing cells, stained with crystal violet. Scale bar=100 μm. (B) Crystal violet staining BxPC-3 control and Sema6C-OE cells. Scale bar=30 μm. (C) BxPC-3 control, Sema6C-silenced (shSema6C), and Sema6C-OE cells were kept in serum free medium for 21 days; then fresh complete medium was added to surviving OE cells. Scale bar=100 μm.

Intriguingly, long-term culture survival in human embryonic stem cells (hESCs) has been associated with YAP/TAZ transcriptional regulators (Ohgushi, Minaguchi, and Sasai 2015). YAP/TAZ activity is dependent on their nuclear localization, which can be induced by several pathways, often related to shape changes and mechanotransduction of extracellular signals. Normally, upon cell confluency and contact inhibition, YAP/TAZ nuclear localization is inhibited and these proteins tend to accumulate in the cytosol, where they may undergo proteasome-mediated degradation (Zeng and Dong 2021). The findings above led us to consider YAP/TAZ as a candidate effector of Sema6C signaling. Thus, we decided to investigate subcellular YAP localization and its potential functional relevance in our cellular models. To this end, we performed immunostaining analyses in control and overexpressing cells, revealing YAP/TAZ nuclear accumulation in Sema6C-overexpressing cells (**Fig. 18**).

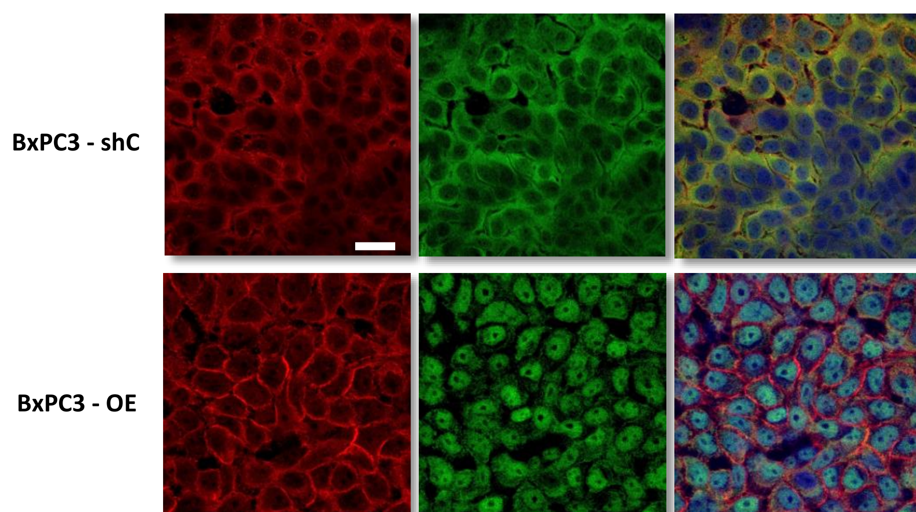



**Figure 18.** Immunofluorescence staining of BxPC3 cells, either control or Sema6C-OE, to analyze the localization of E-Cadherin (red), YAP (green), and DAPI to reveal nuclei (blue). Scale bar= 25 µm.

We also checked YAP expression in cell lysates by immunoblotting, and it turned out that its levels well correlated with Sema6C expression (**Fig. 19**).

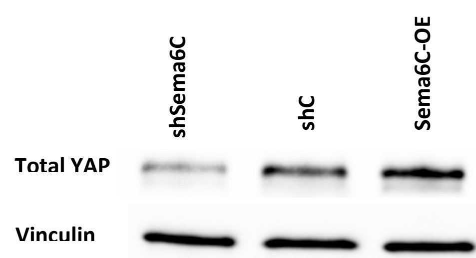



**Figure 19.** Western Blotting of total YAP in BxPC-3 control (shC), Sema6C-overexpressing (OE), and Sema6C silenced (ShSema6C) cells.

In order to confirm the functional role of YAP in Sema6C-regulated phenotypes, we applied the commonly used YAP inhibitor Verteporfin to our cells. Indeed, when treated with Verteporfin for 24 hours, OE cells converted their phenotype to that of controls, and began to reconnect to each other. YAP-inhibited control cells, in turn, acquired a phenotype similar to Sema6C-silenced cells, appearing flattered, multinuclear, and senescent-like (**Fig. 20**).

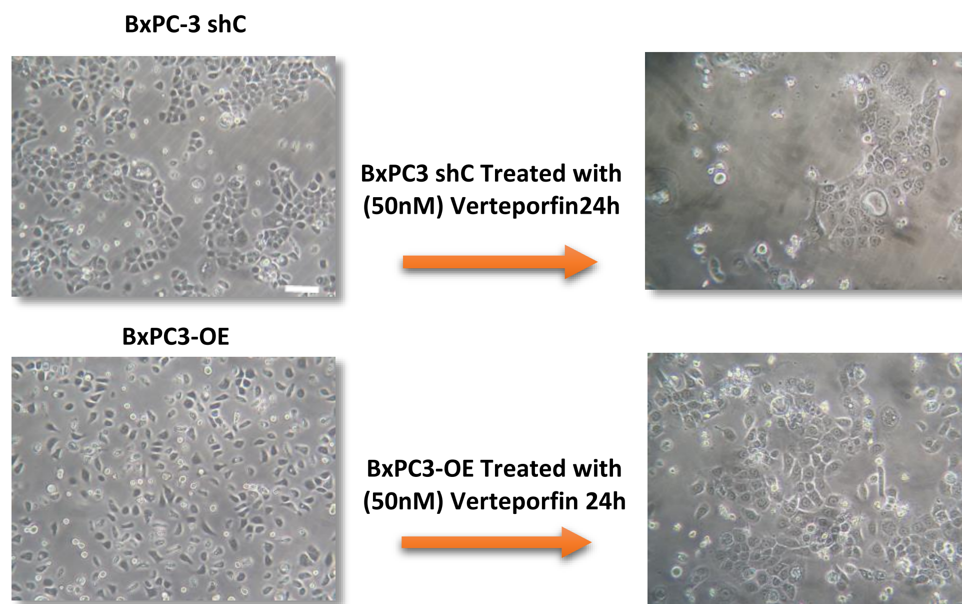



**Figure 20.** BxPC-3 control and Smea6C-overexpressing cells were treated with 50nM Verteporfin to inhibit YAP signalling, and phenotypic changes were assessed after 24 hours. Scale bar= 50µm.

## Sema6C-dependent regulation of cancer cells is dependent on Focal Adhesion Kinase

Then, we asked about signal transducers potentially implicated in Sema6C-dependent activation of ERK, rpS6 and YAP, likely accountable for the observed promotion of cancer cell growth and resistance to metabolic stress. By interrogating scientific literature, we found previous reports indicating Focal Adhesion Kinase (FAK) as an upstream activator of both rpS6 and ERK, with negative regulatory function on p21 and p27 (Gan, Yoo, and Guan 2006; Bryant, Zheng, and Pumiglia 2006; Sawai et al. 2005); moreover, YAP/TAZ activation is induced downstream of FAK (Taniguchi et al. 2015). Notably, FAK tyrosine kinase is often overexpressed in advanced human malignancies, and while it has been associated with integrin signaling, it is also considered a signaling hub in cancer cells, controlling a spectrum

of biological functions, from cell migration to metabolism (Murphy et al. 2020). In particular, FAK has been implicated in a pathway leading to ERK activation, cyclin D1 increase, and p21/p27 downregulation (J.H. Zhao, Reiske, and Guan 1998; Ding et al. 2005). In contrast, FAK inhibition impaired cell proliferation, DNA synthesis, caused cell cycle arrest (mostly in the G2/M stages), and enhanced apoptosis in multiple cell lines (Aboubakar Nana et al. 2019). It was furthermore shown that FAK inhibition in glioblastoma cells augmented p27 protein levels causing proliferative arrest, associated with flattened cell phenotype and β-gal positive staining, indicating cellular senescence (Alza et al. 2020). Autophagy was also reported to be induced upon FAK inhibition (Sandilands et al. 2011; Pham et al. 2018).

In order to investigate the potential involvement of FAK kinase downstream of Sema6C, we firstly assessed the levels of active phosphorylated FAK in our cancer cell models (subjected to gene knock-down or overexpression) by immunoblotting and immunofluorescence analysis; this revealed a striking and consistent association of pFAK levels with Sema6C expression (**Fig. 21**).

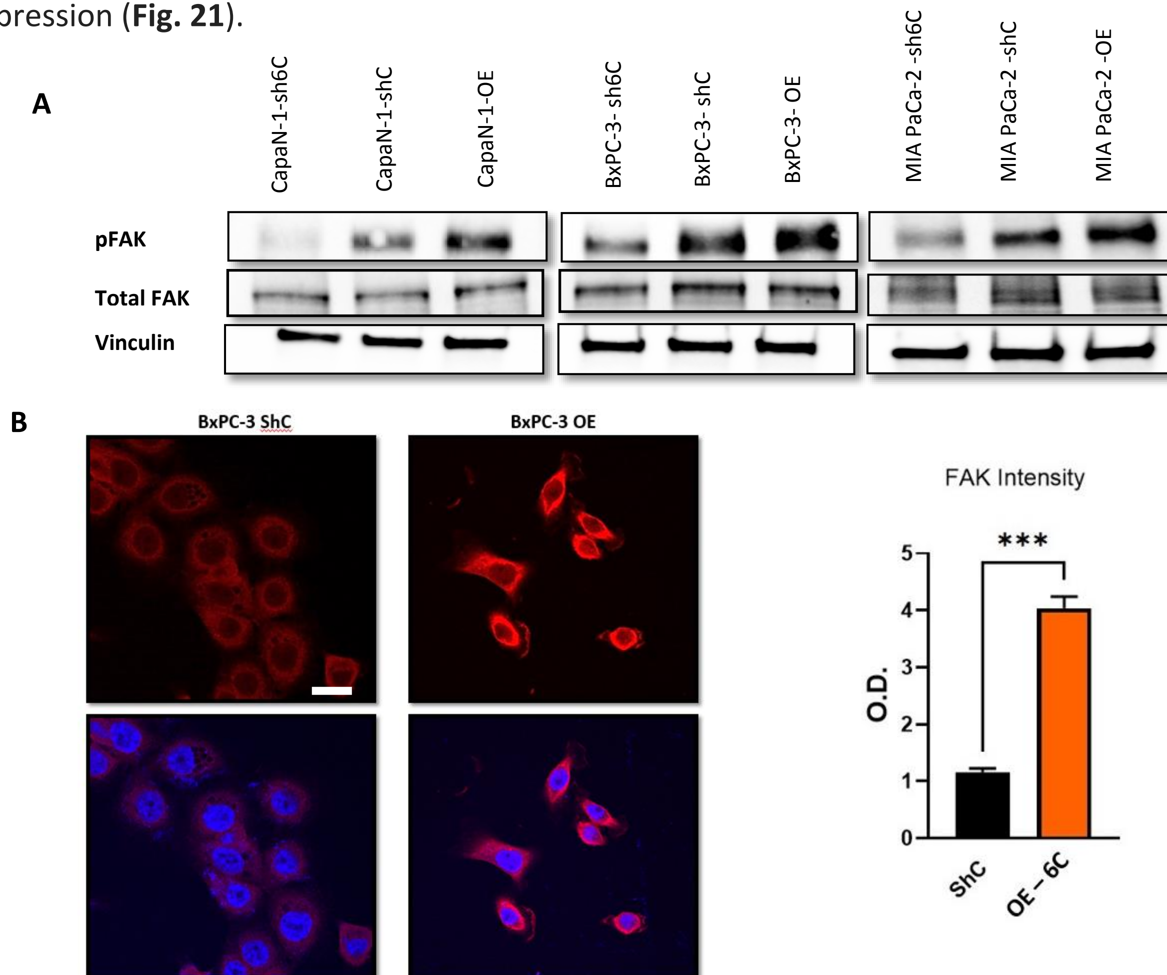


**Figure 21.** (A) Western Blotting for phospho-FAK in diverse PDAC cells (BxPC-3, Capan-1, MIA PaCa-2), either control (shC), Sema6C-overexpressing (OE), or silenced (shSema6C) cells. (B) Immunofluorescence staining for pFAK and DAPI in BxPC-3 control and Sema6C overexpressing cells (pFAK showed in red and DAPI blue); the intensity of fluorescent signal was quantified via ImageJ software. Scale bar= 25 µm

To verify the hypothesis that the FAK activity could play a major role in the observed Sema6C-dependent phenotypic changes, we treated Sema6C-OE cells with the selective FAK inhibitor PF-573228, which resulted in phenotype reversion to that of control cells in as short as 1 hour (**Fig. 22**).

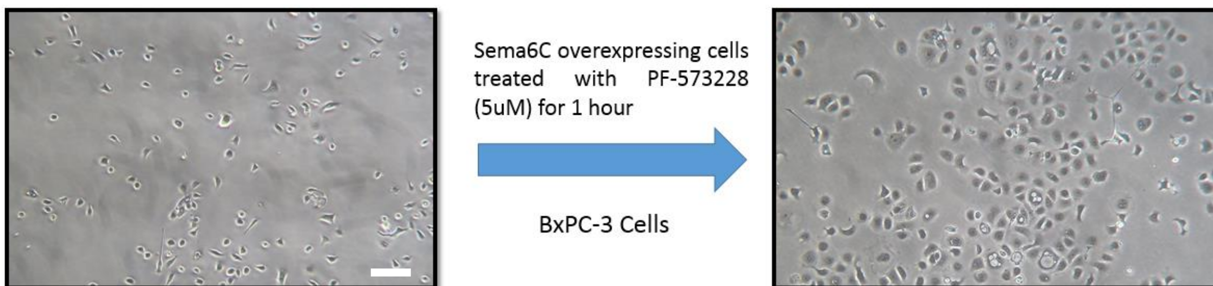



**Figure 22.** BxPC-3 Sema6C-overexpressing cells untreated (left) and treated cells for 1 hour (right) with 5µM PF-573228 (FAK inhibitor). Scale bar=50 µm.

Notably, after 48 hours of treatment of control cells with PF-573228, we observed a significant shape remodeling and enlargement in the cell size similar to that induced by

Sema6C knock-down. Notably, p21 protein levels increased in cancer cells upon treatment with PF-573228, consistent with growth inhibition (**Fig. 23**).

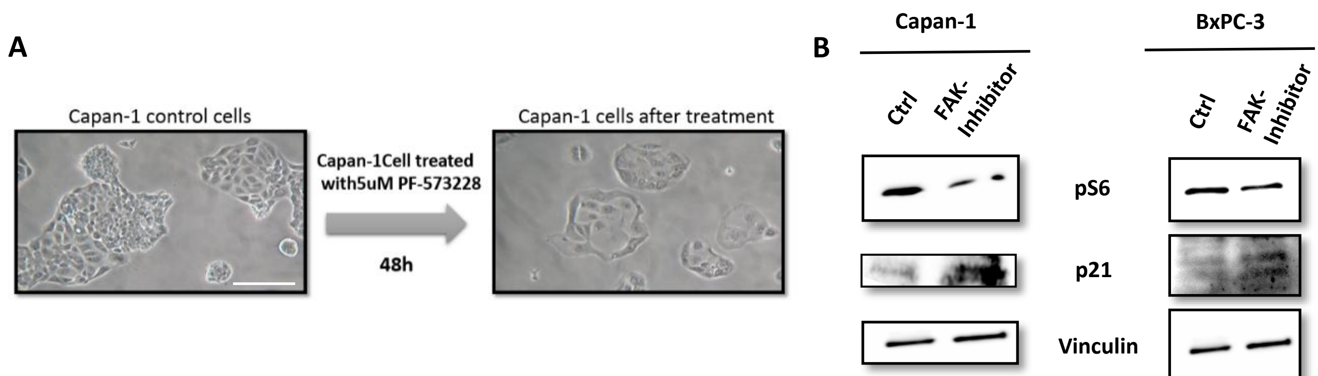



**Figure 23.** A) Capan-1 cells left untreated (left) or treated for 48 hours with 5µM PF-573228 (right). B) Western Blotting of pS6 and p21 in both BxPC-3 and Capan-1 cells treated with 5µM of PF-573228 for 48 hours. Scale bar=50 µm.

As mentioned above, it was demonstrated in intestinal epithelial cells and breast cancer cells that FAK kinase can activate YAP (Song et al. 2021; Lachowski et al. 2018). Moreover, while FAK signaling contributes to YAP activation in colorectal carcinogenesis, the treatment with FAK inhibitors upregulated YAP phosphorylation (which is responsible for its inactivation and degradation), dramatically lowering YAP/TAZ levels and target gene expression (H. Ma et al. 2020).

