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FILOPODIA AND STROMAL EXTRACELLULAR MATRIX AS REGULATORS OF CANCER CELL INVASION AND GROWTH

by

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To my family and friends

Riina Kaukonen
Filopodia and stromal extracellular matrix as regulators of cancer cell invasion and growth

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ABSTRACT

Bidirectional exchange of information between the cancer cells and their environment is essential for cancer to evolve. Cancer cells lose the ability to regulate their growth, gain the ability to detach from neighboring cells and finally some of the cells disseminate from the primary tumor and invade to the adjacent tissue. During cancer progression, cells acquire features that promote cancer motility and proliferation one of them being increased filopodia number. Filopodia are dynamic actin-rich structures extending from the leading edge of migrating cells and the main function of these structures is to serve as environmental sensors. It is nowadays widely appreciated, that not only the cancer cells, but also the surrounding of the tumor – the tumor microenvironment- contribute to cancer cell dissemination and tumor growth. Activated stromal fibroblasts, also known as cancer-associated fibroblasts (CAFs) actively participate on tumor progression. CAFs are the most abundant cell type surrounding the cancer cells and they are the main cell type producing the extracellular matrix (ECM) within tumor stroma. CAFs secrete growth factors to promote tumor growth, direct cancer cell invasion as well as modify the stromal ECM architecture.

The aim of this thesis was to investigate the function of filopodia, particularly the role of filopodia-inducing protein Myosin-X (Myo10), in breast cancer cell invasion and metastasis. We found that Myo10 is an important regulator of basal type breast cancer spreading downstream of mutant p53. In addition, I investigated the role of CAFs and their secreted matrix on tumor growth. According to the results, CAF-derived matrix has altered organization and stiffness which induces the carcinoma cell proliferation via epigenetic mechanisms. I identified histone demethylase enzyme JMJD1a to be regulated by the stiffness and to participate in stiffness induced growth control.

Keywords: Filopodia, invasion, migration, p53, ECM, fibroblast, stroma, JMJD1a, mechanotrasduction

Riina Kaukonen
Filopodiat ja strooman soluväliaine syöpäsolujen leviämisen ja kasvun säätelijöinä

Turun yliopisto, Biolääketieteen laitos, Lääketieteellisen Biokemian ja genetiikan oppiaine, Turun Biotekniikan keskus, VTT Lääkekehityksen biotekniikka, Molekyylilääketieteen tohtoriohjelma ja Turun Biolääketieteellinen tutkijakoulu, Turku, Suomi
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TIIVISTELMÄ

Normaalin kudoksen epiteelisolut ja sidekudos eivät ole suorassa kontaktissa, vaan niitä erottaa soluväliaineesta muodostunut tyvikalvo. Syövän muodostuessa ja sen edetessä syöpäsolut invasoivat tyvikalvon läpi sidekudokseen. Tyvikalvon alainen sidekudos sisältää pääosin sidekudossoluja (fibroblasteja) sekä niiden erittämää soluväliainetta. Soluväliaine on tärkeä solujen normaalin homeostasian säätelijä. Se säätelee solujen kiinnittymistä toisiinsa, solujen jakautumista sekä erilaistumista, eli prosesseja joiden säätely on eteenkin syövässä häiriintynyt.

Syövän ympärillä on havaittu ns. syöpäfibroblasteja, joiden tiedetään erittävän solujen kasvua sekä uudisveristuonten muodostusta lisääviä kasvutekijöitä ja sitä kautta vaikuttavan aktiivisesti syövän etenemiseen. Tutkimukseni tarkoitus oli tutkia muuttuuko sidekudossolujen soluväliaineen rakenne normaalien fibroblastien aktivoituessa syöpäfibroblasteiksi. Havaitsimme, että syöpäfibroblastien tuottama soluväliaineen rakenne oli muuttunut sekä sen jäykkyys lisääntynyt. Nämä muutokset yhdessä johtivat syöpäsolujen lisääntyneeseen kasvuun syöpäfibroblastien tuottamalla soluväliaineella normaaliin soluväliaineeseen verrattuna. Havaitsimme tämän eron johtuvan epigeneettisiä muutoksia säätelevän JMJD1a-entsyymin muuntuneesta säätelystä eri soluväliaineilla.

Syövän edetessä syöpäsolut leviävät lopulta veren- tai imusuonikierron kautta muualle elimistöön ja muodostavat etäpesäkkeitä. Solun sormimaissilla rakenteilla, filopodioilla, on todettu olevan tärkeä rooli etäpesäkkeiden muodostuksessa, sillä filopodioiden määrän on havaittu lisääntyvän syövässä ja vaikuttavan etenkin syöpäsolujen kykyyn liikkua. Myosin- X (Myo10) geenin tiedetään säätelevän filopodioiden muodostusta, mutta sen merkitystä syövän leviämisen säätelyssä ei ole aiemmin tutkittu. Havaitsimme, että Myo10 säätelee rintasyöpäsolujen liikkumista sekä solujen kykyä levitä ja muodostaa etäpesäkkeitä.

Avainsanat: Filopodia, invaasio, migraatio, p53, soluväliaine, fibroblasti, strooma, JMJD1a

TABLE OF CONTENTS

ABBREVIATIONS

2D, 3D	Two dimensional, three dimensional
AFM	Atomic force microscope
AMOT	Angiomotin
Arp2/3	Actin-Related Protein 2/3
β-TrCP	beta-transducin repeat containing E3 ubiquitin protein ligase
CAF	Cancer associated fibroblast
CAM	Chorioallantoic membrane
cAMP	Cyclic adenosine monophosphate
CDM	Cell derived matrix
ChIP	Chromatin immunoprecipitation
CK1	Casein kinase 1
CTGF	Connective tissue growth factor
DCC	Deleted in colorectal cancer
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGR-1	Early growth response protein-1
ERK1/2	Extracellular-signal-regulated kinase 1/2
FA	Focal adhesion
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FERM	4.1 ezrin, radixin, moesin domain
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GPCR	G-protein coupled receptor
H3	Histone 3
H3K9me2	Histone 3 lysine 9 dimethyl
H3K4me3	Histone 3 lysine 9 trimethyl
HCC	Hepatocellular carcinoma
HIF-1α	Hypoxia induced protein 1α
HNSCC	Head and neck Squamous cell carcinoma
IRSp53	Insulin receptor tyrosine kinase p53
JMJD1a	Jumonji Domain-Containing Protein 1A
KDM3a	Lysine (K) -specific demethylase 3a
KO	Knock-out
LATS1/2	Large tumor suppressor 1/2
LINC	Linker of Nucleoskeleton and Cytoskeleton
LOX	Lysyl oxidase
LPA	Lysophosphatidic acid
MAF	Melanoma associated fibroblast

MLC	Myosin light chain
MRCK	Myotonic Dystrophy Protein Kinase-Like-α
mRNA	Messenger RNA
miRNA	microRNA
MMP	Matrix metalloproteinase
Myo10	Myosin-X
MyTH4	Myosin Tail Homology 4
NF	Normal fibroblast
p21	Cyclin-dependent kinase inhibitor p21
Pa	Pascal
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PH	Pleckstrin homology
PHD2	Prolyl Hydroxylase Domain-Containing Protein
PI3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PTEN	Phosphate and tensin homolog
RNA	Ribonucleic acid
RNAi	RNA interference
ROCK	Rho kinase
RT-PCR	Reverse transcription PCR
SEM	Scanning electron microscope
SCC	Squamous cell carcinoma
siRNA	Small interfering RNA
shRNA	Short hairpin RNA
SMA-α	Smooth muscle actin-α
TAZ	Transcriptional Coactivator with PDZ-Binding Motif
TGF-α/β	Transforming growth factor-α/β
THBS1	Thrombospondin 1
TIFF	Telomerase immortalized foreskin fibroblast
TIMP	Tissue inhibitor of metalloproteinase
TMA	Tissue microarray
TP53	Tumor protein p53
uPAR	Urokinase-type plasminogen activator receptor
VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular endothelial growth factor
WASP	Wiskott–Aldrich syndrome protein
WAVE	Wiskott-Aldrich Syndrome Protein Family Member 1
WT	Wild type
YAP	Yes-associated protein
qPCR	Quantitative PCR

LIST OF ORIGINAL PUBLICATIONS

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

I **Riina Kaukonen**, Anja Mai, Maria Georgiadou, Markku Saari, Nicola De Franceschi, Timo Betz, Harri Sihto, Sami Ventelä, Laura Elo, Eija Jokitalo, Jukka Westermarck, Pirkko Kellokumpu-Lehtinen, Heikki Joensuu, Reidar Grenman and Johanna Ivaska. Normal stroma suppresses cancer cell proliferation via JMJD1a-mediated mechanosensitive regulation of YAP/TAZ transcription. *Under revision in Nat. Commun.*