We therefore, asked in our experimental model if FAK could be held responsible for enhancing YAP/TAZ activity. When treating Sema6C-OE cells with the FAK inhibitor PF-573228, we found that YAP mostly relocalized into the cytoplasm, indicating its functional inactivation (**Fig. 24**). In sum, our data were consistent with a signaling cascade driven by Sema6C, via FAK kinase, leading to both pERK/pS6 and YAP activation in cancer cells.

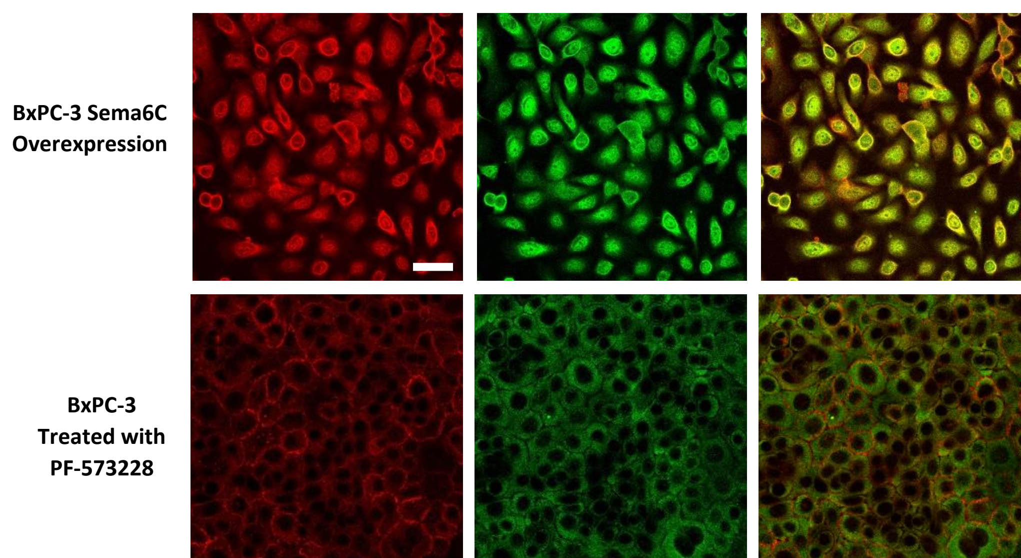



**Figure 24.** Immunofluorescence staining of YAP (green) and E-Cadherin (red) localization, in BxPC-3 Sema6C-overexpressing cells treated (or not) with 5µM of PF-573228 (FAK inhibitor). Scale bar= 30 µm.

The relevance of FAK signaling and YAP activation downstream to Sema6C was further confirmed by transcriptomic analysis of different human tumor samples, which revealed significant correlation of Sema6C levels with FAK- and YAP-regulated gene expression signatures (as defined in GSEA Molecular Signatures Database) (**Fig. 25**).

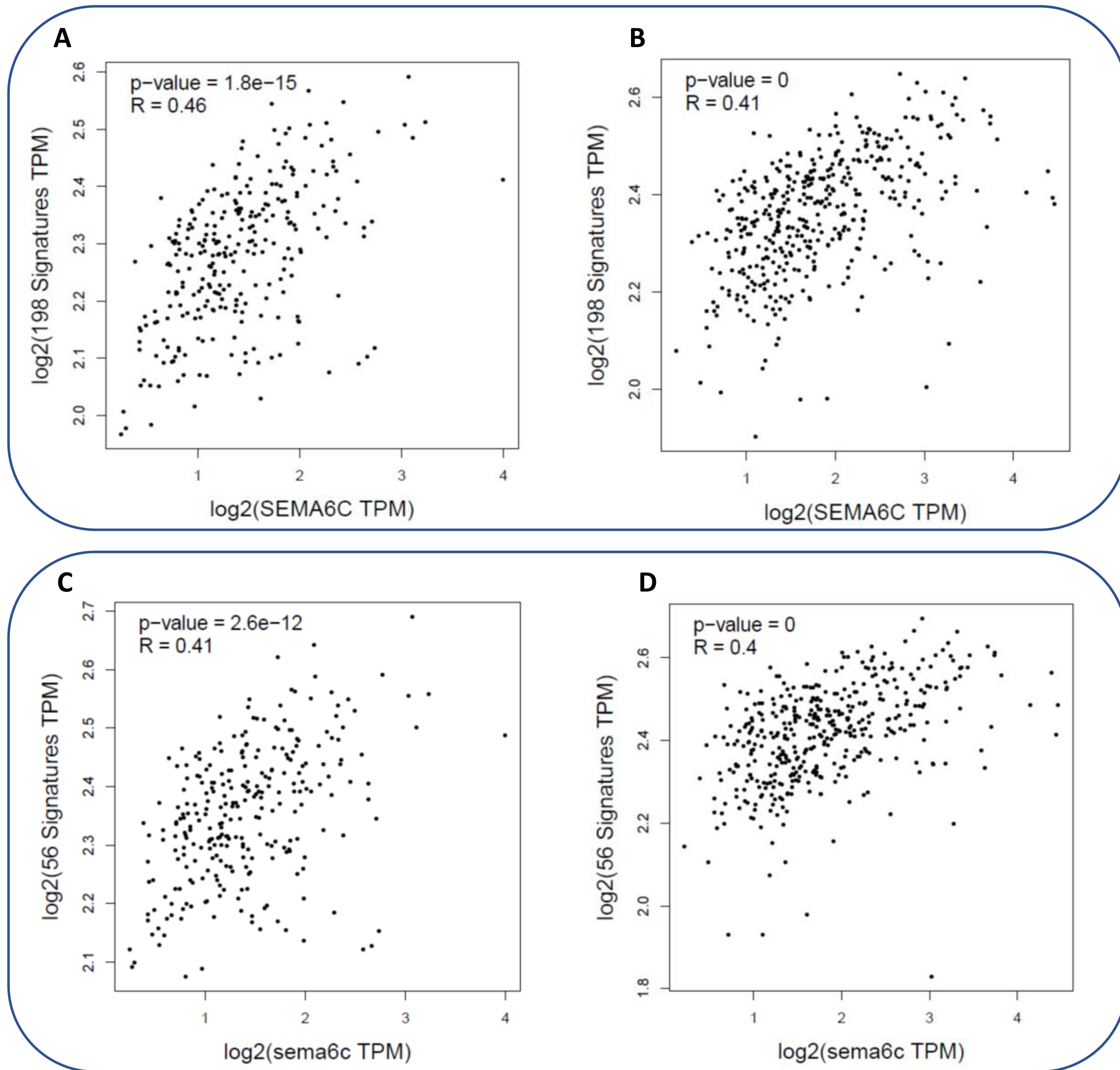



**Figure 25.** Gene Expression Profiling Interactive Analysis (GEPIA2, www.gepia2.cancer-pku.cn) was used to assess the correlation between Sema6C levels and gene expression molecular signatures (GSEA Database v7.5.1, www.gsea-msigdb.org), in human tumor samples (TCGA datasets). Panels A-B show Sema6C correlation with FAK pathway (KEGG_FOCAL_ADHESION/PID_FAK_PATHWAY), while panels C-D refer to correlation with YAP activation signature (CORDENONSI_YAP_CONSERVED_SIGNATURE), in colon (panels A, C) or stomach adenocarcinoma (panels B, D).

## Sema6C-dependent regulation of cancer cells depends on its intracellular portion

In order to elucidate the molecular mechanism linking Sema6C to FAK activation, we aimed at dissecting the signaling pathway of Sema6C in cancer cells.

It is known that transmembrane semaphorins like Sema6C can mediate either forward signaling, via their extracellular moiety acting in autocrine/paracrine or juxtacrine manner; or

reverse signaling, elicited by the intracellular portion of the molecule. To discriminate this point, we first tested the activity of a soluble recombinant Sema6C ectodomain, either overexpressed in cancer cells or added to the culture medium of parental BxPC-3 cancer cells (**Fig. 26**).

These approaches were totally ineffective in inducing the same changes observed in response to transmembrane Sema6C, implying that reverse signaling, mediated by the intracellular portion, is likely involved in the observed effects in cancer cells.

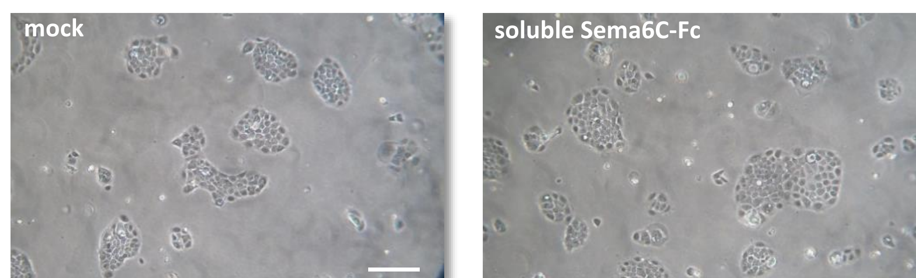



**Figure 26.** Inverted phase contrast micrographs of BxPC-3 cells, either control or overexpressing secretable Sema6C ectodomain (Fc-tagged). Similar results were obtained by addition of the recombinant molecule to the culture medium (data not shown). Scale bar=50µm.

The intracellular portion of Sema6C contains motifs similar to those found in other class 6 semaphorins, and previously implicated in the interaction with non-receptor tyrosine kinases c-Src and c-Abl, and with the adaptor Mena (Klostermann et al. 2000; Godenschwege et al. 2002; Toyofuku et al., 2004; Perez-Branguli et al., 2016). However, no intracellular interactors of Sema6C have been reported so far.

The tyrosine kinase c-Abl may be found in the cytoplasm, in association with the plasma membrane, and in nucleus, and lines of evidence have indicated that it can regulate the activity of FAK kinase (Gotoh et al. 1995; Plattner et al. 1999). Moreover, c-Abl and c-Src have been reported to act in complex by the cell membrane, getting activated in response to the same stimuli, and following the same phosphorylation kinetics (Plattner et al. 1999). Of

interest, we found that c-Abl phosphorylation was prominently increased in Sema6C overexpressing cells (**Fig. 27**).

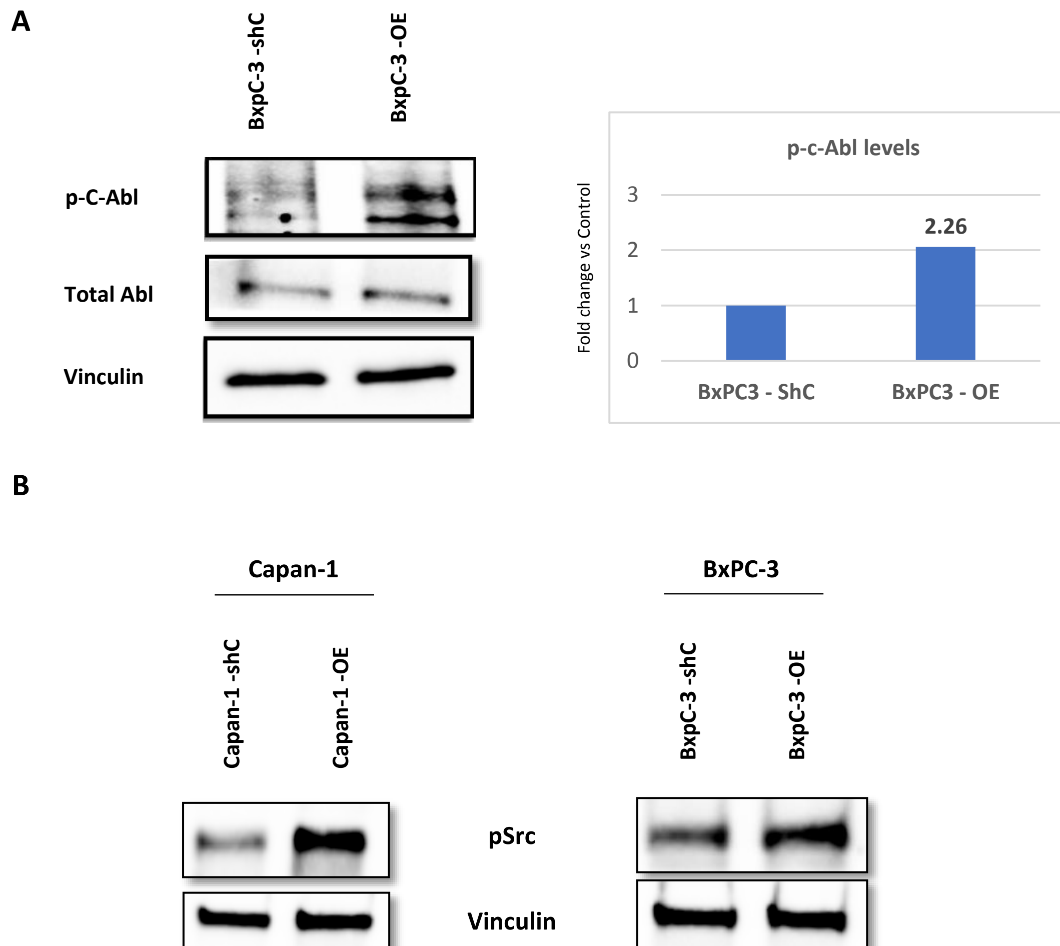



**Figure 27**. (A) Western Blotting analysis of p-Abl in BxPC-3 control and Sema6C-overexpressing cells. (B) Western Blotting analysis of pSrc in BxPC-3 control and Sema6C-overexpressing cells

The homologous class 6 semaphorin members Sema6D and Sema6A were previously reported to recruit Abl tyrosine kinase to their intracellular portions (Toyofuku et al., 2004; Perez-Branguli et al., 2016), putatively due to proline-rich motifs docking to the SH3 domain of the kinase (Hou et al. 2006).

Importantly, when we performed immunoprecipitation experiments in our Sema6C-overexpressing cancer cells, we observed a clear and specific interaction between Sema6C and endogenous c-Abl tyrosine kinase (**Fig. 28**).

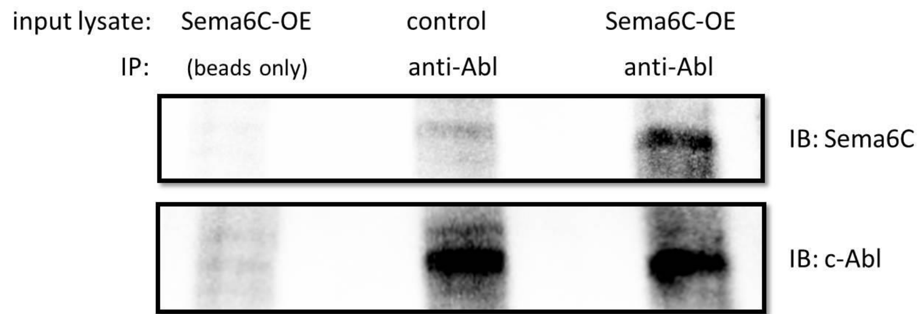
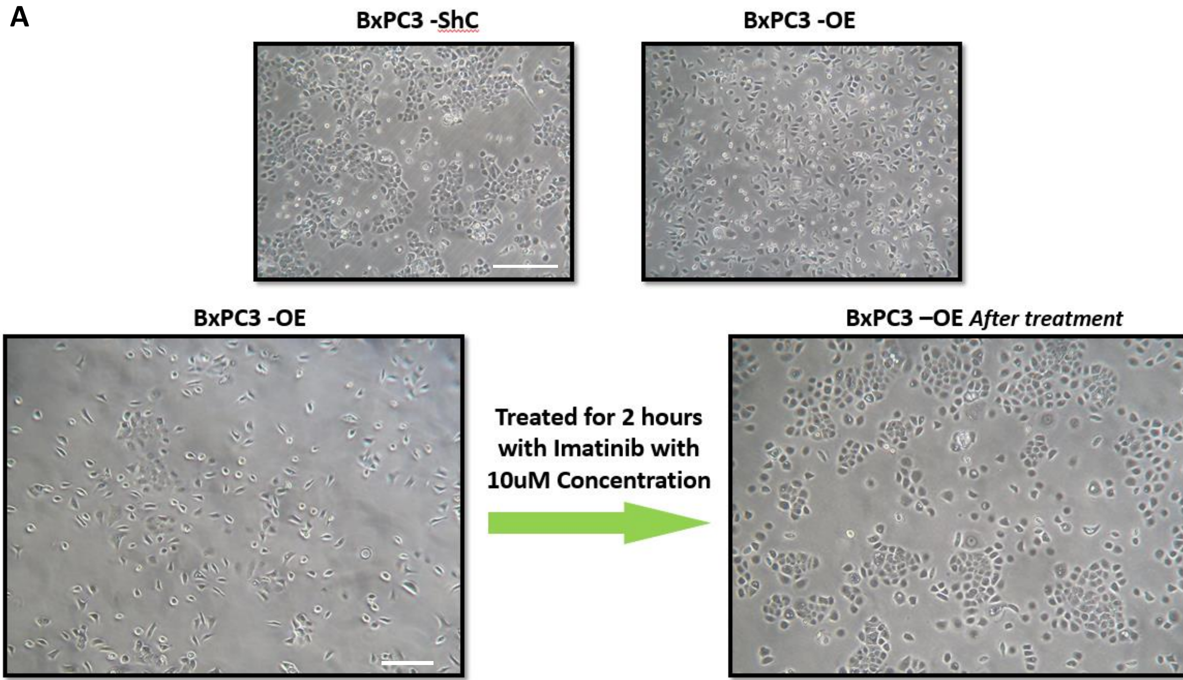



**Figure 28.** Co-immunoprecipitation of c-Abl with Sema6C in BxPC3 cells (either control or Sema6C-OE). A specificity control was provided by incubating Sema6C-overexpressing cell lysates with beads only, without anti-Abl antibodies.

As anticipated above, previous evidence indicates that proteins associated with focal adhesions, and FAK kinase in particular, may be regulated by the Abl/Src signaling complex, activated in response to growth factor receptor stimulation (Plattner et al. 2004; Fu et al. 2015; Gotoh et al. 1995; Salgia et al. 1995).

This kinase signaling cascade impacts on the regulation of cytoskeletal dynamics, cell shape, cell-cell and cell-matrix connections, as well as cellular motility, proliferation and survival, because it can furthermore affect gene expression control through mechano-transduction pathways like the Hippo-YAP/TAZ.

Based on the phenotype observed in Sema6C overexpressing cells, we wondered about the potential role of c-Abl upstream this signaling pathway. Indeed, when we applied the Abl kinase inhibitor Imatinib (10 µM) to BxPC3 Sema6C-OE cells, the phenotype reverted to control, in 2 hours.
Moreover, we investigated by western blotting the impact of Abl inhibition on FAK, Src, and ERK phosphorylation in our cellular models, and verified their dramatic decrease, strongly indicating that these kinases are placed downstream to Abl in the Sema6C-driven signaling cascade that we have unveiled (**Fig. 29**).




B)

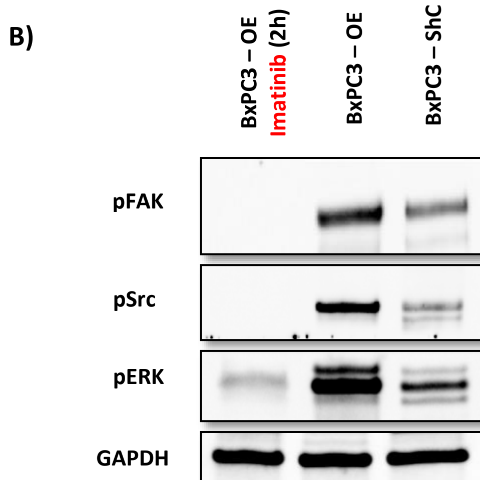



**Figure 29.** (A) BxPC-3 overexpressing cells treated with 10µM of Imatinib and phenotypic change was monitored after 2 hours. Scale bars=90µm (upper) and scale bars=50µm (lower). (B) Western Blotting of pFAK, pSrc and pERK, to reveal the impact of treatment with Imatinib in BxPC-3 Sema6C-OE cells.

It has been shown that the cytoplasmic tail of Sema6C-paralogue Sema6A binds to Ena/VASP-like protein Evl via a C-terminal motif (Klostermann et al. 2000), while both Sema6D and Sema1a (in flies) have been found to interact with Enabled or its murine orthologous Mena, respectively (Godenschwege et al. 2002; Toyofuku, Zhang, Kumanogoh, Takegahara, Yabuki, et al. 2004). Human Mena was found to be overexpressed in more than 70% of primary breast cancers as well as benign breast lesions with a significant chance of transformation. Moreover, Mena is a cellular regulator of the assembly and dynamic of cytoplasmic actin networks, especially involved in the nucleation and polymerization of actin filaments (Gurzu et al. 2013).

Therefore, in order to investigate the potential involvement of Mena in Sema6C signaling, we performed western blot analysis in Sema6C-silenced and overexpressing cells. Intriguingly, Mena protein levels correlated with Sema6C expression (**Fig. 30**), similar to what observed for pAbl.

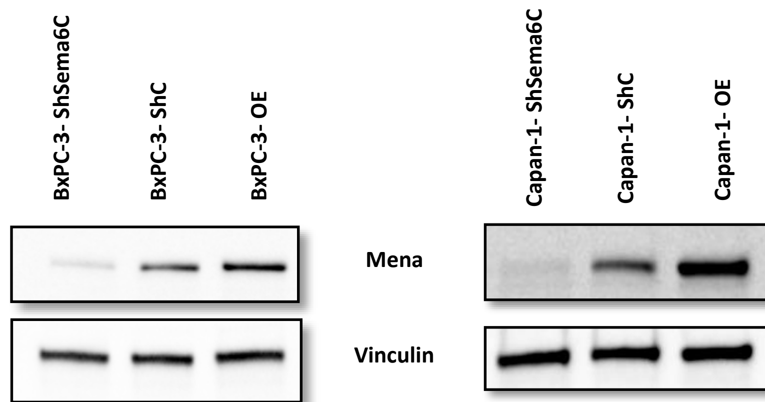



**Figure 30.** Western blotting to assess Mena protein levels in BxPC-3 and Capan-1, either control, Sema6C-silenced (shSema6C), or Sema6C-overexpressing (OE).

Consistent with a mechanistic role of Mena in Sema6C signaling, the treatment of Sema6C-overexpressing cells with siRNAs targeted against Mena specifically reverted their phenotype to that of controls cells (**Fig. 31**).

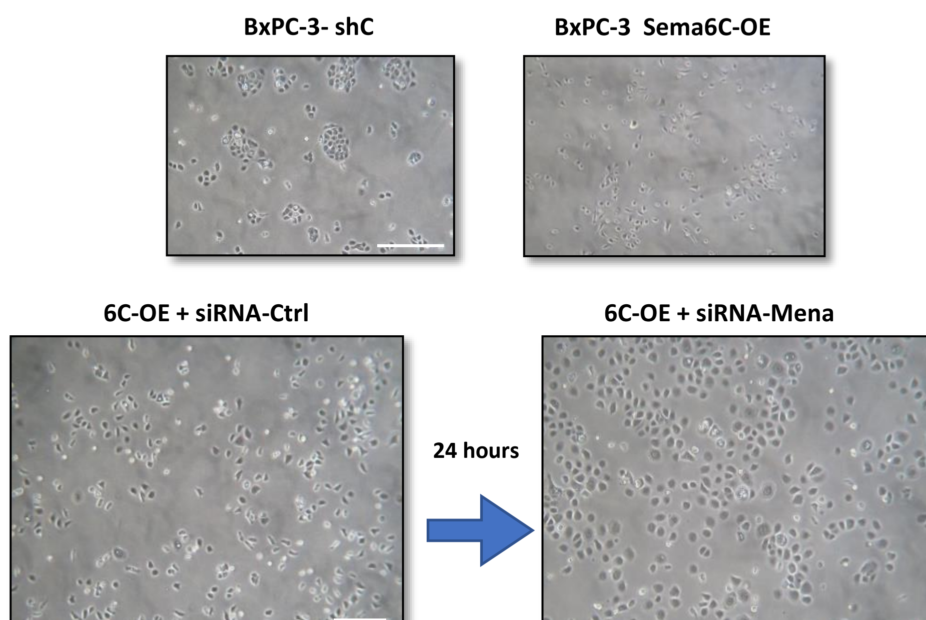
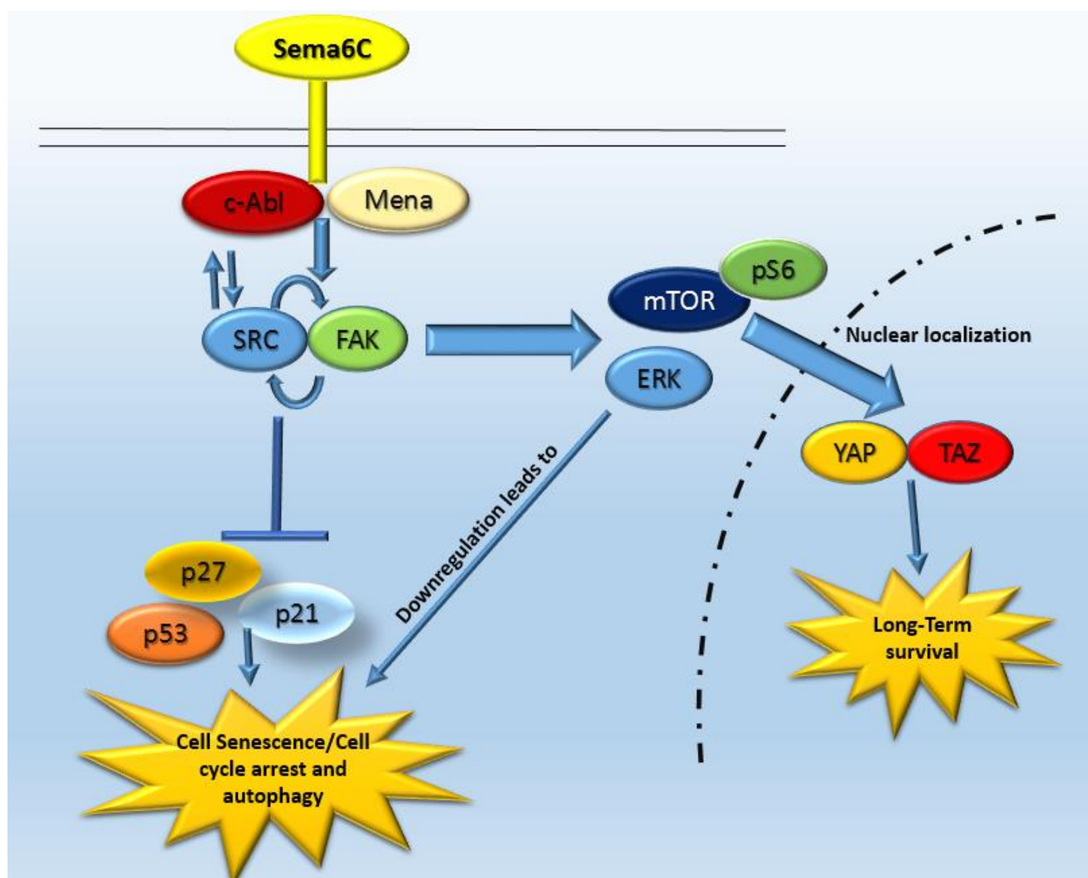



**Figure 31.** BxPC-3 cells overexpressing Sema6C were transfected with Mena-targeted siRNAs, which reverted the phenotype to that of control cells after 24 hours; control siRNAs provided an internal specificity control. Scale bars=100µm (upper) and 60µm (lower).

In sum, our data indicate that Sema6C signaling in cancer cells elicits Abl kinase activation and the upregulation of Abl functional partner Mena. Moreover, Abl inhibition prevented the activation of putative downstream effectors of Sema6C, like FAK, ERK and YAP, strongly suggesting a signaling cascade controlling cytoskeletal dynamics and gene expression reprogramming consistent with phenotypic and functional changes observed in Sema6C-overexpressing cells.

**Signaling cascade overview**



# DISCUSSION

The Semaphorin family comprises around 20 membrane-bound or secreted proteins, initially discovered as axon guidance cues, but later linked to a wide range of biological functions and developmental processes, including cell migration and cell-cell interactions (Alto and Terman 2017). Moreover, semaphorins are emerging as pivotal regulators of tumor development, due to their capacity to control cancer cell behavior and the tumor microenvironment (Tamagnone 2012).

Interestingly, transmembrane semaphorins are bidirectional signals, and mediate both conventional “forward” and and “reverse” communication (Battistini and Tamagnone 2016). Although less extensively studied than forward signaling, semaphorin reverse signaling has a crucial role in neural and cardiac development, and it is increasingly appraised in adult physiology and human diseases. Yet, in most of the cases, the underlying processes are unknown, and further research is needed to understand these complex bidirectional communication networks.

In this thesis work, I focused on Sema6C, a poorly studied transmembrane semaphorin that was first discovered as an axonal chemorepellent (Kikuchi et al. 1999), enriched in skeletal muscles and neuromuscular junctions (Svensson, Libelius, and Tågerud 2008), and implicated in ovarian follicles maturation (Yan et al. 2019), while it has never been studied in cancer context. We noticed that Sema6C expression seemed to correlate with poor patient survival in multiple, though not all, human tumors. However, the molecular mechanisms underlying Sema6C activity in cancer cells were totally unknown. Therefore, this project aimed at specifically investigating the functional role and signaling mechanisms of Sema6C in cancer cells.

In my work, I mostly used pancreatic adenocarcinoma cells as experimental model, though major findings were also replicated in cells derived from other tumor types, suggesting conservation of the signaling mechanisms. Notably, pancreatic ductal adenocarcinoma currently remains an overarching clinical challenge, due to poor efficacy of available therapeutic options (Borchardt et al. 2019). Thus, the identification of novel molecular targets and clinical biomarkers is warranted. Interestingly, our data indicate that Sema6C depletion

dramatically impacts on cancer cell behavior, leading to growth arrest, autophagy, and senescence. These findings were entirely novel, and previous studies on this semaphorin did not provide any hints to hypothesize the underlying molecular mechanisms. Therefore, in order to elucidate the signaling pathway elicited by Sema6C in cancer cells, I undertook gain of function approaches.

Notably, although Sema6C, like other semaphorins, has been found to inhibit axonal extension, we have not observed inhibitory effects of Sema6C in cancer cells. Instead, its overexpression resulted in increased cell motility and long-term survival under metabolic stress. In fact, divergent semaphorin functions in neurons and in other cell types have often been seen (e.g. for Sema3A, Sema3E, Sema4D, etc.), likely reflecting different receptor complexes and signaling pathways.

Actually, Sema6C signaling mechanisms are almost unaddressed in previous literature. In the newborn mouse ovary, Sema6C was found to inhibit primordial follicles activation by controlling PI3K-AKT pathway (Zhou et al. 2018). However, in my experiments, I have not observed pAKT regulation depending on Sema6C in cancer cells, which seems to implicate diverse mechanisms of Sema6C signaling in ovarian physiology and in cancer. Sema6C-induced phenotypic changes that we observed in cancer cells were reminiscent of a classical EMT, but this mechanism was not confirmed at molecular level in our Sema6C-regulated models. Moreover, we did not observe a significant association between Sema6C and the expression of cell-cell adhesion molecules. However, I noticed that Sema6C-overexpressing cells tended to avoid strict cell-cell contacts, including at confluency, a feature that may deserve further investigation.

We thus speculated that Sema6C-dependent regulation of cell phenotype and viability could be accounted by a pathway impacting both cell cycle control and cytoskeletal remodeling. Interestingly, the phenotype produced by gene silencing was not rescued by treatment with a soluble extracellular domain of Sema6C, implicating reverse signaling mechanisms mediated by the intracellular tail, that were never investigated previously for this semaphorin. Interestingly, the phenotype produced by Sema6C silencing in cancer cells was

not rescued by treatment with a soluble extracellular domain of Sema6C, implicating reverse signaling mechanisms mediated by the intracellular tail, that were never investigated previously for this semaphorin.

Intriguingly, cytoskeletal remodeling and RhoA signaling are known to promote the nuclear localization and activation of the transcriptional cofactors YAP and TAZ, which mediate one of the major pathways involved in long-term survival of human embryonic stem cells (Ohgushi, Minaguchi, and Sasai 2015), as well as inhibition of autophagy and apoptosis (Pham et al. 2018; Jin et al. 2021). Recent studies have also shown YAP pathway regulation by PlexinB2 (Junqueira Alves et al. 2021); however, the role of semaphorin signals in this cascade was not elucidated. This inspired us to look deeper into the potential regulation of YAP/TAZ pathway in Sema6C overexpressing cells, which led to the evidence that Sema6C promotes YAP nuclear localization in confluent cell monolayers, while YAP inhibition suppresses Sema6C-induced phenotype in cancer cells.