II Antti Arjonen *, **Riina Kaukonen***, Pegah Rouhi, Gunilla Högnäs, Harri Sihto, Brian Miller, Elmar Bucher, Yehai Cao, Owen Sansom, Heikki Joensuu, Johanna Ivaska (2014). Mutant p53-associated myosin-X upregulation promotes breast cancer invasion and metastasis. *Journal of Clinical Investigation* 124(3):1069-82. *Equal contribution

III Antti Arjonen, **Riina Kaukonen**, Johanna Ivaska (2011). Filopodia and adhesion in cancer cell motility. *Cell Adhesion and Migration* 5(5):421-30. doi: 10.4161/cam.5.5.17723. *Systematic Review.*

1. INTRODUCTION

The typical features of cancer cells, the hallmarks of cancer, have been described by Hanahan and Weinberg (Hanahan & Weinberg, 2000). These hallmarks include characteristics that cancer cells typically acquire during tumor evolution such as [1)]uncontrolled regulation of proliferation and [2)]increased motility features, such as invasion and metastasis as well as [3)] anchorage independent growth (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011). The hallmarks are accompanied by genetic instability, which leads to accumulation of new mutations and chromosomal arrangements. In some cases, these chromosomal aberrations can provide a selective advantage for the cancer cells. In addition to the genetic alterations, abnormalities in epigenetic code, such as changes in DNA methylation or histone modifications, occur commonly during cancer development.

Recently, the impact of tumor microenvironment on tumor evolution has been acknowledged. Tumor stroma, also known as the tumor microenvironment, shares many elements with inflammation and fibrosis and thus tumors are sometimes described as a wound that never heals (Rybinski, Franco-Barraza, & Cukierman, 2014). Immune cells, such as macrophages, typically accumulate within tumors and tumors are known to contain excessive amount of extracellular matrix (ECM) which is typically observed in fibrosis. Normal fibroblasts become activated to myofibroblast-type cells, also known as cancer associated fibroblasts (CAFs). CAFs secrete growth factors, which facilitate tumor growth as well as modify and reorganize the stromal extracellular matrix (ECM). CAF induced ECM reorganization is known to increase the stiffness of the tumor stroma as well as enhance cancer cell invasion and therefore increase the capability of cells to metastasize (Acerbi et al., 2015)

Normal adherent cell types have controlled cell cycle and they need to be anchored to other cells and to the ECM to survive and proliferate. Cells are attached to the ECM via their adhesion receptors called integrins. Integrins are transmembrane receptors which localize to and are activated at the sites of adhesion. These sites include stabile focal adhesions as well as more dynamic structures such as in filopodia. Filopodia are finger-like structures extending from the edges of cells. Filopodia are required especially for directional movement and thus they are important structures for cancer cell invasion and migration. The expression of filopodia-related genes is altered in cancer and to be associated with increased burden of metastasis. Indeed their role as metastatic devices is nowadays been well-established.

2. REVIEW OF THE LITERATURE

2.1. Characteristics of cancer cells

One of the most obvious differences between normal and transformed cells is the loss of normal growth control. Normal cells are capable of dividing 40-60 times, while cancer cells can divide endlessly. Tumors are formed when cells acquire the ability to divide without the normal cell cycle control and to evade apoptosis. Cancer cells are capable of stimulating their own proliferation by secreting growth inducing factors such as platelet-derived growth factor (PDGF) and Transforming growth factor-α (TGF-α). This is referred to as "self-sufficiency in growth signals" (Hanahan & Weinberg, 2011). Another hallmark of cancer is the ability to evade apoptosis. Cancer cells exhibit many characteristics, such as activation of cell-cycle checkpoints, which would normally activate apoptosis. Evasion of apoptosis is essential for the tumor development and important also for their therapy resistance.

The dissemination of the cancer cells from the primary tumor and the formation of metastases to distant organs is the leading cause of death among cancer patients. This invasive process, metastasis, is considered to initiate from epithelia to mesenchymal transition (EMT) where the epithelia-type cancer cells lose their normal polarity and acquire characteristics of mesenchymal cells. These characteristics include loss of cell-cell contacts as well as increased invasion and migration capabilities. These features together allow cells to escape from the primary tumor, intravasate into the blood stream or lymphatic vessels and finally metastasize and colonize to distant organs, such as bone or lungs in the case of breast cancer for example. These processes are dependent on the ability of cells to resist anoikis, apoptosis induced by ECM detachment (Frisch & Francis, 1994). Normal homeostasis of cells and tissues is maintained by their anchorage to the ECM and anchorage-independence has been directly linked to cancer progression, especially to cancer spreading and metastasis.

Cancer cells from different origins metastasize preferably to specific organs. Breast cancer cells tend to metastasize to lymph nodes, lungs and bone, while squamous cell carcinoma (SCC) metastasize inefficiently to bone but are highly potent of colonizing lungs (Ferlito, Shaha, Silver, Rinaldo, & Mondin, 2001). This process has been described by Stephen Paget in 1889 and it was named as "Seed and soil"- theory of metastasis (Fidler, 2003). The theory emphasizes the organ-specific colonization of cancer cells named the seeds-during metastasis and underlines the importance of microenvironment, the soil, on metastatic growth of cancer cells. The mechanism of seed and soil theory has been studied especially in the context of squamous cancer cell (SCC) metastasis. It has been shown that TGFβ2 regulates the colonization of

disseminated SCC cells. High TGFβ2 levels in bone tissues inhibits the growth of extravasated SCC cells while in lungs, where the TGFβ2 levels are low, SCC cells are capable to colonize and form metastases (Bragado et al., 2013) indicating that the surrounding of the cancer cells, known as tumor microenvironment, is an important regulator of cancer evolution.

2.2 Tumor microenvironment

Tumor microenvironment is an important regulator of primary tumor growth, since tumors have limited ability to grow without the cooperative effect from their surrounding tissue. In normal homeostasis, epithelial cells are not in direct contact with the underlying connective tissue, but they are separated by the basement membrane. During carcinogenesis, epithelial cells lose their normal polarity, escape from the normal cell cycle control and finally acquire metastatic features and invade into the stroma. Therefore tumors are a mixture of transformed cancer cells and stromal components of the host tissue. Tumors could be considered as malignant organs, rather than clusters of abnormally proliferating cells, where different cell types maintain the homeostasis and synergistically contribute to the function of the complex tissue (Figure 1).

Tumors can grow only to a limited size, approximately to 1 to 2 millimeters without additional supply of the growth factors and oxygen. Therefore the growth of the new blood vessels, angiogenesis, is essential for the efficient growth of the tumor. Cancer cells are known to overexpress and secrete pro-angiogenic factors, which induce the formation of disorganized blood vessels into the tumor microenvironment (Siemann, 2011). As the tumor grows, especially the interior of the tumor becomes hypoxic due to limited blood supply. Paradoxically, cancer cells exploit hypoxia for the growth and metastasis via mechanisms including EMT induction. Thus tumor hypoxia serves as a prognostic factor for advanced disease as well as poor prognosis and reduced disease-free survival (Brizel et al., 1996). This is, at least partially, explained by the fact that hypoxia induces therapy resistance and thus is an interesting target for future therapies.

Tumors are often found to be infiltrated by immune cells mimicking inflammatory conditions seen upon infection. Tumor-associated accumulation of immune cells enhances tumor progression by inducing the amount of cytokines, growth and pro-angiogenic factors, in the tumor microenvironment as well as inhibiting the ability of the host immune-defence to mount an attack on the tumor cells (Hanahan & Weinberg, 2011). Immune cells are also known to secrete ECM-modifying enzymes, which assists in invasion and metastasis of cancer cells via ECM reorganization.

Tumor formation is accompanied with increased plasticity and many of the normal stromal cells which maintain normal homeostasis become activated during tumor formation and are transformed into tumor-promoting cells. Normal macrophages, for instance, are known to evolve into cancer/tumor associated macrophages (CAM/TAM). One of the most abundant cell types in the tumor stoma are fibroblasts. Similarly to macrophages, normal fibroblasts can be activated to become cancer associated fibroblasts (CAFs) which participate actively to cancer progression.