Furthermore, in other experiments, I found that Sema6C overexpression induced the phosphorylation and functional activation of major intracellular signal transducers controlling cell cycle and phenotypic changes FAK, SRC, and ERK kinases, and the mTOR substrate S6 riboprotein; conversely, these effector molecules were deactivated upon Sema6C silencing in cancer cells. Importantly, FAK kinase was reported previously to promote ERK signaling (Cheung et al. 2008), a pathway implicated in tumor cells invasiveness, e.g. in ovarian cancer (Lai et al. 2018).In mice, a FAK-YAP-mTOR signaling cascade controls stem cell-based tissue regeneration (Hu et al. 2017). Moreover, FAK-dependent activation of mTOR kinase has been found to promote basal-like mammary tumors (Paul et al. 2020).Notably, S6K activation and the ensuing rpS6 phosphorylation are usually regarded as readouts of mTOR activation, and pS6 regulation by FAK has also been reported (Gan, Yoo, and Guan 2006). Multiple data imply a synergistic crosstalk between mTORC1 and ERK pathways to govern rpS6 phosphorylation (Mukhopadhyay et al. 1992); furthermore, ERK was found to induce rpS6 phosphorylation via p90 ribosomal S6 kinases (RSK), also independent of mTORC1 signaling (Roux et al. 2007). Notably, FAK kinase has been linked to different regulatory functions in tumor growth and metastasis, and loss of FAK activity has been associated with the inhibition of cancer cell

proliferation, and the induction of autophagy, senescence, and apoptosis (Pham et al. 2018; Chuang et al. 2019; Alza et al. 2020). Moreover, FAK inhibition was associated with the upregulation of p21, p27, and p53 cell cycle inhibitor proteins (Chuang et al. 2019; Alza et al. 2020), and with the loss of YAP nuclear localization (Lachowski et al. 2018). Altogether, these elements suggested a strong correlation between Sema6C-dependent phenotypes observed in our experiments and known activities mediated by FAK kinase.

Indeed, by the treatment with a selective inhibitor, I could place FAK kinase activity upstream of Sema6C-induced SRC, ERK, and S6 phosphorylation, as well as YAP nuclear localization. However, experiments assaying the physical association between FAK and Sema6C were inconclusive.

Intriguingly, other semaphorin members of class 6, such as Sema6A and Sema6D, have been shown to recruit non-receptor tyrosine kinases to their intracellular domains (Perez-Branguli et al. 2016; Kang et al. 2018). For instance, Abl was found in association with Sema6A cytoplasmic tail, and the activity of this tyrosine kinase is linked to the regulation of both cell cycle and cytoskeletal dynamics (Huang et al. 2008; Plattner et al. 1999).

Actually, in this study, we unveiled that Sema6C recruits c-Abl to its cytoplasmic domain, and elicits Abl kinase activity, through mechanisms awaiting clarification. Moreover, I found that Sema6C-induced activation of c-Abl is responsible for FAK phosphorylation, which is consistent with previous evidence linking the two intracellular kinases(Gotoh et al. 1995), and could potentially represent the initial step of Sema6C signaling cascade in cancer cells.

In sum, we observed that the overexpression of Sema6C leads to overt shape change, associated with increased cell motility and long-term survival upon nutrient deprivation. Although we could not fully elucidate the implicated pathway, we found Sema6C in association with the intracellular tyrosine kinase c-Abl. Moreover, upon Sema6C overexpression, Abl phosphorylation increased, responsible for FAK kinase activation. Abl and FAK were further implicated to activate downstream effectors of Sema6C-induced phenotype, such as ERK, mTOR/pS6 and YAP. Notably the association of Sema6C expression with FAK signaling and YAP-induced transcriptional signature was confirmed in human tumor

samples. On the other hand, the involvement of plexins for triggering Sema6C reverse signaling in trans needs to be investigated further.

Since multiple tumor cells proved to depend on Sema6C signaling for proliferation, and its high expression promoted an aggressive phenotype in culture, our data support the idea that Sema6C-targeting may have beneficial effects for cancer therapy. However, several aspects need to be investigated further. First, since Sema6C-deficient cancer cells could not be propagated in culture, we could not investigate their tumorigenic potential in mouse models. Alternative approaches should be undertaken in this perspective, such as the application of drug-inducible Sema6C knock-down constructs, to be activated after the establishment of tumor xenografts. Moreover, it is unknown the potential impact of Sema6C-inhibitors in normal adult tissues. One intriguing element to consider is that Sema6C knock-out mice have been generated (MGI:3604574). The mutant mice are viable and fertile, and the deposited description of their phenotype does not reveal major defects in tissue homeostasis and function. It may be therefore hypothesized that, different from what we observed in cancer cells, Sema6C expression is not essential during development, being compensated by redundant functions of other family members. If it will be confirmed that Sema6C blockade has no major impact on adult physiology, this may support systemic Sema6C-targeting in mouse cancer models, for therapeutic purposes.

# MATERIALS AND METHODS

**Cell culture and reagents**

Cell lines were grown in a monolayer at 37° C in 5% CO2 atmosphere. BxPC-3 and MIA PaCa-2 pancreatic adenocarcinoma cells were cultured in RPMI-1640 medium supplemented with 10% FBS, while Capan-1 in Iscove medium with 20% FBS; culture media were supplemented with 2mM glutamine, 100U/mL penicillin, and 100µg/mL streptomycin.

**Antibodies and other reagents**

Anti-Sema6C antibodies were from R&D Company (Cat.AF2108). The following antibodies were from Cell Signaling Technology: anti FAK (Cat. #3285), anti pFAK (Y925) (Cat. #3284), anti-pSrc (Tyr416)(Cat. #59548), anti-LC3A/B (Cat. #4108), anti-p44/42 MAPK (Erk1/2, 137F5)(Cat. #4695), anti-p21 Waf1/Cip1 (Cat. #2947), anti-SQSTM1/p62 (Cat. #5114), anti-phospho-c-Abl (Tyr245) (Cat. #2861). Santa Cruz Biotechnology provided: anti c-Abl (8E9) (Cat. sc-56887), anti-YAP (63.7) (Cat. sc-101199). Anti-p27 KIP 1 [Y236] was from Abcam company (cat. ab32034); anti-vinculin from Sigma-Aldrich Company (cat. V9131); anti-Mena from BETHYL-laboratories (cat. A301-500A), anti-E-cadherin from Proteintech (cat. 20874-1-AP). Moreover, PF-573228 (Tocris Bioscience cat. 3239), Verteporfin (Sigma-Aldrich, cat. SML0534), Imatinib (Cell signaling, cat. #9084).

**Gene knock-down and overexpression**

For SEMA6C knock-down, we applied the following shRNA-expressing constructs provided by Sigma-Aldrich: TRCN0000005666 (indicated as shSema6C#1, applied in most experiments) and TRCN0000005664 (shSema6C#2). Mena was knocked-down by oligofection of siRNA sequences provided by Santa Cruz Biotechnology (sc-43496).

To achieve SEMA6C overexpression, the respective human cDNA was produced by gene synthesis by BioCat GmbH (Heidelberg, Germany), and subcloned in pLVX-puro lentiviral transfer plasmid (carrying puromycin resistance). Sema6C-Fc construct (Plasmid: #72167 from Addgene repository) was subcloned into the same lentiviral transfer plasmid.

**Gene transfer with lentiviral vectors**

HEK-293T packaging cells were used to produce non-replicating viral particles with shRNAs or cDNA expressing constructs, as described previously (Follenzi and Naldini, 2002). As previously described, cell supernatants containing lentiviral particles were incubated with cultured cells in the presence of polybrene 8µg/ml. (Follenzi and Naldini, 2002). Cells transduced with vectors containing puromycin resistance were consequently selected in the presence of 1 µg/ml puromycin in the medium.

**mRNA expression analysis**

The RNeasy Mini kit (Qiagen) was used to get total mRNA extracts. In a final volume of 20µL, 1µg of RNA was retrotranscribed using the Improm-II Reverse Transcription Set (Promega), according to the manufacturer's protocol. Real-time PCR was used to evaluate gene expression using specific SYBR Green primers provided by Sigma-Aldrich Company (see table below). According to the formula below, the fold change was calculated: Fold increase= 2^-(CT of target gene – CT of the house-keeping gene).

| | |
|---|---|
| h-Sema6C-S | 5'-CTTCGGCTCAACTGCTCTGT |
| h-Sema6C-AS | 5'-AACCCACGCTCAATCTCATC |
| h-GAPDH-S | 5'-TTGTTGCCATCAATGACCC |
| h-GAPDH-AS | 5'-CCTCCCGTTCTCAGCCTTG |

**Protein immunoprecipitation and Western blot analysis**

Cells were lysed in TritonX100-containig LB buffer, in presence of PMSF (phenylmethylsulfonyl fluoride), and Protease Inhibitor Cocktail (all provided by Cell Signaling Technology). Bradford assays were used to determine protein concentration in cell lysates. For the analysis of total cell lysates, equal amounts of total proteins were denatured by boiling in Laemmli buffer (2% SDS, 50 mM Tris-HCl, pH 7.4, 20% mercaptoethanol, and 20%

glycerol) and analyzed by SDS-PAGE and Western blotting, according to standard methods and manufacturers' instructions. For protein immunoprecipitation, equal amounts of cell lysates were incubated with Protein G-Sepharose beads coated with specific antibodies for 2 hours at 4°C with. After rinsing the beads, the immunoprecipitated proteins were denatured by boiling in Laemmli buffer and subjected to SDS-PAGE and Western blotting analysis.

**Cell senescence assay**

Cells were seeded in 6-well plates and upon reaching 50-60% confluency, the medium was removed. The cells were washed in PBS before being fixed with the 1x fixative solution provided with the senescence-galactosidase staining kit (Cell Signaling Technology; #9860). Following the manufacturer's instructions, a fresh -galactosidase staining solution was made. After washing with PBS twice, cells in each well were stained with a 1 mL staining solution. After a 16-hour incubation at 37°C in a dry incubator, senescent cells were identified for a positive beta-galactosidase-dependent dye conversion.

**Immunofluorescence analysis**

Cells were seeded on glass coverslips, then fixed for 15 minutes in 4% paraformaldehyde, permeabilized with (0.1% or 0.3%, as indicated in specific experiments) Triton/phosphate-buffered saline for 10 minutes at room temperature, and blocked by incubation with 5% normal donkey serum for 30 min or 10% BSA for 1 hour. After incubation with primary antibodies overnight and rinses, fluorochrome-conjugated secondary anti-mouse or anti-rabbit antibodies were added for 1 hour at room temperature. Fluorescent-Phalloidin conjugates were used to stain F-actin, and 4, 6-diamidino-2-phenylindole (DAPI) was used to reveal cell nuclei. The coverslips were finally washed and mounted on slides. Images were acquired at room temperature with a confocal laser scanning microscope (SP5 Leica) equipped with a 63×/1.30 HCX Plan-Apochromat oil immersion objective lens (ACS APO 63×/1.30 oil CS 0.17/E, 0.16) using Leica LAS AF software. Images were analyzed with LAS AF Lite software.

### Cell proliferation analysis

The growth curve was determined as follows: the cells were seeded in multiple 96-well plastic culture plates (1000 and 2000 cells/well), which were fixed with 4% PAF for 15 minutes on subsequent days. Cell nuclei were then stained with DAPI, visualized by an inverted fluorescence microscope (Leica), and counted at different time points.

### Boyden chamber migration assays

Transwell chambers with 8 µM pore size (Corning Costar Incorporated, NY, USA) were used to analyze cell migration. In brief, the lower side of the filter was precoated with 10 µg/ml fibronectin and blocked in presence of 1% BSA; then $5 \times 10^4$ cells resuspended in serum-free medium were included in the upper chamber and allowed to migrate through the filter separating the bottom chamber, which contained 1% FBS-containing medium. To ensure comparable cell inputs, the same volume of cell suspension was seeded in cell culture multiwell dishes in parallel and eventually quantified. At the end of the assay, after 12 or 24 hours, non-migrated cells on the upper side of the filter were removed with a cotton swab. The insert was then fixed with 4% paraformaldehyde and stained with crystal violet. The integrated pixel values of light microscopy images of the inserts were then measured using ImageJ (NIH). Experiments were replicated at least three times in order to ensure results consistency.

### Immunoprecipitation

Cells were lysed in LB buffer containing lysis buffer, PMSF (phenylmethylsulfonyl fluoride), Protease Inhibitor Cocktail (Cell Signaling Technology). Bradford assays were used to determine protein concentration in cell lysates. Equal amounts of cell lysates were incubated with Protein G-Sepharose beads (Abcam company #ab193259) coated with specific

antibodies. After 2 hours of incubation at 4°C, the beads were washed and immunoprecipitated proteins were denaturated with boiling in Laemmli buffer.

**Wound healing Assay**

The cells were seeded at $1 \times 10^6$ cells per well in a six-well cluster (Corning Costar Incorporated, NY, USA), allowing them to attach and spread in order to obtain a confluent monolayer. After 24h, the scratch was made in the confluent monolayer by using a sharp object, such as a 200 µl pipette tip. After scratching, the monolayer washed with PBS. Then, the cells were replenished with a fresh medium. Then the cells were placed in a cell culture incubator at 37 °C for 24h, allowing the cells to fill the gap. After this incubation, the cell monolayer was washed with PBS, fixed with 4% paraformaldehyde for 15 minutes, and finally stained with 1% crystal violet for 30 minutes. Then, the images were acquired by phase-contrast microscopy and analyzed quantitatively by ImageJ software. We identified the area to be measured in T0 images for each condition, and the same area was analyzed in the other time points to achieve quantitative analysis and determine the percentage of wound closure.

**Cell cycle analysis by cytofluorimetry**

Harvested cells ($10^5$ cells per condition) were fixed overnight in ethanol (70%)/PBS solution at 4 °C. The cells were then incubated for 3 hours at room temperature in a solution containing 0.1 mg/ml propidium iodide and 200 units/ml RNase before being examined by flow cytometry.

**Time-lapse Videomicroscopy**

Cells were stained with cell tracker (Invitrogen by thermos Fisher Scientific) and seeded at low density on 8-well glass-bottom plates (ibidi GmbH) coated with 1 µg/ml fibronectin (Sigma-Aldrich). The plate was then placed on an inverted microscope with a 37 °C humidified

chamber containing 5% $CO_2$, and monitored by a fluorescence microscope (Leica) overnight. Every 11 minutes, photographs are taken to monitor cell motility.

### Statistics

Average values of at least three independent experimental replicates are usually shown, together with standard deviation (SD). The statistical significance of comparisons was calculated by Student's T-test; p values, indicated by asterisks in figures, have been specified in figure legends.

# BIBLIOGRAPHY

Aboubakar Nana, F., M. Lecocq, M. Z. Ladjemi, B. Detry, S. Dupasquier, O. Feron, P. P. Massion, Y. Sibille, C. Pilette, and S. Ocak. 2019. "Therapeutic Potential of Focal Adhesion Kinase Inhibition in Small Cell Lung Cancer." *Mol Cancer Ther* 18 (1): 17-27. https://doi.org/10.1158/1535-7163.MCT-18-0328. https://www.ncbi.nlm.nih.gov/pubmed/30352800.

Adi, S. D., N. Eiza, J. Bejar, H. Shefer, S. Toledano, O. Kessler, G. Neufeld, E. Toubi, and Z. Vadasz. 2019. "Semaphorin 3A Is Effective in Reducing Both Inflammation and Angiogenesis in a Mouse Model of Bronchial Asthma." *Front Immunol* 10: 550. https://doi.org/10.3389/fimmu.2019.00550. https://www.ncbi.nlm.nih.gov/pubmed/30967873.

Alessio, N., D. Aprile, S. Cappabianca, G. Peluso, G. Di Bernardo, and U. Galderisi. 2021. "Different Stages of Quiescence, Senescence, and Cell Stress Identified by Molecular Algorithm Based on the Expression of Ki67, RPS6, and Beta-Galactosidase Activity." *Int J Mol Sci* 22 (6). https://doi.org/10.3390/ijms22063102. https://www.ncbi.nlm.nih.gov/pubmed/33803589.

Allegra, M., A. Zaragkoulias, E. Vorgia, M. Ioannou, G. Litos, H. Beug, and G. Mavrothalassitis. 2012. "Semaphorin-7a reverses the ERF-induced inhibition of EMT in Ras-dependent mouse mammary epithelial cells." *Mol Biol Cell* 23 (19): 3873-81. https://doi.org/10.1091/mbc.E12-04-0276. https://www.ncbi.nlm.nih.gov/pubmed/22875994.

Alto, L. T., and J. R. Terman. 2017. "Semaphorins and their Signaling Mechanisms." *Methods Mol Biol* 1493: 1-25. https://doi.org/10.1007/978-1-4939-6448-2_1. https://www.ncbi.nlm.nih.gov/pubmed/27787839.

Alza, L., M. Nàger, A. Visa, C. Cantí, and J. Herreros. 2020. "FAK Inhibition Induces Glioblastoma Cell Senescence-Like State through p62 and p27." *Cancers (Basel)* 12 (5). https://doi.org/10.3390/cancers12051086. https://www.ncbi.nlm.nih.gov/pubmed/32349327.

Battistini, C., and L. Tamagnone. 2016. "Transmembrane semaphorins, forward and reverse signaling: have a look both ways." *Cell Mol Life Sci* 73 (8): 1609-22. https://doi.org/10.1007/s00018-016-2137-x. https://www.ncbi.nlm.nih.gov/pubmed/26794845.