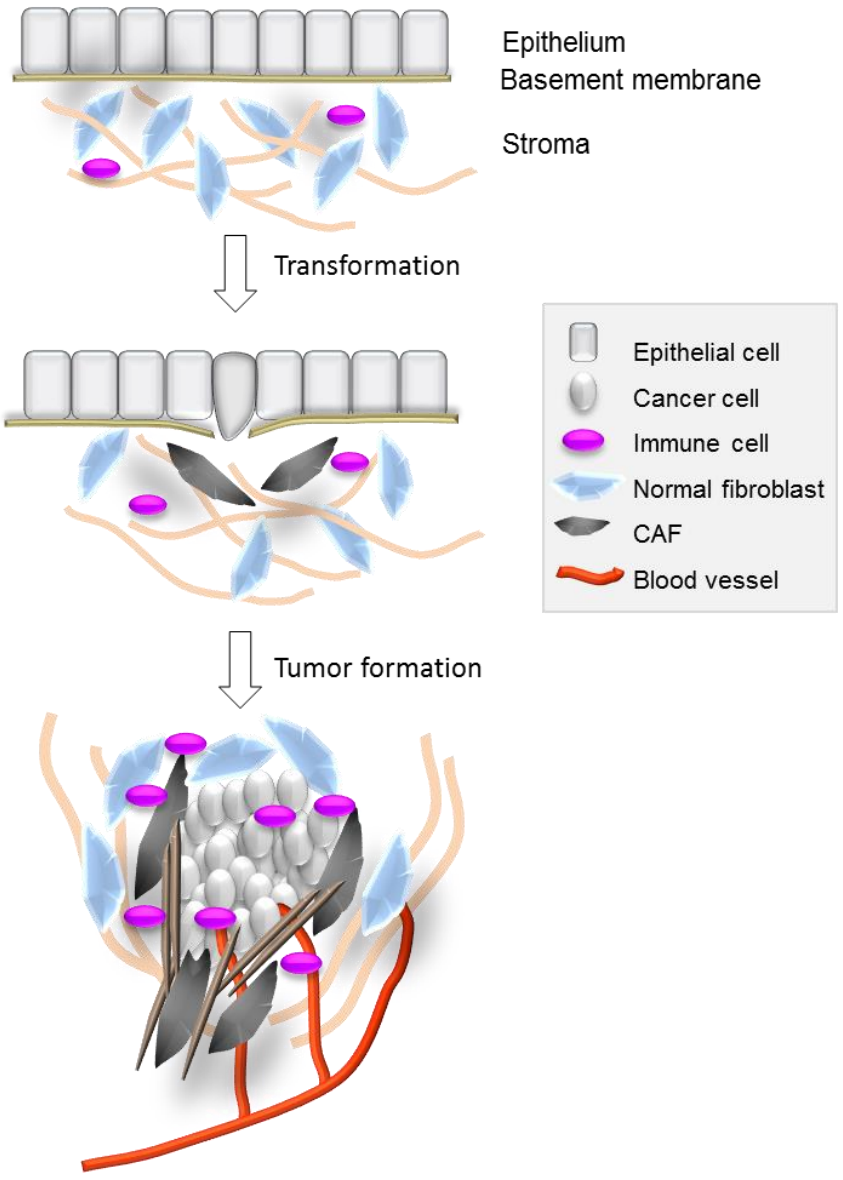

Figure 1. Schematic representation of tumor development. 1) In normal homeostasis, epithelial cells are organized as a monolayer on the basement membrane. Basement membrane separates epithelial cells from the underlying stroma. 2) Transformation of normal epithelial cell into carcinoma cell is accompanied with changes in underlying stroma. 3) Tumor is composed of transformed cancer cells, stromal fibroblast, extracellular matrix and immune cells and blood vessels.

Some cancer types, such as pancreatic cancer, are composed mainly on stromal components. It has been evaluated that approximately 90 % of the tumor is stroma and only the remaining 10 % is composed of actual transformed tumor cells (Von Hoff et al., 2011). Interestingly several studies have shown that genetic alterations in stromal cells are as frequent as in cancer cells (Ellsworth et al., 2004; Fukino et al., 2004; Kurose et al., 2002; Tuhkanen et al., 2004). Mutations in tumor suppressor genes, such as tumor protein 53 (TP53) and phosphate and tensin homolog (PTEN), occur frequently in cancer (Vogelstein, Lane, & Levine, 2000). In the study of Kurose et al., 50 % (25/50) of the tumors had mutations in the TP53 gene. Nine of the tumors (9/25) had the mutation in the stromal compartment and eleven (11/25) in the epithelium. The remaining five tumors contained mutation in both compartments. These studies imply that there is a significant correlation between stromal alterations and tumor progression.

Indeed, the functional role of stroma on tumor growth and metastasis has been studied in various cancer types. It has been shown that stromal gene expression signature has a prognostic role at least in colorectal cancer. Colorectal cancer is categorized into three different subtypes (CSS1-3) based on their ability to resist therapy as well as the level of prognosis. It was recently discovered that the prognostic power of these different subtypes depend more on stromal cells rather than the actual tumor cells (Calon et al., 2015). In the case of breast cancer, stromal collagen alignment is a prognostic factor for human breast carcinoma patients (Conklin et al., 2011) and stromal Type III collagen has shown to suppress tumor microenvironment evolvement by regulating fibroblasts mediated ECM organization and inhibiting tumor growth and invasion (Brisson et al., 2015). In addition to Brisson et al. several other studies have recognized the importance of ECM composition and organization in tumorigenesis (Acerbi et al., 2015; Levental et al., 2009; Provenzano et al., 2006a).

2.3. Extracellular matrix

Extracellular matrix (ECM) is abundant in the normal mesenchyma as well as within tumor stroma. The main function of ECM is to connect cells and tissues, bind growth factors and other signalling proteins and provide physical support (Vlodavsky et al., 1990). ECM is composed over 300 proteins, 200 glycoproteins and 30 proteoglycans (Hynes & Naba, 2012). Cells adhere to the ECM via transmembrane receptors, such as syndecans and most importantly via integrins. Integrins are heterodimeric cell surface receptors, which are composed of α- and β-subunits and these subunits are known to form 24 different integrin heterodimers. Integrin heterodimers bind to their ligands via the extracellular C-terminal end (Harburger & Calderwood, 2009). Cell-ECM interactions support cell proliferation, survival and other essential processes by supplying biomechanical and biochemical signals and by providing physical support for the cells.

The ECM exists roughly in two distinct forms called [1)] basement membrane and [2)] stromal ECM. The basement membrane, which is mostly produced by the epithelial cells, is a sheet-like structure composed mainly of laminins, collagen IV and various proteoglycans. The basement membrane is located on the basal surface of epithelial and endothelial cell layer and its function is to separate epithelial cells from the mesenchyma as well as to regulate the apical-basal polarity of the epithelial cells. Stromal ECM is produced mainly by fibroblasts and it serves as an important regulator of tissue homeostasis (Bhowmick & Moses, 2005). The most abundant proteins in the stroma are collagen I, collagen III and fibronectin (Badylak, Freytes, & Gilbert, 2009).

The mechanical properties of the ECM depend primarily on elastic fibers (e.g. elastic, fibrillin and fibulin), collagens and glycosaminoglycans. There are more than 30 different collagens and collagen-related proteins and the most highly expressed types are collagens I and II. Collagen is the main protein providing the material stiffness. Collagen I and II are capable of forming fibrils spontaneously *in vitro*. However, collagen I-containing ECM fibers do not form in the absence of fibronectin, integrins, and collagen V *in vivo* (Kadler, Hill, & Canty-Laird, 2008). The assembly of collagen triple-helices is dependent on ascorbic acid, since ascorbic acid is an important cofactor for prolyl and lysyl hydroxylases and the proper hydroxylation of collagen is essential for triple-helicase formation and fibrillogenesis (Kypreos, Birk, Trinkaus-Randall, Hartmann, & Sonenshein, 2000).

Matrix metalloproteinases (MMPs) and MMP inhibitors called Tissue inhibitors of metalloproteinases (TIMPs) are main proteins modifying and degrading collagen and collagen related proteins such as gelatins. MMPs include Collagenases (MMP-1, MMP-2, MMP-13) , gelatinases (MMP-2, MMP-9) and the membrane type MMPs MT-1, -2 and -3, which are able to degrade broad

range of different collagens and other ECM proteins such as laminins and fibronectin (Visse & Nagase, 2003).

Fibronectin fibrillogenesis is thought to require mechanical tension and direct binding to integrin receptors (Mao & Schwarzbauer, 2005; Scott, Mair, Narang, Feleke, & Lemmon, 2015). It has been proposed that fibronectin binds to integrin and is stretched by the cells. Pulling on the fibronectin molecules opens otherwise hidden domains and the revealing of these domains induces the assembly of the fibronectin molecules into fibrils and thus tension and mechanical forces are important regulators of fibrillogenesis and formation of ECM in general (Baneyx, Baugh, & Vogel, 2002; Zhong et al., 1998). Since the fibroblasts are the main cell type producing the ECM proteins and thus defining the biochemical composition and the organization of the ECM architecture, the impact of CAFs on cancer development is partially mediated by matrix alterations induced by them.

2.4. Cancer associated fibroblasts (CAFs)

2.4.1. The origin and maintenance of CAFs

Fibroblasts are the main cell type in the mesenchyme.They are known to be incorporated into the growing tumors and, most importantly, have an active role in regulation of tumor growth and cancer cell metastasis. Normal fibroblasts can inhibit the growth of certain cancer cells (Flaberg et al., 2011; Sadlonova et al., 2007), while this ability is reduced from CAFs (Surowiak et al., 2007a). Even though CAFs are known to exist in tumor stroma for over a decade the origin of them is still under active investigation.

Increased plasticity is a general hallmark of cancer (Vicente-Duenas, Gutierrez de Diego, Rodriguez, Jimenez, & Cobaleda, 2009). While epithelial cells transform into mesenchymal-type cells via EMT, normal tissue-resident fibroblasts are considered to get activated into myofibroblasts via process that is sometimes called mesenchymal to mesenchymal transition (MMT) (Werth et al., 2008). It has been proposed that increased secretion of growth factor, such as TGF-β of cancer cells could promote this process. Indeed, normal fibroblasts have been transformed into CAFs *in vitro* by TGF-β induction (Calon, Tauriello, & Batlle, 2014; Calon et al., 2015).

CAFs may also originate from transformed epithelial cells since normal epithelial and endothelial cells have shown to be activated to myofibroblasts via EMT and endothelial to mesenchymal transition (EndMT) respectively during fibrosis and tumor formation (Zeisberg, Potenta, Xie, Zeisberg, & Kalluri, 2007). Mesenchymal stem cells (MSC) which have potential to differentiate into

several different cell types are also an important source of CAFs (Spaeth et al., 2009) (Spaeth et al. 2009). It was found that approximately 20% of CAFs originate from bone marrow MSCs (Quante et al., 2011)

CAF induction is associated with increased CAF-marker gene expression. Several proteins have found to be upregulated when normal fibroblast transform into CAFs. One of the most commonly used markers is increased expression of smooth muscle actin-α (SMA-α) which is normally expressed only by myofibroblasts (Ha, Yeo, Xuan, & Kim, 2014). Other mesenchymal proteins which are upregulated upon CAF activation are fibroblast-specific protein (FSP-1), also known as S100A4 and the fibroblast-activating protein (FAP). The expression pattern of known CAF-marker genes is heterogenous and considered to depend on the origin of CAFs as well as the tissue they are derived from (Sugimoto, Mundel, Kieran, & Kalluri, 2006). The molecular mechanism of how the CAF phenotype, and CAF-specific gene expression, is maintained is not entirely known but it has been proposed to be mediated by epigenetic changes (Hu et al., 2005) as well as dependent on genetic alterations (Kurose et al., 2002).

In addition, CAF-phenotype is known to be maintained by their ability to remodel their surrounding ECM. It has been reported that signalling molecules such as Lysophosphatidic acid (LPA) and TGF-β can induce matrix stiffening and affect the contractility of the actin cytoskeleton of the CAFs (Calvo et al., 2013). Stiff actomyosin network of the fibroblasts leads to stress fiber formation, Src-kinase activation and importantly facilitates the stiffening of the fibroblast derived ECM (Calvo et al., 2013). It has been recently shown that transcription factors YAP and TAZ are important contributors of CAF maintenance as well as their contractile and pro-fibrotic phenotype (Figure 2) (Liu et al. 2015).

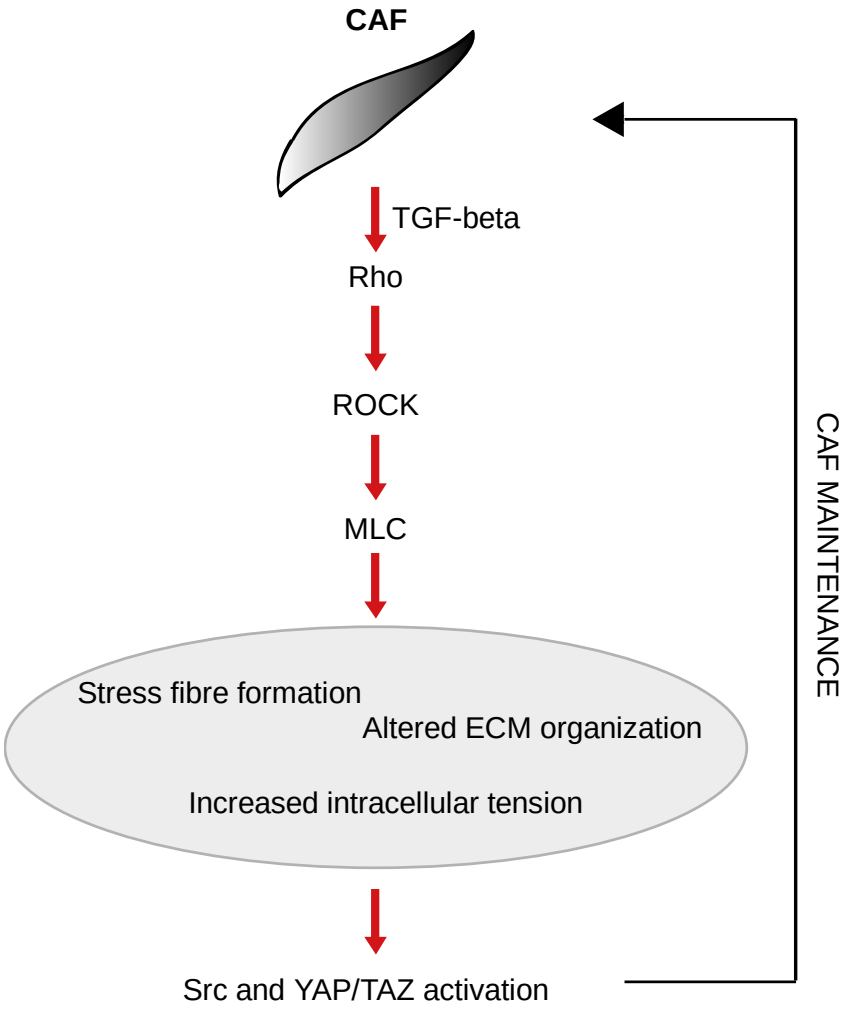

Figure 2. CAF activation and maintenance. Modified from Calvo et al. 2013. The expression and secretion of TGF-β is increased when normal fibroblasts transform to CAFs. Especially TGF-beta has an important function in CAF maintenance, since it has been shown to activate the RhoA-ROCK-MLC signalling pathway. Increased RhoA activity leads to formation of actin stress fibers, increased actomyosin contractility and tension as well as alter ECM organization. All these processes have shown to be important for CAF maintenance.

2.4.2. CAFs as promoters of cancer progression

The presence of CAF within tumor stoma has been connected to increased tumor progression (reviewed in Karagiannis et al., 2012). This is partially dependent of increased secretion of tumor promoting growth factors, cytokines and chemokines (Kuperwasser et al., 2004). These soluble factors can activate CAFs via autocrine signalling as well as stimulate the angiogenesis, growth and other oncogenic functions of cancer cells. It has been shown that CAFs produce excessive amounts of chemokines such as Interleukin 6 (IL-6), C-X-C motif chemokine 12 (CXCL12) and growth factors including vascular endothelial-derived growth factor (VEGF), TGF-β, hepatocyte growth factor (HGF), epidermal growth factor (EGF), or fibroblast growth factor (FGF) (Feig et al., 2013; Jia et al., 2013; McDonald et al., 2015; Nagasaki et al., 2014) all which have been linked to enhancement of tumor growth or metastasis when dysregulated (Erez, Glanz, Raz, Avivi, & Barshack, 2013; Franco et al., 2011; Su, Sung, Beebe, & Friedl, 2012).

CAFs are known to promote cancer invasion by operating as leader cells during invasion and directing cancer cells away from the primary tumor (Gaggioli et al., 2007). Cancer cells follow CAFs via the ECM cues that the fibroblasts make while invading through the 3D matrix. Integrin α3 and α5 induced RhoA GTPase and ROCK activation and MLC phosphorylation was shown to mediate the invasion of CAFs while Cdc42 and Myotonic Dystrophy Protein Kinase-Like-α (MRCK) were activated in the following cancer cells (Gaggioli et al., 2007). It was recently shown that hypoxia inactivates CAF and therefore cancer cell invasion via mechanism, which involves inhibition of Prolyl Hydroxylase Domain-Containing Protein (PHD2). PHD2 inhibition reduced the contractility of CAFs and thus affected their ability to organize the matrix in a way that would favor cancer cell invasion and metastasis. PHD2 inhibition decreased tumor stiffness, CAF activation and, finally, the inhibition of PHD2 in fibroblasts reduced the CAF-induced metastasis to the distal organs when CAF were co-injected with cancer cells (Madsen et al., 2015). In addition to altering primary tumor growth and local invasion, CAFs are found from the blood stream where they are incorporated to cell clusters together with metastatic cancer cells. CAF depletion reduced the incidence of metastasis indicating that CAFs assist cancer cell colonization and growth at the distal metastatic sites (Duda et al., 2010).