Bhatt, K. V., L. S. Spofford, G. Aram, M. McMullen, K. Pumiglia, and A. E. Aplin. 2005. "Adhesion control of cyclin D1 and p27Kip1 levels is deregulated in melanoma cells through BRAF-MEK-ERK signaling." *Oncogene* 24 (21): 3459-71. https://doi.org/10.1038/sj.onc.1208544. https://www.ncbi.nlm.nih.gov/pubmed/15735667.

Blagosklonny, M. V. 2012. "Cell cycle arrest is not yet senescence, which is not just cell cycle arrest: terminology for TOR-driven aging." *Aging (Albany NY)* 4 (3): 159-65.

https://doi.org/10.18632/aging.100443. https://www.ncbi.nlm.nih.gov/pubmed/22394614.

Boczek, N. J., D. Ye, E. K. Johnson, W. Wang, L. Crotti, D. J. Tester, F. Dagradi, Y. Mizusawa, M. Torchio, M. Alders, J. R. Giudicessi, A. A. Wilde, P. J. Schwartz, J. M. Nerbonne, and M. J. Ackerman. 2014. "Characterization of SEMA3A-encoded semaphorin as a naturally occurring Kv4.3 protein inhibitor and its contribution to Brugada syndrome." *Circ Res* 115 (4): 460-9. https://doi.org/10.1161/CIRCRESAHA.115.303657. https://www.ncbi.nlm.nih.gov/pubmed/24963029.

Borchardt, H., A. Schulz, K. Datta, M. H. Muders, and A. Aigner. 2019. "Silencing of Neuropilins and GIPC1 in pancreatic ductal adenocarcinoma exerts multiple cellular and molecular antitumor effects." *Sci Rep* 9 (1): 15471. https://doi.org/10.1038/s41598-019-51881-8. https://www.ncbi.nlm.nih.gov/pubmed/31664117.

Bryant, P., Q. Zheng, and K. Pumiglia. 2006. "Focal adhesion kinase controls cellular levels of p27/Kip1 and p21/Cip1 through Skp2-dependent and -independent mechanisms." *Mol Cell Biol* 26 (11): 4201-13. https://doi.org/10.1128/MCB.01612-05. https://www.ncbi.nlm.nih.gov/pubmed/16705171.

Burgaya, F., X. Fontana, A. Martínez, M. Montolio, A. Mingorance, S. Simó, J. A. del Río, and E. Soriano. 2006. "Semaphorin 6C leads to GSK-3-dependent growth cone collapse and redistributes after entorhino-hippocampal axotomy." *Mol Cell Neurosci* 33 (3): 321-34. https://doi.org/10.1016/j.mcn.2006.08.008. https://www.ncbi.nlm.nih.gov/pubmed/17029982.

Burkhardt, C., M. Müller, A. Badde, C. C. Garner, E. D. Gundelfinger, and A. W. Püschel. 2005. "Semaphorin 4B interacts with the post-synaptic density protein PSD-95/SAP90 and is recruited to synapses through a C-terminal PDZ-binding motif." *FEBS Lett* 579 (17): 3821-8. https://doi.org/10.1016/j.febslet.2005.05.079. https://www.ncbi.nlm.nih.gov/pubmed/15978582.

Cagnoni, G., and L. Tamagnone. 2014. "Semaphorin receptors meet receptor tyrosine kinases on the way of tumor progression." *Oncogene* 33 (40): 4795-802. https://doi.org/10.1038/onc.2013.474. https://www.ncbi.nlm.nih.gov/pubmed/24213581.

Cai, H., and R. R. Reed. 1999. "Cloning and characterization of neuropilin-1-interacting protein: a PSD-95/Dlg/ZO-1 domain-containing protein that interacts with the cytoplasmic domain of neuropilin-1." *J Neurosci* 19 (15): 6519-27. https://www.ncbi.nlm.nih.gov/pubmed/10414980.

Capparuccia, L., and L. Tamagnone. 2009. "Semaphorin signaling in cancer cells and in cells of the tumor microenvironment--two sides of a coin." *J Cell Sci* 122 (Pt 11): 1723-36. https://doi.org/10.1242/jcs.030197. https://www.ncbi.nlm.nih.gov/pubmed/19461072.

Carulli, D., F. de Winter, and J. Verhaagen. 2021. "Semaphorins in Adult Nervous System Plasticity and Disease." *Front Synaptic Neurosci* 13: 672891. https://doi.org/10.3389/fnsyn.2021.672891. https://www.ncbi.nlm.nih.gov/pubmed/34045951.

Casazza, A., V. Finisguerra, L. Capparuccia, A. Camperi, J. M. Swiercz, S. Rizzolio, C. Rolny, C. Christensen, A. Bertotti, I. Sarotto, M. Risio, L. Trusolino, J. Weitz, M. Schneider, M. Mazzone, P. M. Comoglio, and L. Tamagnone. 2010. "Sema3E-Plexin D1 signaling drives human cancer cell invasiveness and metastatic spreading in mice." *J Clin Invest* 120 (8): 2684-98. https://doi.org/10.1172/JCI42118. https://www.ncbi.nlm.nih.gov/pubmed/20664171.

Casazza, A., B. Kigel, F. Maione, L. Capparuccia, O. Kessler, E. Giraudo, M. Mazzone, G. Neufeld, and L. Tamagnone. 2012. "Tumour growth inhibition and anti-metastatic activity of a mutated furin-resistant Semaphorin 3E isoform." *EMBO Mol Med* 4 (3): 234-50. https://doi.org/10.1002/emmm.201100205. https://www.ncbi.nlm.nih.gov/pubmed/22247010.

Chambard, J. C., R. Lefloch, J. Pouysségur, and P. Lenormand. 2007. "ERK implication in cell cycle regulation." *Biochim Biophys Acta* 1773 (8): 1299-310. https://doi.org/10.1016/j.bbamcr.2006.11.010. https://www.ncbi.nlm.nih.gov/pubmed/17188374.

Chen, L. H., C. Y. Liao, L. C. Lai, M. H. Tsai, and E. Y. Chuang. 2019. "Semaphorin 6A Attenuates the Migration Capability of Lung Cancer Cells via the NRF2/HMOX1 Axis." *Sci Rep* 9 (1): 13302. https://doi.org/10.1038/s41598-019-49874-8. https://www.ncbi.nlm.nih.gov/pubmed/31527696.

Cheung, P. F., C. K. Wong, W. K. Ip, and C. W. Lam. 2008. "FAK-mediated activation of ERK for eosinophil migration: a novel mechanism for infection-induced allergic inflammation." *Int Immunol* 20 (3): 353-63. https://doi.org/10.1093/intimm/dxm146. https://www.ncbi.nlm.nih.gov/pubmed/18182379.

Choi, Y. I., J. S. Duke-Cohan, W. B. Ahmed, M. A. Handley, F. Mann, J. A. Epstein, L. K. Clayton, and E. L. Reinherz. 2008. "PlexinD1 glycoprotein controls migration of positively selected thymocytes into the medulla." *Immunity* 29 (6): 888-98. https://doi.org/10.1016/j.immuni.2008.10.008. https://www.ncbi.nlm.nih.gov/pubmed/19027330.

Chuang, H. H., P. H. Wang, S. W. Niu, Y. Y. Zhen, M. S. Huang, M. Hsiao, and C. J. Yang. 2019. "Inhibition of FAK Signaling Elicits Lamin A/C-Associated Nuclear Deformity and Cellular Senescence." *Front Oncol* 9: 22. https://doi.org/10.3389/fonc.2019.00022. https://www.ncbi.nlm.nih.gov/pubmed/30761269.

Conrotto, P., D. Valdembri, S. Corso, G. Serini, L. Tamagnone, P. M. Comoglio, F. Bussolino, and S. Giordano. 2005. "Sema4D induces angiogenesis through Met recruitment by Plexin

B1." *Blood* 105 (11): 4321-9. https://doi.org/10.1182/blood-2004-07-2885. https://www.ncbi.nlm.nih.gov/pubmed/15632204.

Dacquin, R., C. Domenget, A. Kumanogoh, H. Kikutani, P. Jurdic, and I. Machuca-Gayet. 2011. "Control of bone resorption by semaphorin 4D is dependent on ovarian function." *PLoS One* 6 (10): e26627. https://doi.org/10.1371/journal.pone.0026627. https://www.ncbi.nlm.nih.gov/pubmed/22046317.

Delmas, C., S. Manenti, A. Boudjelal, C. Peyssonnaux, A. Eychène, and J. M. Darbon. 2001. "The p42/p44 mitogen-activated protein kinase activation triggers p27Kip1 degradation independently of CDK2/cyclin E in NIH 3T3 cells." *J Biol Chem* 276 (37): 34958-65. https://doi.org/10.1074/jbc.M101714200. https://www.ncbi.nlm.nih.gov/pubmed/11418594.

Delorme, G., F. Saltel, E. Bonnelye, P. Jurdic, and I. Machuca-Gayet. 2005. "Expression and function of semaphorin 7A in bone cells." *Biol Cell* 97 (7): 589-97. https://doi.org/10.1042/BC20040103. https://www.ncbi.nlm.nih.gov/pubmed/15859945.

Dimri, G. P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E. E. Medrano, M. Linskens, I. Rubelj, and O. Pereira-Smith. 1995. "A biomarker that identifies senescent human cells in culture and in aging skin in vivo." *Proc Natl Acad Sci U S A* 92 (20): 9363-7. https://doi.org/10.1073/pnas.92.20.9363. https://www.ncbi.nlm.nih.gov/pubmed/7568133.

Ding, Q., J. R. Grammer, M. A. Nelson, J. L. Guan, J. E. Stewart, and C. L. Gladson. 2005. "p27Kip1 and cyclin D1 are necessary for focal adhesion kinase regulation of cell cycle progression in glioblastoma cells propagated in vitro and in vivo in the scid mouse brain." *J Biol Chem* 280 (8): 6802-15. https://doi.org/10.1074/jbc.M409180200. https://www.ncbi.nlm.nih.gov/pubmed/15557280.

Dong, Z., X. Liang, X. Wu, X. Kang, Y. Guo, S. Shen, J. Liang, and W. Guo. 2019. "Promoter hypermethylation-mediated downregulation of tumor suppressor gene SEMA3B and lncRNA SEMA3B-AS1 correlates with progression and prognosis of esophageal squamous cell carcinoma." *Clin Exp Metastasis* 36 (3): 225-241. https://doi.org/10.1007/s10585-019-09964-3. https://www.ncbi.nlm.nih.gov/pubmed/30915595.

Eckhardt, F., O. Behar, E. Calautti, K. Yonezawa, I. Nishimoto, and M. C. Fishman. 1997. "A novel transmembrane semaphorin can bind c-src." *Mol Cell Neurosci* 9 (5-6): 409-19. https://doi.org/10.1006/mcne.1997.0644. https://www.ncbi.nlm.nih.gov/pubmed/9361278.

Eickholt, B. J., F. S. Walsh, and P. Doherty. 2002. "An inactive pool of GSK-3 at the leading edge of growth cones is implicated in Semaphorin 3A signaling." *J Cell Biol* 157 (2): 211-7. https://doi.org/10.1083/jcb.200201098. https://www.ncbi.nlm.nih.gov/pubmed/11956225.

Evans, E. E., A. S. Jonason, H. Bussler, S. Torno, J. Veeraraghavan, C. Reilly, M. A. Doherty, J. Seils, L. A. Winter, C. Mallow, R. Kirk, A. Howell, S. Giralico, M. Scrivens, K. Klimatcheva, T. L. Fisher, W. J. Bowers, M. Paris, E. S. Smith, and M. Zauderer. 2015. "Antibody Blockade of Semaphorin 4D Promotes Immune Infiltration into Tumor and Enhances Response to Other Immunomodulatory Therapies." *Cancer Immunol Res* 3 (6): 689-701. https://doi.org/10.1158/2326-6066.CIR-14-0171. https://www.ncbi.nlm.nih.gov/pubmed/25614511.

Fard, D., and L. Tamagnone. 2021. "Semaphorins in health and disease." *Cytokine Growth Factor Rev* 57: 55-63. https://doi.org/10.1016/j.cytogfr.2020.05.006. https://www.ncbi.nlm.nih.gov/pubmed/32900601.

Feiner, L., A. L. Webber, C. B. Brown, M. M. Lu, L. Jia, P. Feinstein, P. Mombaerts, J. A. Epstein, and J. A. Raper. 2001. "Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption." *Development* 128 (16): 3061-70. https://www.ncbi.nlm.nih.gov/pubmed/11688556.

Foster, J. S., R. I. Fernando, N. Ishida, K. I. Nakayama, and J. Wimalasena. 2003. "Estrogens down-regulate p27Kip1 in breast cancer cells through Skp2 and through nuclear export mediated by the ERK pathway." *J Biol Chem* 278 (42): 41355-66. https://doi.org/10.1074/jbc.M302830200. https://www.ncbi.nlm.nih.gov/pubmed/12904306.

Fridlyanskaya, I., L. Alekseenko, and N. Nikolsky. 2015. "Senescence as a general cellular response to stress: A mini-review." *Exp Gerontol* 72: 124-8. https://doi.org/10.1016/j.exger.2015.09.021. https://www.ncbi.nlm.nih.gov/pubmed/26435346.

Fu, P., P. V. Usatyuk, A. Lele, A. Harijith, C. C. Gregorio, J. G. Garcia, R. Salgia, and V. Natarajan. 2015. "c-Abl mediated tyrosine phosphorylation of paxillin regulates LPS-induced endothelial dysfunction and lung injury." *Am J Physiol Lung Cell Mol Physiol* 308 (10): L1025-38. https://doi.org/10.1152/ajplung.00306.2014. https://www.ncbi.nlm.nih.gov/pubmed/25795725.

Fujimaki, K., R. Li, H. Chen, K. Della Croce, H. H. Zhang, J. Xing, F. Bai, and G. Yao. 2019. "Graded regulation of cellular quiescence depth between proliferation and senescence by a lysosomal dimmer switch." *Proc Natl Acad Sci U S A* 116 (45): 22624-22634. https://doi.org/10.1073/pnas.1915905116. https://www.ncbi.nlm.nih.gov/pubmed/31636214.

Gan, B., Y. Yoo, and J. L. Guan. 2006. "Association of focal adhesion kinase with tuberous sclerosis complex 2 in the regulation of s6 kinase activation and cell growth." *J Biol Chem* 281 (49): 37321-9. https://doi.org/10.1074/jbc.M605241200. https://www.ncbi.nlm.nih.gov/pubmed/17043358.

Gao, Y., M. Li, W. Chen, and M. Simons. 2000. "Synectin, syndecan-4 cytoplasmic domain binding PDZ protein, inhibits cell migration." *J Cell Physiol* 184 (3): 373-9. https://doi.org/10.1002/1097-4652(200009)184:3<373::AID-JCP12>3.0.CO;2-I. https://www.ncbi.nlm.nih.gov/pubmed/10911369.

Garcia-Areas, R., S. Libreros, S. Amat, P. Keating, R. Carrio, P. Robinson, C. Blieden, and V. Iragavarapu-Charyulu. 2014. "Semaphorin7A promotes tumor growth and exerts a pro-angiogenic effect in macrophages of mammary tumor-bearing mice." *Front Physiol* 5: 17. https://doi.org/10.3389/fphys.2014.00017. https://www.ncbi.nlm.nih.gov/pubmed/24550834.

Giordano, S., S. Corso, P. Conrotto, S. Artigiani, G. Gilestro, D. Barberis, L. Tamagnone, and P. M. Comoglio. 2002. "The semaphorin 4D receptor controls invasive growth by coupling with Met." *Nat Cell Biol* 4 (9): 720-4. https://doi.org/10.1038/ncb843. https://www.ncbi.nlm.nih.gov/pubmed/12198496.

Godenschwege, T. A., H. Hu, X. Shan-Crofts, C. S. Goodman, and R. K. Murphey. 2002. "Bi-directional signaling by Semaphorin 1a during central synapse formation in Drosophila." *Nat Neurosci* 5 (12): 1294-301. https://doi.org/10.1038/nn976. https://www.ncbi.nlm.nih.gov/pubmed/12436113.

Goldmuntz, E., and B. S. Emanuel. 1997. "Genetic disorders of cardiac morphogenesis. The DiGeorge and velocardiofacial syndromes." *Circ Res* 80 (4): 437-43. https://doi.org/10.1161/01.res.80.4.437. https://www.ncbi.nlm.nih.gov/pubmed/9118473.

Good, P. F., D. Alapat, A. Hsu, C. Chu, D. Perl, X. Wen, D. E. Burstein, and D. S. Kohtz. 2004. "A role for semaphorin 3A signaling in the degeneration of hippocampal neurons during Alzheimer's disease." *J Neurochem* 91 (3): 716-36. https://doi.org/10.1111/j.1471-4159.2004.02766.x. https://www.ncbi.nlm.nih.gov/pubmed/15485501.