In contrast, it has also been shown that CAF depletion in pancreatic ductal adenocarcinoma (PDAC) model promotes tumor growth and invasion (Ozdemir et al., 2014). It was found that CAF deletion enhances hypoxia within tumor stroma and induces the immunosuppression due to reduced peri-tumoral T and B-cell infiltration (Ozdemir et al., 2014) indicating that CAFs may have more versatile role in tumor stroma than previously thought.

2.4.3. CAFs induce desmoplasia and alter tumor stiffness

In addition to the biochemical differences between the normal and tumor stroma, the biophysical changes, such as altered stiffness, has been acknowledged. CAFs remodel the connective tissue, particularly ECM proteins, which lead to generation of forces within the stroma. The remodeling does not only affect their own functions, such as CAF maintenance (Figure 1), but also promotes cancer cell growth and their ability to invade and metastasize (Calvo et al., 2013; Gaggioli et al., 2007; Levental et al., 2009; Stanisavljevic et al., 2015; Yamaguchi et al., 2014).

Activated fibroblasts are known to regulate ECM assembly by releasing abnormal amounts of matrix proteases, such as MMP14, inducing changes in the biochemical and biophysical properties of the ECM (Rosenthal et al 2004, Zhang et al. 2006). ECM re-organization, especially collagen accumulation and desmoplasia, are connected to tumor development. Desmoplasia, the growth of a connective tissue, is typical in fibrosis but is also common in cancer. Different stages of collagen accumulation have been classified into three different subgroups called tumor-associated collagen signatures (TACS). Increased collagen density is an indicator of TACS-1, while TACS-2 is accompanied with stretched collagen fibers around the tumor. TACS-3 is typically found from the regions of cancer cell invasion and is associated with aligned collagen fibres (Provenzano et al., 2006a).

Alterations in the stromal collagen composition and alignment are in fact powerful prognostic markers and stromal alterations correlate with poor prognosis (Levental et al., 2009). As an example, human breast cancer evolution has shown to be accompanied by linearization and bundling of thick collagen fibers. (Acerbi et al., 2015). These fibers are visualized merely at the invasive edge of the tumor and their presence correlated with increased stiffness. Interestingly, the stiffness at the invasive front was found to the highest in most aggressive breast cancer types, known as basal-type and Her2, compared to less aggressive Luminal A and Luminal B type tumor subtypes. Acerbi et al. could also show that ECM organization and stiffness affected other stromal components such as macrophages. Infiltrated tumor macrophages were shown to localize to the sites of increased stiffness where they are known to contribute to tumor progression by secreting growth factors, such as TGF-β. Moreover, it has been shown that crosslinking of the stromal collagen leads to ECM stiffening and, most importantly, increased oncogenic signalling in tumor cells (Levental et al., 2009).

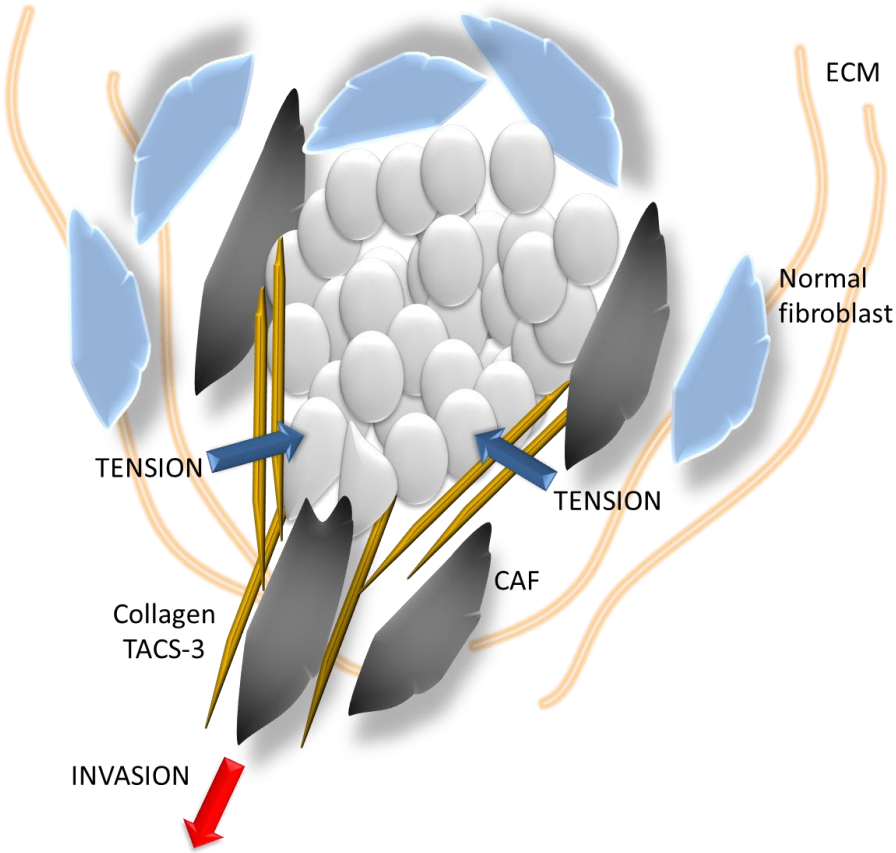

Figure 3. The impact of stroma on tumor development. Tumor progression is accompanied by changes in biophysical and biochemical propertied of the tumor. CAFs increase the tension within tumor stroma by modifying collagen structure and creating tumor-associated collagen signatures 1-3 (TACS 1-3). TACS-3 are aligned thick collagen bundles, which are typically found from the regions of cancer cell invasion.

2.4.5. The clinical relevance of CAFs

The expression of SMA-α in fibroblasts, indicating the presence of CAFs, correlates with poor survival in oesophageal adenocarcinoma as well as increased growth of tumor cells and relapse among breast cancer patients (Shimosato et al., 1980; Surowiak et al., 2007b). In addition, high expression of another CAF marker FAP together with desmoplastic stroma correlates with the reduced survival among NSCLC patients (Liao, Ni, He, Liu, & Du, 2013; Shimosato et al., 1980). Recently CAFs were shown to exhibit a prognostic role also in colorectal cancer (CRC). CRC-patients are classified into three different subtypes based on their predicted prognosis and therapy resistance. It was found that patients, who were considered to belong into “good prognosis”-

group, but relapsed after the therapy, exhibited gene expression which was typical for CAFs. In fact the CAFs increased the frequency of tumor-initiating cells due to increased secretion of TGF-β (Calon et al., 2015). In addition to TGF-β, CAFs are known to promote tumor growth and lymphatic metastasis by secreting growth factors such as osteopontin and stromal cell-derived factor 1 (SDF1) (Orimo et al., 2005; Ota et al., 2014).

Interestingly, accumulation of stroma is directly connected to the survival among breast cancer patients. It has been shown that when stroma covers over half of the tumor volume, the survival is significantly reduced compared to the patients whose tumors are mainly composed of tumor cells (de Kruijf et al., 2011). Indeed, stromal depletion, as a part of cancer therapy, has been studied especially in the context of pancreatic cancer. Certain chemotherapeutics, such as combination of albumin-bound Paclitaxel and Gentamicine, led to stromal depletion, increased vasculature as well as increased Gentamicine amount in the tumor. Importantly, this led to reduced tumor growth and increased survival among pancreatic cancer patients (Von Hoff et al., 2011).

As discussed earlier, tumor development is associated with increased tissue stiffness. Stiff tumor stroma is known to enhance tumor growth and lead to accumulation of tumor-associated macrophages (TAM) and induce tumor-related inflammation (Acerbi et al., 2015). According to recent *in vivo* studies, targeting of CAFs could lead to softening of tumor. Indeed, albumin-bound Paclitaxel treatment reduced the number of CAFs in tumor stoma and led to softening of the tumor (Alvarez et al., 2013) indicating that depletion of stromal components, such as CAFs, may improve cancer patient therapy and survival in the future by creating a tumor-resistant microenvironment (Zhou, Yang, Andl, Wickett, & Zhang, 2015). In controversy, it was recently shown that the presence of CAFs within tumor stroma enhances the immunosurveillance and CAFs depletion inhibited immunosuppression and reduced Gemcitabine respond in PDAC mouse model (Ozdemir et al., 2014).

To conclude, CAFs are known both to promote and suppress tumor formation. However, in vast majority of studies, CAF have been shown to enhance tumor progression via enhanced paracrine signalling and increased desmoplasia and tumor stiffness within tumor stoma and thus they are considered as possible targets for targeted cancer therapy.

2.5. Stiffness and mechanotransduction

2.5.1. Extracellular mechanical forces

The cell-ECM interactions are not only controlled by the molecular composition of the ECM but also affected by its mechanical properties such as stiffness. Cells can sense the properties of the ECM, neighboring cells and tension caused by the mechanical stress by converting the physical forces to a biochemical signals. This process is called mechanotransduction. Depending on the tissue, the normal environment of cells can be soft, such as breast or brain or rigid such as bone or muscle tissue. Cells respond to changes in tension in a cell type dependent manner. Mechanosensitive regulation affects various cellular functions such as proliferation, migration and differentiation, many of which are important for normal homeostasis as well as several pathologies such as fibrosis and cancer (Paszek et al., 2005).

Changes in the tissue stiffness have been linked to for instance to breast cancer pathogenesis. It has been shown that increased breast tissue stiffness is a risk factor for breast cancer formation. Women who have higher mammographic density have 4- to 6-fold greater risk of developing breast cancer (Boyd et al., 2014). Increased mammographic density has also been linked not only to increased cell number but also to increased collagen content in the breast tissue (T. Li et al., 2005). It has been shown that both tumor cells and stroma acquire increased stiffness compared to tissue of normal homeostasis (Paszek et al., 2005). Tissue stiffening has been linked to increased collagen crosslinking by lysyl oxidase (LOX). LOX is secreted by tumor cells upon hypoxic condition and its levels correlate with poor prognosis (Erler et al., 2009). The crosslinking of the collagen, which led to stiffening of the ECM and increased metastatic potential of the breast carcinoma cells (Levental et al., 2009). Recently, pharmacological inhibition LOX was found to inhibit pancreatic ductal adenocarcinoma (PDAC) metastasis and increase the chemotherapy efficacy and thus LOX is considered as good target for future therapies (B. W. Miller et al., 2015).

Sensing of mechanical extracellular forces is mediated by plasma membrane receptors such as [1)]Integrins (Tzima et al., 2005), [2)]mechanosensitive ion channels (Hayakawa, Tatsumi, & Sokabe, 2008) and [3)]G-protein coupled receptors (GPCR) (Meyer et al., 2000). GPCRs have shown to be activated by integrin activation in combination with mechanical stress. GPCR mediated induction of cyclic AMP (cAMP) leads to activation of protein kinase A (PKA) and PKA mediated signaling pathways (Alenghat, Tytell, Thodeti, Derrien, & Ingber, 2009; Meyer et al., 2000). Mechanosensitive ion channels exist in specific cell types such as in neurons. Mechanical stretching of an actin cytoskeleton can induce opening of certain ion channels and induced influx of

Ca2+ (Hayakawa et al., 2008). However, the most established plasma membrane receptors in the context of mechanosensing are integrin adhesion receptors.

2.5.2. Integrins and mechanical forces within focal adhesions

Cells adhere to the extracellular proteins via adhesion complexes such as focal adhesions, fibrillar adhesions and nascent adhesions (Kanchanawong et al., 2010). These different focal complexes are different in their protein composition and stability (Parsons, Horwitz, & Schwartz, 2010). Focal adhesions (FAs) are the most stabile focal complexes and their role in mechanotransduction is well established. The size of the FAs is increased and thus the signaling from the FAs is activated when cells are exposed to higher stiffness (Levental et al., 2009). The functional role of FAs has been mostly studied in 2D environment but there are studies suggesting that FAs could be formed also in 3D environment (Chiu et al., 2014; Cukierman, Pankov, Stevens, & Yamada, 2001; Tolde, Rosel, Janostiak, Vesely, & Brabek, 2012).

FAs are populated by the integrin receptors, which connect the ECM to the cytoskeleton of the cell. The intracellular N-terminus of the integrin is the binding site of various proteins, such as talin, which regulate either the integrin activity or mediate the signals from the extracellular space inside the cells (Figure 4). Integrins exist in different conformations which reflect their activity. Inactive, bent conformations, become extended when integrins are activated. Integrin activation can be induced by ligand binding (outside-in activation) or by binding of activators like talin or kindlin to the cytoplasmic tail of β-integrins (inside-out activation) (Harburger & Calderwood, 2009).

Talin is the main integrin activator and acts also as a mehanosensitive scaffold and actin-linking protein in FAs. It binds to β-integrins via the N-terminal FERM domain and has up to 11 binding sites for vinculin as well as several actin-binding sites (Thompson et al., 2014). Tension regulates the availability of several vinculin-binding sites on talin and this is one of the first steps of converting the mechanical forces into biochemical signals (Austen et al., 2015). Talin-integrin interactions are weak when focal complexes start to form. During the maturation of FAs, talin is stretched and binding sites for other FA proteins, such as vinculin, are exposed (del Rio et al., 2009). Vinculin conformation is also regulated by stretching. Vinculin adopts an autoinhibited closed conformation, which is opened by stretching, resembling the mechanosensitive regulation of talin (Carisey et al., 2013; Pasapera, Schneider, Rericha, Schlaepfer, & Waterman, 2010).

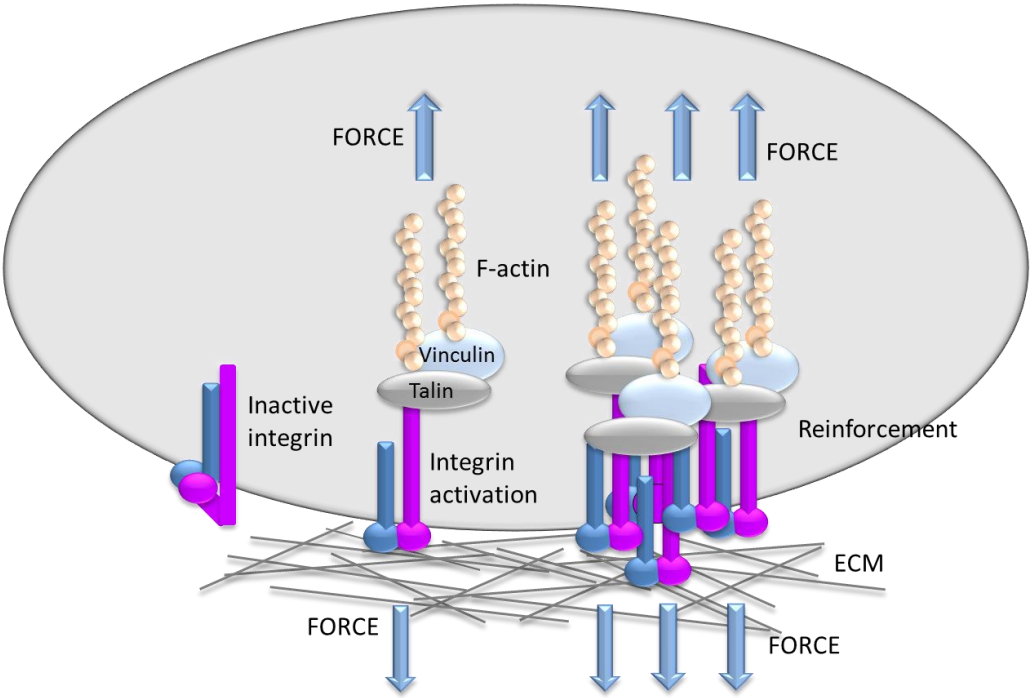

Figure 4. Force generation within focal adhesions. Inactive, bent conformations, become extended when integrins are activated. Integrin activation can be induced by ligand binding (outside-in activation) or by talin or kindlin binding to the cytoplasmic tail of β-integrins (inside-out activation). Integrin activation induces talin-mediated recruitment of vinculin, which induces the strengthening and stabilization of the integrin-ligand connection. This process is called reinforcement.

When force is applied to the integrins, for example by stretching, talin mediated recruitment of vinculin takes place. The integrin-ligand connection is strengthened and gets stabilized and this process is called reinforcement. Interestingly, plasma membrane tension has recently found to activate integrins even in the absence of ligand binding (Ferraris et al., 2014). It was shown that activation of urokinase-type plasminogen activator receptor (uPAR) can indirectly lead to integrin activation via increased plasma membrane tension (Ferraris et al., 2014). Ligand independent activation of integrins was shown to be accompanied by talin and kindlin binding to the cytoplasmic tails of integrins.