Gotoh, A., K. Miyazawa, K. Ohyashiki, T. Tauchi, H. S. Boswell, H. E. Broxmeyer, and K. Toyama. 1995. "Tyrosine phosphorylation and activation of focal adhesion kinase (p125FAK) by BCR-ABL oncoprotein." *Exp Hematol* 23 (11): 1153-9. https://www.ncbi.nlm.nih.gov/pubmed/7556524.

Gras, C., B. Eiz-Vesper, Y. Jaimes, S. Immenschuh, R. Jacobs, T. Witte, R. Blasczyk, and C. Figueiredo. 2014. "Secreted semaphorin 5A activates immune effector cells and is a biomarker for rheumatoid arthritis." *Arthritis Rheumatol* 66 (6): 1461-71. https://doi.org/10.1002/art.38425. https://www.ncbi.nlm.nih.gov/pubmed/24585544.

Gurrapu, S., G. Franzolin, D. Fard, M. Accardo, E. Medico, I. Sarotto, A. Sapino, C. Isella, and L. Tamagnone. 2019. "Reverse signaling by semaphorin 4C elicits SMAD1/5- and ID1/3-dependent invasive reprogramming in cancer cells." *Sci Signal* 12 (595). https://doi.org/10.1126/scisignal.aav2041. https://www.ncbi.nlm.nih.gov/pubmed/31431542.

Gurrapu, S., E. Pupo, G. Franzolin, L. Lanzetti, and L. Tamagnone. 2018. "Sema4C/PlexinB2 signaling controls breast cancer cell growth, hormonal dependence and tumorigenic potential." *Cell Death Differ* 25 (7): 1259-1275. https://doi.org/10.1038/s41418-018-0097-4. https://www.ncbi.nlm.nih.gov/pubmed/29555978.

Gurzu, S., D. Ciortea, I. Ember, and I. Jung. 2013. "The possible role of Mena protein and its splicing-derived variants in embryogenesis, carcinogenesis, and tumor invasion: a systematic review of the literature." *Biomed Res Int* 2013: 365192. https://doi.org/10.1155/2013/365192. https://www.ncbi.nlm.nih.gov/pubmed/23956979.

Hayashi, M., T. Nakashima, M. Taniguchi, T. Kodama, A. Kumanogoh, and H. Takayanagi. 2012. "Osteoprotection by semaphorin 3A." *Nature* 485 (7396): 69-74. https://doi.org/10.1038/nature11000. https://www.ncbi.nlm.nih.gov/pubmed/22522930.

Herranz, N., and J. Gil. 2018. "Mechanisms and functions of cellular senescence." *J Clin Invest* 128 (4): 1238-1246. https://doi.org/10.1172/JCI95148. https://www.ncbi.nlm.nih.gov/pubmed/29608137.

Hota, P. K., and M. Buck. 2012. "Plexin structures are coming: opportunities for multilevel investigations of semaphorin guidance receptors, their cell signaling mechanisms, and functions." *Cell Mol Life Sci* 69 (22): 3765-805. https://doi.org/10.1007/s00018-012-1019-0. https://www.ncbi.nlm.nih.gov/pubmed/22744749.

Hu, J. K., W. Du, S. J. Shelton, M. C. Oldham, C. M. DiPersio, and O. D. Klein. 2017. "An FAK-YAP-mTOR Signaling Axis Regulates Stem Cell-Based Tissue Renewal in Mice." *Cell Stem Cell* 21 (1): 91-106.e6. https://doi.org/10.1016/j.stem.2017.03.023. https://www.ncbi.nlm.nih.gov/pubmed/28457749.

Huang, Y., E. O. Comiskey, R. S. Dupree, S. Li, A. J. Koleske, and J. K. Burkhardt. 2008. "The c-Abl tyrosine kinase regulates actin remodeling at the immune synapse." *Blood* 112 (1): 111-9. https://doi.org/10.1182/blood-2007-10-118232. https://www.ncbi.nlm.nih.gov/pubmed/18305217.

Ieda, M., H. Kanazawa, K. Kimura, F. Hattori, Y. Ieda, M. Taniguchi, J. K. Lee, K. Matsumura, Y. Tomita, S. Miyoshi, K. Shimoda, S. Makino, M. Sano, I. Kodama, S. Ogawa, and K. Fukuda. 2007. "Sema3a maintains normal heart rhythm through sympathetic innervation patterning." *Nat Med* 13 (5): 604-12. https://doi.org/10.1038/nm1570. https://www.ncbi.nlm.nih.gov/pubmed/17417650.

Jiang, C., A. Javed, L. Kaiser, M. M. Nava, R. Xu, D. T. Brandt, D. Zhao, B. Mayer, J. Fernández-Baldovinos, L. Zhou, C. Höß, K. Sawmynaden, A. Oleksy, D. Matthews, L. S. Weinstein, H. Hahn, H. J. Gröne, P. L. Graumann, C. M. Niessen, S. Offermanns, S. A. Wickström, and T. Worzfeld. 2021. "Mechanochemical control of epidermal stem cell divisions by B-

plexins." *Nat Commun* 12 (1): 1308. https://doi.org/10.1038/s41467-021-21513-9. https://www.ncbi.nlm.nih.gov/pubmed/33637728.

Jin, L., Y. Chen, D. Cheng, Z. He, X. Shi, B. Du, X. Xi, Y. Gao, and Y. Guo. 2021. "YAP inhibits autophagy and promotes progression of colorectal cancer via upregulating Bcl-2 expression." *Cell Death Dis* 12 (5): 457. https://doi.org/10.1038/s41419-021-03722-8. https://www.ncbi.nlm.nih.gov/pubmed/33963173.

Junqueira Alves, C., R. Dariolli, J. Haydak, S. Kang, T. Hannah, R. J. Wiener, S. DeFronzo, R. Tejero, G. L. Gusella, A. Ramakrishnan, R. Alves Dias, A. Wojcinski, S. Kesari, L. Shen, E. A. Sobie, J. P. Rodrigues Furtado de Mendonça, E. U. Azeloglu, H. Zou, and R. H. Friedel. 2021. "Plexin-B2 orchestrates collective stem cell dynamics via actomyosin contractility, cytoskeletal tension and adhesion." *Nat Commun* 12 (1): 6019. https://doi.org/10.1038/s41467-021-26296-7. https://www.ncbi.nlm.nih.gov/pubmed/34650052.

Kang, S., and A. Kumanogoh. 2013. "Semaphorins in bone development, homeostasis, and disease." *Semin Cell Dev Biol* 24 (3): 163-71. https://doi.org/10.1016/j.semcdb.2012.09.008. https://www.ncbi.nlm.nih.gov/pubmed/23022498.

Kang, S., Y. Nakanishi, Y. Kioi, D. Okuzaki, T. Kimura, H. Takamatsu, S. Koyama, S. Nojima, M. Nishide, Y. Hayama, Y. Kinehara, Y. Kato, T. Nakatani, T. Shimogori, J. Takagi, T. Toyofuku, and A. Kumanogoh. 2018. "Semaphorin 6D reverse signaling controls macrophage lipid metabolism and anti-inflammatory polarization." *Nat Immunol* 19 (6): 561-570. https://doi.org/10.1038/s41590-018-0108-0. https://www.ncbi.nlm.nih.gov/pubmed/29777213.

Kerkhoff, E., and U. R. Rapp. 1997. "Induction of cell proliferation in quiescent NIH 3T3 cells by oncogenic c-Raf-1." *Mol Cell Biol* 17 (5): 2576-86. https://doi.org/10.1128/MCB.17.5.2576. https://www.ncbi.nlm.nih.gov/pubmed/9111327.

Kikuchi, K., A. Chédotal, H. Hanafusa, Y. Ujimasa, F. de Castro, C. S. Goodman, and T. Kimura. 1999. "Cloning and characterization of a novel class VI semaphorin, semaphorin Y." *Mol Cell Neurosci* 13 (1): 9-23. https://doi.org/10.1006/mcne.1998.0732. https://www.ncbi.nlm.nih.gov/pubmed/10049528.

Kim, J., W. J. Oh, N. Gaiano, Y. Yoshida, and C. Gu. 2011. "Semaphorin 3E-Plexin-D1 signaling regulates VEGF function in developmental angiogenesis via a feedback mechanism." *Genes Dev* 25 (13): 1399-411. https://doi.org/10.1101/gad.2042011. https://www.ncbi.nlm.nih.gov/pubmed/21724832.

Klionsky, et al., and C. K. Tong. 2021. "Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition)." *Autophagy* 17 (1): 1-382.

https://doi.org/10.1080/15548627.2020.1797280. https://www.ncbi.nlm.nih.gov/pubmed/33634751.

Klostermann, A., B. Lutz, F. Gertler, and C. Behl. 2000. "The orthologous human and murine semaphorin 6A-1 proteins (SEMA6A-1/Sema6A-1) bind to the enabled/vasodilator-stimulated phosphoprotein-like protein (EVL) via a novel carboxyl-terminal zyxin-like domain." *J Biol Chem* 275 (50): 39647-53. https://doi.org/10.1074/jbc.M006316200. https://www.ncbi.nlm.nih.gov/pubmed/10993894.

Koh, J. M., B. Oh, J. Y. Lee, J. K. Lee, K. Kimm, G. S. Kim, B. L. Park, H. S. Cheong, H. D. Shin, J. M. Hong, T. H. Kim, E. K. Park, and S. Y. Kim. 2006. "Association study of semaphorin 7a (sema7a) polymorphisms with bone mineral density and fracture risk in postmenopausal Korean women." *J Hum Genet* 51 (2): 112-7. https://doi.org/10.1007/s10038-005-0331-z. https://www.ncbi.nlm.nih.gov/pubmed/16372136.

Koropouli, E., and A. L. Kolodkin. 2014. "Semaphorins and the dynamic regulation of synapse assembly, refinement, and function." *Curr Opin Neurobiol* 27: 1-7. https://doi.org/10.1016/j.conb.2014.02.005. https://www.ncbi.nlm.nih.gov/pubmed/24598309.

Kortylewski, M., P. C. Heinrich, M. E. Kauffmann, M. Böhm, A. MacKiewicz, and I. Behrmann. 2001. "Mitogen-activated protein kinases control p27/Kip1 expression and growth of human melanoma cells." *Biochem J* 357 (Pt 1): 297-303. https://doi.org/10.1042/0264-6021:3570297. https://www.ncbi.nlm.nih.gov/pubmed/11415463.

Kumanogoh, A., S. Marukawa, K. Suzuki, N. Takegahara, C. Watanabe, E. Ch'ng, I. Ishida, H. Fujimura, S. Sakoda, K. Yoshida, and H. Kikutani. 2002. "Class IV semaphorin Sema4A enhances T-cell activation and interacts with Tim-2." *Nature* 419 (6907): 629-33. https://doi.org/10.1038/nature01037. https://www.ncbi.nlm.nih.gov/pubmed/12374982.

Kumanogoh, A., T. Shikina, K. Suzuki, S. Uematsu, K. Yukawa, S. Kashiwamura, H. Tsutsui, M. Yamamoto, H. Takamatsu, E. P. Ko-Mitamura, N. Takegahara, S. Marukawa, I. Ishida, H. Morishita, D. V. Prasad, M. Tamura, M. Mizui, T. Toyofuku, S. Akira, K. Takeda, M. Okabe, and H. Kikutani. 2005. "Nonredundant roles of Sema4A in the immune system: defective T cell priming and Th1/Th2 regulation in Sema4A-deficient mice." *Immunity* 22 (3): 305-16. https://doi.org/10.1016/j.immuni.2005.01.014. https://www.ncbi.nlm.nih.gov/pubmed/15780988.

Kumanogoh, A., T. Shikina, C. Watanabe, N. Takegahara, K. Suzuki, M. Yamamoto, H. Takamatsu, D. V. Prasad, M. Mizui, T. Toyofuku, M. Tamura, D. Watanabe, J. R. Parnes, and H. Kikutani. 2005. "Requirement for CD100-CD72 interactions in fine-tuning of B-cell antigen receptor signaling and homeostatic maintenance of the B-cell compartment." *Int Immunol* 17 (10): 1277-82. https://doi.org/10.1093/intimm/dxh307. https://www.ncbi.nlm.nih.gov/pubmed/16113236.

Kumanogoh, A., K. Suzuki, E. Ch'ng, C. Watanabe, S. Marukawa, N. Takegahara, I. Ishida, T. Sato, S. Habu, K. Yoshida, W. Shi, and H. Kikutani. 2002. "Requirement for the lymphocyte semaphorin, CD100, in the induction of antigen-specific T cells and the maturation of dendritic cells." *J Immunol* 169 (3): 1175-81. https://doi.org/10.4049/jimmunol.169.3.1175. https://www.ncbi.nlm.nih.gov/pubmed/12133937.

Kumanogoh, A., C. Watanabe, I. Lee, X. Wang, W. Shi, H. Araki, H. Hirata, K. Iwahori, J. Uchida, T. Yasui, M. Matsumoto, K. Yoshida, H. Yakura, C. Pan, J. R. Parnes, and H. Kikutani. 2000. "Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling." *Immunity* 13 (5): 621-31. https://doi.org/10.1016/s1074-7613(00)00062-5. https://www.ncbi.nlm.nih.gov/pubmed/11114375.

Lachowski, D., E. Cortes, B. Robinson, A. Rice, K. Rombouts, and A. E. Del Río Hernández. 2018. "FAK controls the mechanical activation of YAP, a transcriptional regulator required for durotaxis." *FASEB J* 32 (2): 1099-1107. https://doi.org/10.1096/fj.201700721R. https://www.ncbi.nlm.nih.gov/pubmed/29070586.

Lai, H., X. Zhao, Y. Qin, Y. Ding, R. Chen, G. Li, M. Labrie, Z. Ding, J. Zhou, J. Hu, D. Ma, Y. Fang, and Q. Gao. 2018. "FAK-ERK activation in cell/matrix adhesion induced by the loss of apolipoprotein E stimulates the malignant progression of ovarian cancer." *J Exp Clin Cancer Res* 37 (1): 32. https://doi.org/10.1186/s13046-018-0696-4. https://www.ncbi.nlm.nih.gov/pubmed/29458390.

Li, Z., R. Jagadapillai, E. Gozal, and G. Barnes. 2019. "Deletion of Semaphorin 3F in Interneurons Is Associated with Decreased GABAergic Neurons, Autism-like Behavior, and Increased Oxidative Stress Cascades." *Mol Neurobiol* 56 (8): 5520-5538. https://doi.org/10.1007/s12035-018-1450-9. https://www.ncbi.nlm.nih.gov/pubmed/30635860.

Loria, R., G. Bon, V. Perotti, E. Gallo, I. Bersani, P. Baldassari, M. Porru, C. Leonetti, S. Di Carlo, P. Visca, M. F. Brizzi, A. Anichini, R. Mortarini, and R. Falcioni. 2015. "Sema6A and Mical1 control cell growth and survival of BRAFV600E human melanoma cells." *Oncotarget* 6 (5): 2779-93. https://doi.org/10.18632/oncotarget.2995. https://www.ncbi.nlm.nih.gov/pubmed/25576923.

Luchino, J., M. Hocine, M. C. Amoureux, B. Gibert, A. Bernet, A. Royet, I. Treilleux, P. Lécine, J. P. Borg, P. Mehlen, S. Chauvet, and F. Mann. 2013. "Semaphorin 3E suppresses tumor cell death triggered by the plexin D1 dependence receptor in metastatic breast cancers." *Cancer Cell* 24 (5): 673-85. https://doi.org/10.1016/j.ccr.2013.09.010. https://www.ncbi.nlm.nih.gov/pubmed/24139859.

Ma, B., E. L. Herzog, C. G. Lee, X. Peng, C. M. Lee, X. Chen, S. Rockwell, J. S. Koo, H. Kluger, R. S. Herbst, M. Sznol, and J. A. Elias. 2015. "Role of chitinase 3-like-1 and semaphorin 7a in

pulmonary melanoma metastasis." *Cancer Res* 75 (3): 487-96.
https://doi.org/10.1158/0008-5472.CAN-13-3339.
https://www.ncbi.nlm.nih.gov/pubmed/25511377.

Ma, H., J. Wang, X. Zhao, T. Wu, Z. Huang, D. Chen, Y. Liu, and G. Ouyang. 2020. "Periostin Promotes Colorectal Tumorigenesis through Integrin-FAK-Src Pathway-Mediated YAP/TAZ Activation." *Cell Rep* 30 (3): 793-806.e6.
https://doi.org/10.1016/j.celrep.2019.12.075.
https://www.ncbi.nlm.nih.gov/pubmed/31968254.

Movassagh, H., L. Shan, J. Chakir, J. F. McConville, A. J. Halayko, L. Koussih, and A. S. Gounni. 2017. "Expression of semaphorin 3E is suppressed in severe asthma." *J Allergy Clin Immunol* 140 (4): 1176-1179. https://doi.org/10.1016/j.jaci.2017.04.031.
https://www.ncbi.nlm.nih.gov/pubmed/28506853.