When integrins are activated at the focal adhesions, several different signalling and adaptor proteins are recruited to the focal adhesions. Signaling proteins include Focal adhesion kinase (FAK) and proto-oncogene non-receptor tyrosine-protein kinase Src (Src). Both FAK and Src are known to mediate mechanosignals and are activated downstream of integrin signaling. Src binds

to integrins via its SH3-domain and the binding leads to Src activation. It was recently shown that mechanobiological role of Src is mediated via p130Crk-associated substrate (p130Cas). Src phosphorylates p130Cas upon mechanical stimulus, such as stretching and phosphorylated p130Cas is further needed for mechanosignalling (Janostiak, Pataki, Brabek, & Rosel, 2014). These results have been confirmed also *in vivo*, since LOX induced collagen crosslinking is known to increase p130Cas immunostaining in fibroblast of the mouse mammary glands (Levental et al., 2009).

Increased tension induces growth of the focal adhesions and recruitment of FAK and Paxillin to the adhesion sites (Burridge & Guilluy, 2015). There are studies showing that FAK could actually be recruited to FAs even in the absence of talin and furthermore, FAK could even promote talin recruitment to FAs (Lawson et al., 2012; Lawson & Schlaepfer, 2012). FAK is activated via autophosphorylation at tyrosine Y379 (Y379) after integrin ligation. In addition, FAK is phosphorylated by Src at Y574 and Y577 which leads to full activation of FAK (Ruest, Shin, Polte, Zhang, & Hanks, 2001). FAK activates several downstream effectors but one of the most important for mechanosensing is small GTPase RhoA activity is important for mechanosensing since it regulates the organization of actomyosin network. RhoA activation induces stress fiber organization and thus intracellular tension. Interestingly, FAK can both activate and inactivate the Rho GTPases. FAK-mediated phosphorylation of p190RhoGEF correlates with enhanced RhoA activity. Controversially, FAK is known to inhibit RhoA-activity via phosphorylation of p190RhoGAP. Transient RhoA inactivation by FAK is reported to lead to increased FA turnover which facilitates migration (Ren et al., 2000). Given the many signalling outputs of FAK it has an important, yet complex and certainly context dependent role on regulation of cell adhesion and mechanoresponsiveness of cells.

Taken together, integrins and several of their downstream effectors, such as talin and vinculin, are known to be regulated by tesion, leading to increased integrin signalling on stiffer supports.

2.5.3. Intracellular mechanical forces

Mechanosensors, such as stabilized focal adhesions, transmit the physical signals from the extracellular space to the interior of the cell (Kong et al., 2013). This process is mediated mainly by the cellular actomyosin network and other cytoskeletal components such as microtubules and intermediate filaments (Yusko & Asbury, 2014). RhoA GTPase activates Rho-kinase (ROCK), which further increases the phosphorylation of myosin-light chain (MLC) of non-muscle myosin II by inhibiting the MLC-phosphatase. Phosphorylated MLC and actin together form actin stress fibers which are

known to induce intracellular tension (Amano et al., 1996). The number of actin and myosin fibers was shown to increase intracellular stiffness depicted by increased elastic modulus. In addition, actin stress fibers are known to form and orient towards the direction of force and based on a mathematic model, the speed of this process correlates with the amount of extracellular force (Walcott & Sun, 2010).

In addition to providing a links to the ECM, the cellular cytoskeleton connects the focal adhesions physically to the nucleus. The actin cytoskeleton is directly connected to the nucleoskeleton via complex called linker of nucleoskeleton and cytoskeleton (LINC). Adhesion-cytoskeleton-nucleus interplay allows rapid transport of mechanical signals to the nucleus. In fact mechanotransduction from the plasma membrane to the nucleus is fast, occurring only within 5 milliseconds, which is approximately 40 times faster than other known signal transducing pathways (Na et al., 2008; Wang, Tytell, & Ingber, 2009).

It has become evident that the nucleus has an active role on sensing of the mechanosignals and translating these signals into gene expression alterations. Mutations in the nuclear envelope protein coding genes, such as *lamin*, *nesprin* or *emerin*, are known to result in defects in mechanosensing, suggesting that nuclear envelope is an important mechanosensor among its other well-known functions (Dahl, Ribeiro, & Lammerding, 2008; Swift & Discher, 2014). In some cases the mutation is nuclear envelope coding genes manifest as human diseases like progeria and other laminopathies, even though at present the role of the mechanosignalling on the onset of these diseases is poorly understood. Interestingly it has been shown that the stiffness of the nucleus itself is regulated by force, since the exposure of force to the isolated nucleus induces stiffening of the nucleus (Guilluy et al., 2014). It was shown that nuclear stiffening is induced by phosphorylation of Emerin which becomes phosphorylated in response to tension. In addition to the nuclear envelope proteins, the mechanosensor proteins YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) are important mediators of biophysical signals to the nucleus (Dupont et al., 2011a).

2.5.4. Mechanosensitive transcription factors YAP and TAZ

As discussed earlier, mechanical forces are translated into biochemical signals. These signalling pathways mediate signals from the plasma membrane to the nucleus in order to affect nuclear functions such as transcription. The main mediators of mechanotransduction are transcription factors YAP and TAZ, which were earlier known to be regulated by the Hippo-pathway. YAP/TAZ function as transcription factors which regulate the expression of wide variety of target genes together with other co-factors such as TEAD family of transcription factors (Zanconato et al., 2015).

YAP/TAZ have an established role as regulators of organ growth, cell proliferation, motility, EMT and pluripotency (Guo & Zhao, 2013; Yu & Guan, 2013) and thus it is not surprising that this pathway is commonly inactivated in cancer (Lai et al., 2005; Tapon et al., 2002). Indeed, YAP amplification and overexpression in head and neck squamous cell carcinoma (HNSCC) patients was associated with cetuximab resistance (Jerhammar et al., 2014a). Additionally high TAZ expression correlates with poor clinical outcome in breast cancer patients, is highly upregulated in cancer stem cells (CSCs) and is important for chemoresistance of breast cancer stem cells (Bartucci et al., 2015).

2.5.4.1. Regulation of YAP/TAZ by Hippo-pathway

The Hippo-pathway is activated downstream of several different plasma membrane receptors such as E-cadherin and G-protein coupled receptors (GPCR) (N. G. Kim, Koh, Chen, & Gumbiner, 2011) leading to activation of Mst1/2 kinase. Mst1/2 together with a scaffold protein Sav1 or Mob1 phosphorylates and activates Large tumor suppressor 1/2 (LATS1/2) –kinase which further phosphorylates several conserved HXRXXS motifs of YAP and TAZ (Zhao, Li, Tumaneng, Wang, & Guan, 2010). Phosphorylated YAP and TAZ (serine 127) are translocated from the nucleus to the cytoplasm, where they are bound to 14-3-3 protein. Binding to 14-3-3 retains YAP and TAZ in the cytoplasm where they cannot accomplish their role transcription factors.

Alternatively YAP can be phosphorylated on serine 331 or 381 by LATS1/2. This site induces further phosphorylation by casein kinase 1 (CK1). Latter phosphorylation recruits and promotes ubiquitination of YAP/TAZ by β-transducin repeat containing E3 ubiquitin protein ligase (β-TrCP) and ubiquitinated YAP/TAZ are primed for proteasomal degradation (C. Y. Liu et al., 2010). In addition, members of Angiomotin (AMOT) protein family inhibit YAP/TAZ activity. They directly bind to YAP/TAZ, retain them in the cytoplasm and induce the phosphorylation via Hippo-pathway (Zhao et al., 2011).

2.5.4.2. Mechanosensitive regulation of YAP/TAZ

Recently YAP/TAZ localization and protein stability were found to be regulated by tension independently of Hippo-pathway (Dupont et al., 2011a). Dupont et al. showed that YAP/TAZ translocate from the nucleus to the cytoplasm softer substrates. Additionally, cell spreading regulates YAP/TAZ localization. On bigger micropatterns (3200 μm^2), when cells were spread, YAP/TAZ was prominently nuclear while on smaller micropatterns (300 μm^2), when cell morphology was more round, YAP/TAZ is fully cytoplasmic. The mediators of

Hippo-pathway, such as LATS-kinases, are not responsible for tension-induced cytoplasmic translocation, but rather this is regulated by ROCK and actomyosin contractility (Figure 5).

RhoA GTPase activity, as well as actin cytoskeletal tension, is required for YAP/TAZ nuclear maintenance, since inhibition of ROCK or disruption of actin cytoskeleton by Latrunculin A, induced YAP/ATZ cytoplasmic localization already on stiff substrates. Stiffness mediated regulation of YAP/TAZ was shown to be independent of Hippo-pathway since the silencing of LATS-kinases could not rescue the stiffness induced cytoplasmic translocation of YAP/TAZ (Dupont et al., 2011a).