Movassagh, H., L. Shan, A. Mohammed, A. J. Halayko, and A. S. Gounni. 2017. "Semaphorin 3E Deficiency Exacerbates Airway Inflammation, Hyperresponsiveness, and Remodeling in a Mouse Model of Allergic Asthma." *J Immunol* 198 (5): 1805-1814.
https://doi.org/10.4049/jimmunol.1601514.
https://www.ncbi.nlm.nih.gov/pubmed/28108561.

Mumblat, Y., O. Kessler, N. Ilan, and G. Neufeld. 2015. "Full-Length Semaphorin-3C Is an Inhibitor of Tumor Lymphangiogenesis and Metastasis." *Cancer Res* 75 (11): 2177-86.
https://doi.org/10.1158/0008-5472.CAN-14-2464.
https://www.ncbi.nlm.nih.gov/pubmed/25808871.

Murphy, J. M., Y. A. R. Rodriguez, K. Jeong, E. E. Ahn, and S. S. Lim. 2020. "Targeting focal adhesion kinase in cancer cells and the tumor microenvironment." *Exp Mol Med* 52 (6): 877-886. https://doi.org/10.1038/s12276-020-0447-4.
https://www.ncbi.nlm.nih.gov/pubmed/32514188.

Nakagawa, Y., H. Takamatsu, T. Okuno, S. Kang, S. Nojima, T. Kimura, T. R. Kataoka, M. Ikawa, T. Toyofuku, I. Katayama, and A. Kumanogoh. 2011. "Identification of semaphorin 4B as a negative regulator of basophil-mediated immune responses." *J Immunol* 186 (5): 2881-8.
https://doi.org/10.4049/jimmunol.1003485.
https://www.ncbi.nlm.nih.gov/pubmed/21270411.

Nakano, Y., K. Chayama, H. Ochi, M. Toshishige, Y. Hayashida, D. Miki, C. N. Hayes, H. Suzuki, T. Tokuyama, N. Oda, K. Suenari, Y. Uchimura-Makita, K. Kajihara, A. Sairaku, C. Motoda, M. Fujiwara, Y. Watanabe, Y. Yoshida, K. Ohkubo, I. Watanabe, A. Nogami, K. Hasegawa, H. Watanabe, N. Endo, T. Aiba, W. Shimizu, S. Ohno, M. Horie, K. Arihiro, S. Tashiro, N. Makita, and Y. Kihara. 2013. "A nonsynonymous polymorphism in semaphorin 3A as a risk factor for human unexplained cardiac arrest with documented ventricular fibrillation." *PLoS Genet* 9 (4): e1003364. https://doi.org/10.1371/journal.pgen.1003364.
https://www.ncbi.nlm.nih.gov/pubmed/23593010.

Nasarre, P., S. Kusy, B. Constantin, V. Castellani, H. A. Drabkin, D. Bagnard, and J. Roche. 2005. "Semaphorin SEMA3F has a repulsing activity on breast cancer cells and inhibits E-cadherin-mediated cell adhesion." *Neoplasia* 7 (2): 180-9. https://doi.org/10.1593/neo.04481. https://www.ncbi.nlm.nih.gov/pubmed/15802023.
Negishi-Koga, T., M. Shinohara, N. Komatsu, H. Bito, T. Kodama, R. H. Friedel, and H. Takayanagi. 2011. "Suppression of bone formation by osteoclastic expression of semaphorin 4D." *Nat Med* 17 (11): 1473-80. https://doi.org/10.1038/nm.2489. https://www.ncbi.nlm.nih.gov/pubmed/22019888.
Neufeld, G., and O. Kessler. 2008. "The semaphorins: versatile regulators of tumour progression and tumour angiogenesis." *Nat Rev Cancer* 8 (8): 632-45. https://doi.org/10.1038/nrc2404. https://www.ncbi.nlm.nih.gov/pubmed/18580951.
Neufeld, G., Y. Mumblat, T. Smolkin, S. Toledano, I. Nir-Zvi, K. Ziv, and O. Kessler. 2016. "The role of the semaphorins in cancer." *Cell Adh Migr* 10 (6): 652-674. https://doi.org/10.1080/19336918.2016.1197478. https://www.ncbi.nlm.nih.gov/pubmed/27533782.
Nguyen, H., V. S. Ivanova, L. Kavandi, G. C. Rodriguez, G. L. Maxwell, and V. Syed. 2011. "Progesterone and 1,25-dihydroxyvitamin $D_3$ inhibit endometrial cancer cell growth by upregulating semaphorin 3B and semaphorin 3F." *Mol Cancer Res* 9 (11): 1479-92. https://doi.org/10.1158/1541-7786.MCR-11-0213. https://www.ncbi.nlm.nih.gov/pubmed/21933904.
Nishide, M., S. Nojima, D. Ito, H. Takamatsu, S. Koyama, S. Kang, T. Kimura, K. Morimoto, T. Hosokawa, Y. Hayama, Y. Kinehara, Y. Kato, T. Nakatani, Y. Nakanishi, T. Tsuda, J. H. Park, T. Hirano, Y. Shima, M. Narazaki, E. Morii, and A. Kumanogoh. 2017. "Semaphorin 4D inhibits neutrophil activation and is involved in the pathogenesis of neutrophil-mediated autoimmune vasculitis." *Ann Rheum Dis* 76 (8): 1440-1448. https://doi.org/10.1136/annrheumdis-2016-210706. https://www.ncbi.nlm.nih.gov/pubmed/28416516.
Nkyimbeng-Takwi, E. H., K. Shanks, E. Smith, A. Iyer, M. M. Lipsky, L. J. Detolla, H. Kikutani, A. D. Keegan, and S. P. Chapoval. 2012. "Neuroimmune semaphorin 4A downregulates the severity of allergic response." *Mucosal Immunol* 5 (4): 409-19. https://doi.org/10.1038/mi.2012.18. https://www.ncbi.nlm.nih.gov/pubmed/22472774.
O'Connor, B. P., S. Y. Eun, Z. Ye, A. L. Zozulya, J. D. Lich, C. B. Moore, H. A. Iocca, K. E. Roney, E. K. Holl, Q. P. Wu, H. W. van Deventer, Z. Fabry, and J. P. Ting. 2008. "Semaphorin 6D regulates the late phase of CD4+ T cell primary immune responses." *Proc Natl Acad Sci U S A* 105 (35): 13015-20. https://doi.org/10.1073/pnas.0803386105. https://www.ncbi.nlm.nih.gov/pubmed/18728195.
Ohgushi, M., M. Minaguchi, and Y. Sasai. 2015. "Rho-Signaling-Directed YAP/TAZ Activity Underlies the Long-Term Survival and Expansion of Human Embryonic Stem Cells." *Cell*

*Stem Cell* 17 (4): 448-61. https://doi.org/10.1016/j.stem.2015.07.009. https://www.ncbi.nlm.nih.gov/pubmed/26321201.

Oinuma, I., Y. Ishikawa, H. Katoh, and M. Negishi. 2004. "The Semaphorin 4D receptor Plexin-B1 is a GTPase activating protein for R-Ras." *Science* 305 (5685): 862-5. https://doi.org/10.1126/science.1097545. https://www.ncbi.nlm.nih.gov/pubmed/15297673.

Okuno, T., Y. Nakatsuji, and A. Kumanogoh. 2011. "The role of immune semaphorins in multiple sclerosis." *FEBS Lett* 585 (23): 3829-35. https://doi.org/10.1016/j.febslet.2011.03.033. https://www.ncbi.nlm.nih.gov/pubmed/21420960.

Pan, G., X. Zhang, J. Ren, J. Lu, W. Li, H. Fu, S. Zhang, and J. Li. 2013. "Semaphorin 5A, an axon guidance molecule, enhances the invasion and metastasis of human gastric cancer through activation of MMP9." *Pathol Oncol Res* 19 (1): 11-8. https://doi.org/10.1007/s12253-012-9550-8. https://www.ncbi.nlm.nih.gov/pubmed/22821546.

Pasterkamp, R. J., and R. J. Giger. 2009. "Semaphorin function in neural plasticity and disease." *Curr Opin Neurobiol* 19 (3): 263-74. https://doi.org/10.1016/j.conb.2009.06.001. https://www.ncbi.nlm.nih.gov/pubmed/19541473.

Paul, R., M. Luo, X. Mo, J. Lu, S. K. Yeo, and J. L. Guan. 2020. "FAK activates AKT-mTOR signaling to promote the growth and progression of MMTV-Wnt1-driven basal-like mammary tumors." *Breast Cancer Res* 22 (1): 59. https://doi.org/10.1186/s13058-020-01298-3. https://www.ncbi.nlm.nih.gov/pubmed/32493400.

Peacock, J. W., A. Takeuchi, N. Hayashi, L. Liu, K. J. Tam, N. Al Nakouzi, N. Khazamipour, T. Tombe, T. Dejima, K. C. Lee, M. Shiota, D. Thaper, W. C. Lee, D. H. Hui, H. Kuruma, L. Ivanova, P. Yenki, I. Z. Jiao, S. Khosravi, A. L. Mui, L. Fazli, A. Zoubeidi, M. Daugaard, M. E. Gleave, and C. J. Ong. 2018. "SEMA3C drives cancer growth by transactivating multiple receptor tyrosine kinases via Plexin B1." *EMBO Mol Med* 10 (2): 219-238. https://doi.org/10.15252/emmm.201707689. https://www.ncbi.nlm.nih.gov/pubmed/29348142.

Perez-Branguli, F., Y. Zagar, D. K. Shanley, I. A. Graef, A. Chédotal, and K. J. Mitchell. 2016. "Reverse Signaling by Semaphorin-6A Regulates Cellular Aggregation and Neuronal Morphology." *PLoS One* 11 (7): e0158686. https://doi.org/10.1371/journal.pone.0158686. https://www.ncbi.nlm.nih.gov/pubmed/27392094.

Pham, D. C., Y. C. Chang, S. R. Lin, Y. M. Fuh, M. J. Tsai, and C. F. Weng. 2018. "FAK and S6K1 Inhibitor, Neferine, Dually Induces Autophagy and Apoptosis in Human Neuroblastoma Cells." *Molecules* 23 (12). https://doi.org/10.3390/molecules23123110. https://www.ncbi.nlm.nih.gov/pubmed/30486505.

Piaton, G., M. S. Aigrot, A. Williams, S. Moyon, V. Tepavcevic, I. Moutkine, J. Gras, K. S. Matho, A. Schmitt, H. Soellner, A. B. Huber, P. Ravassard, and C. Lubetzki. 2011. "Class 3 semaphorins influence oligodendrocyte precursor recruitment and remyelination in adult central nervous system." *Brain* 134 (Pt 4): 1156-67. https://doi.org/10.1093/brain/awr022. https://www.ncbi.nlm.nih.gov/pubmed/21421691.

Plattner, R., L. Kadlec, K. A. DeMali, A. Kazlauskas, and A. M. Pendergast. 1999. "c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF." *Genes Dev* 13 (18): 2400-11. https://doi.org/10.1101/gad.13.18.2400. https://www.ncbi.nlm.nih.gov/pubmed/10500097.

Plattner, R., A. J. Koleske, A. Kazlauskas, and A. M. Pendergast. 2004. "Bidirectional signaling links the Abelson kinases to the platelet-derived growth factor receptor." *Mol Cell Biol* 24 (6): 2573-83. https://doi.org/10.1128/MCB.24.6.2573-2583.2004. https://www.ncbi.nlm.nih.gov/pubmed/14993293.

Qu, X., H. Wei, Y. Zhai, H. Que, Q. Chen, F. Tang, Y. Wu, G. Xing, Y. Zhu, S. Liu, M. Fan, and F. He. 2002. "Identification, characterization, and functional study of the two novel human members of the semaphorin gene family." *J Biol Chem* 277 (38): 35574-85. https://doi.org/10.1074/jbc.M206451200. https://www.ncbi.nlm.nih.gov/pubmed/12110693.

Rivard, N., M. J. Boucher, C. Asselin, and G. L'Allemain. 1999. "MAP kinase cascade is required for p27 downregulation and S phase entry in fibroblasts and epithelial cells." *Am J Physiol* 277 (4): C652-64. https://doi.org/10.1152/ajpcell.1999.277.4.C652. https://www.ncbi.nlm.nih.gov/pubmed/10516095.

Rizzolio, S., N. Rabinowicz, E. Rainero, L. Lanzetti, G. Serini, J. Norman, G. Neufeld, and L. Tamagnone. 2012. "Neuropilin-1-dependent regulation of EGF-receptor signaling." *Cancer Res* 72 (22): 5801-11. https://doi.org/10.1158/0008-5472.CAN-12-0995. https://www.ncbi.nlm.nih.gov/pubmed/22986738.

Roux, P. P., D. Shahbazian, H. Vu, M. K. Holz, M. S. Cohen, J. Taunton, N. Sonenberg, and J. Blenis. 2007. "RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation." *J Biol Chem* 282 (19): 14056-64. https://doi.org/10.1074/jbc.M700906200. https://www.ncbi.nlm.nih.gov/pubmed/17360704.

Sadanandam, A., S. S. Sidhu, S. Wullschleger, S. Singh, M. L. Varney, C. S. Yang, A. E. Ashour, S. K. Batra, and R. K. Singh. 2012. "Secreted semaphorin 5A suppressed pancreatic tumour burden but increased metastasis and endothelial cell proliferation." *Br J Cancer* 107 (3): 501-7. https://doi.org/10.1038/bjc.2012.298. https://www.ncbi.nlm.nih.gov/pubmed/22782341.

Sahay, A., C. H. Kim, J. P. Sepkuty, E. Cho, R. L. Huganir, D. D. Ginty, and A. L. Kolodkin. 2005. "Secreted semaphorins modulate synaptic transmission in the adult hippocampus." *J Neurosci* 25 (14): 3613-20. https://doi.org/10.1523/JNEUROSCI.5255-04.2005. https://www.ncbi.nlm.nih.gov/pubmed/15814792.

Salgia, R., B. Brunkhorst, E. Pisick, J. L. Li, S. H. Lo, L. B. Chen, and J. D. Griffin. 1995. "Increased tyrosine phosphorylation of focal adhesion proteins in myeloid cell lines expressing p210BCR/ABL." *Oncogene* 11 (6): 1149-55. https://www.ncbi.nlm.nih.gov/pubmed/7566975.

Sandilands, E., B. Serrels, D. G. McEwan, J. P. Morton, J. P. Macagno, K. McLeod, C. Stevens, V. G. Brunton, W. Y. Langdon, M. Vidal, O. J. Sansom, I. Dikic, S. Wilkinson, and M. C. Frame. 2011. "Autophagic targeting of Src promotes cancer cell survival following reduced FAK signalling." *Nat Cell Biol* 14 (1): 51-60. https://doi.org/10.1038/ncb2386. https://www.ncbi.nlm.nih.gov/pubmed/22138575.

Sandireddy, R., D. M. Cibi, P. Gupta, A. Singh, N. Tee, A. Uemura, J. A. Epstein, and M. K. Singh. 2019. "Semaphorin 3E/PlexinD1 signaling is required for cardiac ventricular compaction." *JCI Insight* 4 (16). https://doi.org/10.1172/jci.insight.125908. https://www.ncbi.nlm.nih.gov/pubmed/31434798.

Sato, M., H. J. Tsai, and H. J. Yost. 2006. "Semaphorin3D regulates invasion of cardiac neural crest cells into the primary heart field." *Dev Biol* 298 (1): 12-21. https://doi.org/10.1016/j.ydbio.2006.05.033. https://www.ncbi.nlm.nih.gov/pubmed/16860789.

Sawai, H., Y. Okada, H. Funahashi, Y. Matsuo, H. Takahashi, H. Takeyama, and T. Manabe. 2005. "Activation of focal adhesion kinase enhances the adhesion and invasion of pancreatic cancer cells via extracellular signal-regulated kinase-1/2 signaling pathway activation." *Mol Cancer* 4: 37. https://doi.org/10.1186/1476-4598-4-37. https://www.ncbi.nlm.nih.gov/pubmed/16209712.

Sawicka, K., A. Pyronneau, M. Chao, M. V. Bennett, and R. S. Zukin. 2016. "Elevated ERK/p90 ribosomal S6 kinase activity underlies audiogenic seizure susceptibility in fragile X mice." *Proc Natl Acad Sci U S A* 113 (41): E6290-E6297. https://doi.org/10.1073/pnas.1610812113. https://www.ncbi.nlm.nih.gov/pubmed/27663742.

Saxena, S., Y. Hayashi, L. Wu, M. Awaji, P. Atri, M. L. Varney, A. Purohit, S. Rachagani, S. K. Batra, and R. K. Singh. 2018. "Pathological and functional significance of Semaphorin-5A in pancreatic cancer progression and metastasis." *Oncotarget* 9 (5): 5931-5943. https://doi.org/10.18632/oncotarget.23644. https://www.ncbi.nlm.nih.gov/pubmed/29464045.

Seyfried, T. N., and L. C. Huysentruyt. 2013. "On the origin of cancer metastasis." *Crit Rev Oncog* 18 (1-2): 43-73. https://doi.org/10.1615/critrevoncog.v18.i1-2.40. https://www.ncbi.nlm.nih.gov/pubmed/23237552.

Shen, C. Y., Y. C. Chang, L. H. Chen, W. C. Lin, Y. H. Lee, S. T. Yeh, H. K. Chen, W. Fang, C. P. Hsu, J. M. Lee, T. P. Lu, P. W. Hsiao, L. C. Lai, M. H. Tsai, and E. Y. Chuang. 2018. "The extracellular SEMA domain attenuates intracellular apoptotic signaling of semaphorin 6A in lung cancer cells." *Oncogenesis* 7 (12): 95. https://doi.org/10.1038/s41389-018-0105-z. https://www.ncbi.nlm.nih.gov/pubmed/30518871.

Sheng, M., and C. Sala. 2001. "PDZ domains and the organization of supramolecular complexes." *Annu Rev Neurosci* 24: 1-29. https://doi.org/10.1146/annurev.neuro.24.1.1. https://www.ncbi.nlm.nih.gov/pubmed/11283303.

Song, X., H. Xu, P. Wang, J. Wang, S. Affo, H. Wang, M. Xu, B. Liang, L. Che, W. Qiu, R. F. Schwabe, T. T. Chang, M. Vogl, G. M. Pes, S. Ribback, M. Evert, X. Chen, and D. F. Calvisi. 2021. "Focal adhesion kinase (FAK) promotes cholangiocarcinoma development and progression via YAP activation." *J Hepatol* 75 (4): 888-899. https://doi.org/10.1016/j.jhep.2021.05.018. https://www.ncbi.nlm.nih.gov/pubmed/34052254.

Suzuki, K., T. Okuno, M. Yamamoto, R. J. Pasterkamp, N. Takegahara, H. Takamatsu, T. Kitao, J. Takagi, P. D. Rennert, A. L. Kolodkin, A. Kumanogoh, and H. Kikutani. 2007. "Semaphorin 7A initiates T-cell-mediated inflammatory responses through alpha1beta1 integrin." *Nature* 446 (7136): 680-4. https://doi.org/10.1038/nature05652. https://www.ncbi.nlm.nih.gov/pubmed/17377534.

Svensson, A., R. Libelius, and S. Tågerud. 2008. "Semaphorin 6C expression in innervated and denervated skeletal muscle." *J Mol Histol* 39 (1): 5-13. https://doi.org/10.1007/s10735-007-9113-6. https://www.ncbi.nlm.nih.gov/pubmed/17605078.

Swiercz, J. M., R. Kuner, J. Behrens, and S. Offermanns. 2002. "Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology." *Neuron* 35 (1): 51-63. https://doi.org/10.1016/s0896-6273(02)00750-x. https://www.ncbi.nlm.nih.gov/pubmed/12123608.

Takagi, S., T. Hirata, K. Agata, M. Mochii, G. Eguchi, and H. Fujisawa. 1991. "The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors." *Neuron* 7 (2): 295-307. https://doi.org/10.1016/0896-6273(91)90268-5. https://www.ncbi.nlm.nih.gov/pubmed/1908252.

Takamatsu, H., N. Takegahara, Y. Nakagawa, M. Tomura, M. Taniguchi, R. H. Friedel, H. Rayburn, M. Tessier-Lavigne, Y. Yoshida, T. Okuno, M. Mizui, S. Kang, S. Nojima, T. Tsujimura, Y. Nakatsuji, I. Katayama, T. Toyofuku, H. Kikutani, and A. Kumanogoh. 2010. "Semaphorins guide the entry of dendritic cells into the lymphatics by activating myosin

II." *Nat Immunol* 11 (7): 594-600. https://doi.org/10.1038/ni.1885. https://www.ncbi.nlm.nih.gov/pubmed/20512151.

Tamagnone, L. 2012. "Emerging role of semaphorins as major regulatory signals and potential therapeutic targets in cancer." *Cancer Cell* 22 (2): 145-52. https://doi.org/10.1016/j.ccr.2012.06.031. https://www.ncbi.nlm.nih.gov/pubmed/22897846.

Tamagnone, L., S. Artigiani, H. Chen, Z. He, G. I. Ming, H. Song, A. Chedotal, M. L. Winberg, C. S. Goodman, M. Poo, M. Tessier-Lavigne, and P. M. Comoglio. 1999. "Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates." *Cell* 99 (1): 71-80. https://doi.org/10.1016/s0092-8674(00)80063-x. https://www.ncbi.nlm.nih.gov/pubmed/10520995.

Tamagnone, L., and M. Mazzone. 2011. "Semaphorin signals on the road of endothelial tip cells." *Dev Cell* 21 (2): 189-90. https://doi.org/10.1016/j.devcel.2011.07.017. https://www.ncbi.nlm.nih.gov/pubmed/21839915.

Tomizawa, Y., Y. Sekido, M. Kondo, B. Gao, J. Yokota, J. Roche, H. Drabkin, M. I. Lerman, A. F. Gazdar, and J. D. Minna. 2001. "Inhibition of lung cancer cell growth and induction of apoptosis after reexpression of 3p21.3 candidate tumor suppressor gene SEMA3B." *Proc Natl Acad Sci U S A* 98 (24): 13954-9. https://doi.org/10.1073/pnas.231490898. https://www.ncbi.nlm.nih.gov/pubmed/11717452.

Toyofuku, T., H. Zhang, A. Kumanogoh, N. Takegahara, F. Suto, J. Kamei, K. Aoki, M. Yabuki, M. Hori, H. Fujisawa, and H. Kikutani. 2004. "Dual roles of Sema6D in cardiac morphogenesis through region-specific association of its receptor, Plexin-A1, with off-track and vascular endothelial growth factor receptor type 2." *Genes Dev* 18 (4): 435-47. https://doi.org/10.1101/gad.1167304. https://www.ncbi.nlm.nih.gov/pubmed/14977921.

Toyofuku, T., H. Zhang, A. Kumanogoh, N. Takegahara, M. Yabuki, K. Harada, M. Hori, and H. Kikutani. 2004. "Guidance of myocardial patterning in cardiac development by Sema6D reverse signalling." *Nat Cell Biol* 6 (12): 1204-11. https://doi.org/10.1038/ncb1193. https://www.ncbi.nlm.nih.gov/pubmed/15543137.

Tse, C., R. H. Xiang, T. Bracht, and S. L. Naylor. 2002. "Human Semaphorin 3B (SEMA3B) located at chromosome 3p21.3 suppresses tumor formation in an adenocarcinoma cell line." *Cancer Res* 62 (2): 542-6. https://www.ncbi.nlm.nih.gov/pubmed/11809707.

Ueda, Y., N. Kondo, M. Ozawa, K. Yasuda, T. Tomiyama, and T. Kinashi. 2016. "Sema3e/Plexin D1 Modulates Immunological Synapse and Migration of Thymocytes by Rap1 Inhibition." *J Immunol* 196 (7): 3019-31. https://doi.org/10.4049/jimmunol.1502121. https://www.ncbi.nlm.nih.gov/pubmed/26921307.

Vaitkienė, P., D. Skiriutė, G. Steponaitis, K. Skauminas, A. Tamašauskas, and A. Kazlauskas. 2015. "High level of Sema3C is associated with glioma malignancy." *Diagn Pathol* 10: 58.

https://doi.org/10.1186/s13000-015-0298-9. https://www.ncbi.nlm.nih.gov/pubmed/26032848.

Valdembri, D., D. Regano, F. Maione, E. Giraudo, and G. Serini. 2016. "Class 3 semaphorins in cardiovascular development." *Cell Adh Migr* 10 (6): 641-651. https://doi.org/10.1080/19336918.2016.1212805. https://www.ncbi.nlm.nih.gov/pubmed/27439112.

Villanueva, J., Y. Yung, J. L. Walker, and R. K. Assoian. 2007. "ERK activity and G1 phase progression: identifying dispensable versus essential activities and primary versus secondary targets." *Mol Biol Cell* 18 (4): 1457-63. https://doi.org/10.1091/mbc.e06-10-0908. https://www.ncbi.nlm.nih.gov/pubmed/17314399.

Vivekanadhan, S., and D. Mukhopadhyay. 2019. "Divergent roles of Plexin D1 in cancer." *Biochim Biophys Acta Rev Cancer* 1872 (1): 103-110. https://doi.org/10.1016/j.bbcan.2019.05.004. https://www.ncbi.nlm.nih.gov/pubmed/31152824.

Vogelstein, B., D. Lane, and A. J. Levine. 2000. "Surfing the p53 network." *Nature* 408 (6810): 307-10. https://doi.org/10.1038/35042675. https://www.ncbi.nlm.nih.gov/pubmed/11099028.

Wei, J. C., J. Yang, D. Liu, M. F. Wu, L. Qiao, J. N. Wang, Q. F. Ma, Z. Zeng, S. M. Ye, E. S. Guo, X. F. Jiang, L. Y. You, Y. Chen, L. Zhou, X. Y. Huang, T. Zhu, L. Meng, J. F. Zhou, Z. H. Feng, D. Ma, and Q. L. Gao. 2017. "Tumor-associated Lymphatic Endothelial Cells Promote Lymphatic Metastasis By Highly Expressing and Secreting SEMA4C." *Clin Cancer Res* 23 (1): 214-224. https://doi.org/10.1158/1078-0432.CCR-16-0741. https://www.ncbi.nlm.nih.gov/pubmed/27401250.

Wen, H., Y. Lei, S. Y. Eun, and J. P. Ting. 2010. "Plexin-A4-semaphorin 3A signaling is required for Toll-like receptor- and sepsis-induced cytokine storm." *J Exp Med* 207 (13): 2943-57. https://doi.org/10.1084/jem.20101138. https://www.ncbi.nlm.nih.gov/pubmed/21098092.

Williams, A., G. Piaton, M. S. Aigrot, A. Belhadi, M. Théaudin, F. Petermann, J. L. Thomas, B. Zalc, and C. Lubetzki. 2007. "Semaphorin 3A and 3F: key players in myelin repair in multiple sclerosis?" *Brain* 130 (Pt 10): 2554-65. https://doi.org/10.1093/brain/awm202. https://www.ncbi.nlm.nih.gov/pubmed/17855378.

Witherden, D. A., M. Watanabe, O. Garijo, S. E. Rieder, G. Sarkisyan, S. J. Cronin, P. Verdino, I. A. Wilson, A. Kumanogoh, H. Kikutani, L. Teyton, W. H. Fischer, and W. L. Havran. 2012. "The CD100 receptor interacts with its plexin B2 ligand to regulate epidermal γδ T cell function." *Immunity* 37 (2): 314-25. https://doi.org/10.1016/j.immuni.2012.05.026. https://www.ncbi.nlm.nih.gov/pubmed/22902232.

Worzfeld, T., and S. Offermanns. 2014. "Semaphorins and plexins as therapeutic targets." *Nat Rev Drug Discov* 13 (8): 603-21. https://doi.org/10.1038/nrd4337. https://www.ncbi.nlm.nih.gov/pubmed/25082288.

Worzfeld, T., J. M. Swiercz, M. Looso, B. K. Straub, K. K. Sivaraj, and S. Offermanns. 2012. "ErbB-2 signals through Plexin-B1 to promote breast cancer metastasis." *J Clin Invest* 122 (4): 1296-305. https://doi.org/10.1172/JCI60568. https://www.ncbi.nlm.nih.gov/pubmed/22378040.

Wu, F., Q. Zhou, J. Yang, G. J. Duan, J. J. Ou, R. Zhang, F. Pan, Q. P. Peng, H. Tan, Y. F. Ping, Y. H. Cui, C. Qian, X. C. Yan, and X. W. Bian. 2011. "Endogenous axon guiding chemorepulsant semaphorin-3F inhibits the growth and metastasis of colorectal carcinoma." *Clin Cancer Res* 17 (9): 2702-11. https://doi.org/10.1158/1078-0432.CCR-10-0839. https://www.ncbi.nlm.nih.gov/pubmed/21349996.

Xie, J., and H. Wang. 2017. "Semaphorin 7A as a potential immune regulator and promising therapeutic target in rheumatoid arthritis." *Arthritis Res Ther* 19 (1): 10. https://doi.org/10.1186/s13075-016-1217-5. https://www.ncbi.nlm.nih.gov/pubmed/28109308.

Xue, D., M. Desjardins, G. N. Kaufman, M. Béland, S. Al-Tamemi, E. Ahmed, S. Tao, R. H. Friedel, W. Mourad, and B. D. Mazer. 2016. "Semaphorin 4C: A Novel Component of B-Cell Polarization in Th2-Driven Immune Responses." *Front Immunol* 7: 558. https://doi.org/10.3389/fimmu.2016.00558. https://www.ncbi.nlm.nih.gov/pubmed/28003812.

Yan, W., S. Zhou, W. Shen, J. Cheng, S. Yuan, S. Ye, Y. Jin, A. Luo, and S. Wang. 2019. "Suppression of SEMA6C promotes preantral follicles atresia with decreased cell junctions in mice ovaries." *J Cell Physiol* 234 (4): 4934-4943. https://doi.org/10.1002/jcp.27294. https://www.ncbi.nlm.nih.gov/pubmed/30256425.

Yang, W. J., J. Hu, A. Uemura, F. Tetzlaff, H. G. Augustin, and A. Fischer. 2015. "Semaphorin-3C signals through Neuropilin-1 and PlexinD1 receptors to inhibit pathological angiogenesis." *EMBO Mol Med* 7 (10): 1267-84. https://doi.org/10.15252/emmm.201404922. https://www.ncbi.nlm.nih.gov/pubmed/26194913.

Yoshida, Y., B. Han, M. Mendelsohn, and T. M. Jessell. 2006. "PlexinA1 signaling directs the segregation of proprioceptive sensory axons in the developing spinal cord." *Neuron* 52 (5): 775-88. https://doi.org/10.1016/j.neuron.2006.10.032. https://www.ncbi.nlm.nih.gov/pubmed/17145500.

Zeng, R., and J. Dong. 2021. "The Hippo Signaling Pathway in Drug Resistance in Cancer." *Cancers (Basel)* 13 (2). https://doi.org/10.3390/cancers13020318. https://www.ncbi.nlm.nih.gov/pubmed/33467099.

Zhao, J. H., H. Reiske, and J. L. Guan. 1998. "Regulation of the cell cycle by focal adhesion kinase." *J Cell Biol* 143 (7): 1997-2008. https://doi.org/10.1083/jcb.143.7.1997. https://www.ncbi.nlm.nih.gov/pubmed/9864370.

Zhao, J., H. Tang, H. Zhao, W. Che, L. Zhang, and P. Liang. 2015. "SEMA6A is a prognostic biomarker in glioblastoma." *Tumour Biol* 36 (11): 8333-40. https://doi.org/10.1007/s13277-015-3584-y. https://www.ncbi.nlm.nih.gov/pubmed/26014517.

Zhou, S., W. Yan, W. Shen, J. Cheng, Y. Xi, S. Yuan, F. Fu, T. Ding, A. Luo, and S. Wang. 2018. "Low expression of SEMA6C accelerates the primordial follicle activation in the neonatal mouse ovary." *J Cell Mol Med* 22 (1): 486-496. https://doi.org/10.1111/jcmm.13337. https://www.ncbi.nlm.nih.gov/pubmed/28881413.

**ACKNOWLEDGEMENTS**

This journey would not have been possible without the support of my professor. Therefore, I want to express my heartfelt gratitude and appreciation to my supervisor prof. Luca Tamagnone, for giving me the opportunity to work in his laboratory, for having stood with me through the ups and downs of my project and my life.

I want to sincerely thank Prof. Silvia Giordano for her insightful remarks and effort spent reviewing my thesis.

I would also like to thank our lab senior members: Prof. Bianca Scicchitano, Prof. Maria Teresa Viscomi, Prof. Alessio D'Alessio, and Prof. Silvia Masciarelli, for their precious help and advices.

I want to express my gratitude to all of my colleagues, from Turin to Rome, for their friendship and moral support, as well as all of the pleasant memories they created for me while I was away from home and family.

Many thanks in particular to Sabrina Rizzolio, Sreeharsha Gurrapu, and Melissa Milan, my former colleagues, for sharing with me their working and scientific expertise.

Furthermore, big thanks to my current labmates: Virginia, Erika, Roberta, Claudia.P, Alessandra, Shishir, Claudia.R.

Finally, my sincere thanks go also to my family, because they are the best family I could ever wish for.