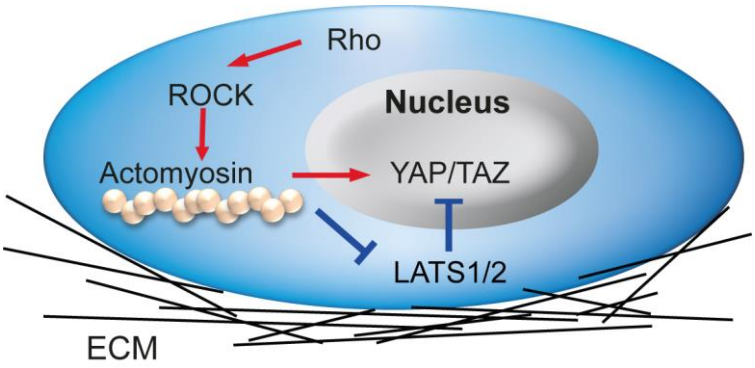

Figure 5. Regulation of YAP/TAZ by mechanotransduction. Modified from Halder, Dupont, Piccolo 2012. A model for the mechanical regulation of YAP and TAZ. Stiff extracellular forces promote cell-ECM adhesions via integrin clustering and the development of intracellular contractile actomyosin forces. RhoA, ROCK and contractile actin stress fibers sustain YAP and TAZ nuclear localization. Additionally, F-actin inhibits YAP and TAZ phosphorylation through inhibition of the kinases Large tumor suppressor homologue 1 and 2 (LATS 1/2).

To conclude, YAP/TAZ cytoplasmic translocation can be activated downstream of variety of receptors and signals. High cell density, serum starvation or low stiffness leads eventually to YAP/TAZ phosphorylation and cytoplasmic translocation in order to render their activity. Inspite of their important role in the Hippo-pathway, the mechanosensitive regulation of YAP and TAZ indicates more widespread role for these transcription factors in the regulation of cellular functions.

2.5.5. Regulation of proliferation by stiffness

Extracellular tension is found to regulate the proliferation of both normal and transformed cells (Bomo et al., 2015; Yeh et al., 2012). It has been demonstrated that 3D rigidity could promote proliferation and spheroid formation of both normal and transformed liver cells (Bomo et al., 2015). In addition, collagen density and matrix rigidity has been shown to drive proliferation of mammary epithelial cells, since mice which are resistant to collagenase and thus have excessive amounts of collagen in their mammary glands, are more susceptible to tumor formation (Provenzano et al., 2008). Stiffness was also shown to modulate the HGF-response and thus affected the growth-induced mitogenic signalling of hepatocellular carcinoma (HCC) cells (Schrader et al., 2011). In addition, the proliferation of normal endothelial cells (ECs) is directed by substrate stiffness indicating that formation of blood vessels is directed by extracellular tension.

The mechanism by which stiffness regulates proliferation involves tension-induced formation of actin stress fibres and increased RhoA activity. Downregulation of RhoA inhibits the formation of stress fibers and proliferation several cell types including ECs and NIH 3T3 mouse fibroblasts (Swant, Rendon, Symons, & Mitchell, 2005; Yeh et al., 2012). Furthermore, integrin heterodimer $\alpha_v\beta_3$ together with its effectors Src/Vav2 kinase functions upstream of RhoA in ECs since inhibition of $\alpha_v\beta_3$ led to attenuation of Src/Vav2 kinase activation and inhibition of RhoA-dependent EC proliferation (Yeh et al., 2012). In addition, since the RhoA–ROCK pathway stimulates the formation of actin stress fibers and integrin clustering, it also promotes MAP-kinase pathway activity. Indeed, it has been shown that pharmacological inhibition of RhoA leads to inhibition of Extracellular-signal-regulated kinase (ERK)-dependent *cyclin D1* induction and thus the progression of mitosis (Swant et al., 2005; Welsh et al., 2001). ERK activation is also found to be associated with increased density, and thus increased stiffness, within tumors and therefore facilitate melanoma cell proliferation and survival (Hirata et al., 2015).

Since YAP/TAZ are known regulators of proliferation, stiffness mediated growth control is largely mediated by these proteins. As discussed above, stiffness regulates YAP/TAZ activity via nuclear export. YAP/TAZ regulate growth by inducing the expression of genes, such as connective tissue growth factor (CTGF), Cysteine-rich angiogenic inducer 61 (Cyr61), Thromposbondin 1 (THBS1), which are important for proliferation (H. Zhang et al., 2009; Zhao et al., 2008). In addition, it has been shown that beta-catenin participates in the regulation of cell-cycle progression together with YAP. Under low mechanical tension YAP localized to the cytoplasm and beta-catenin to the cell-cell contacts. When tension is applied, both proteins re-localize to the nucleus and cell enters to the synthesis (S)-phase of the cell cycle (Benham-Pyle, Pruitt, & Nelson, 2015). Benham-Pyle et al. concluded that tension induced E-cadherin

activation was needed for YAP and beta-catenin nuclear accumulation as well as cell-cycle progression.

2.6. Epigenetic regulation of transcription

The word epigenetics is typically defined as regulation of gene expression which is not coded in the DNA sequence. Epigenetic regulation includes DNA methylation, histone modifications and non-coding RNAs, especially miRNA-mediated regulation of gene expression (Jaenisch & Bird, 2003). Epigenetic regulatory mechanisms are important for the maintenance of tissue- and cell type specific gene expression signatures Epigenetic modifications, DNA methylation and histone modifications in particular, alter chromatin conformation and accessibility without affecting the actual DNA nucleotide sequence.

Chromatin can be found in two conformations: open (euchromatin) or closed (heterochromatin). Closed, more compact conformation is related to transcriptional inactivation while open chromatin is accessible for transcription factors and other regulatory proteins and therefore is considered to be transcriptionally active. DNA is commonly methylated at the CpG-islands at the core promoter of the gene. When the promoter is methylated, the expression of the gene is typically silenced. While DNA methylation has an unambiguous impact on gene expression, the code of the histone modifications is more complex. Histones can be modified for instance by methylation, acetylation, phosphorylation or ubiquitination (Jaenisch & Bird, 2003).

Post-translational modifications of the histones are known to have an effect on DNA-repair and replication but they have been most intensively studied in the context of transcriptional regulation (Jaenisch & Bird, 2003). Depending on the modification and site, histone modification can be transcriptionally inactivating or activating. As an example, methylation of histone 3 lysine 4 (H3K4) activates transcription, while methylation of adjacent lysines such as lysines 9 (K9) or 27 (K27) are transcriptionally inactive. Acetylation of the histones is broadly activating (Bannister & Kouzarides, 2011). The biological role of these histone modifications can be explained by the fact that they form binding sites for other regulatory proteins. Chromatin binding proteins are known to contain bromodomains, which bind to acetylated lysines, and chromatin organization modifier *(*chromo) -domains, which bind to methylated histones. Many chromodomain containing proteins are responsible for heterochromatin formation (Josling, Selvarajah, Petter, & Duffy, 2012; Winston & Allis, 1999).

Abnormalities in epigenetic code are frequently found in cancer (Sharma, Kelly, & Jones, 2010). It has been shown as an example, that DNA methyltransferase 3A (DNMT3A) is important regulator of carcinogenesis in gastric carcinoma

since it methylates the promoter of $p18^{INK4C}$ tumor suppressor gene leading to the inactivation of $p18^{INK4C}$ and increased proliferation of gastric cancer cells (Cui et al., 2015). One of the histone modifying enzymes, histone demethylase JMJD1a, is also found to be upregulated in several cancer types.

2.6.1. Histone demethylase JMJD1a

Enzymes belonging to Jumonji-domain family (Jmj), also known as lysine (K)-specific demethylase (KDM) family, are demethylases which remove methyl groups from histone tails and thus epigenetically regulate the chromatin organization and the expression of their target genes (Labbe, Holowatyj, & Yang, 2013). JMJD1a, also known as KDM3a, is a histone 3 lysine 9 (H3K9) specific demethylase which removes methyl groups from mono- or dimethylated lysines, with a preference towards dimethylated lysines, via oxidative reaction that requires iron and alpha-ketoglutarate (H. Chen et al., 2010; Okada, Scott, Ray, Mishina, & Zhang, 2007).

H3K9me2 is known to functions as a transcription inhibiting mark and the removal of H3K9me2 from the target gene promoter leads to transcriptional activation (Figure 6). JMJD1a, together with other members of JMJD-family named JMJD1b and JMJD1c, are the main enzymes demethylating H3K9me2 (M. Chen et al., 2015; Yamane et al., 2006). Even though JMJD1a, -b and -c have the same target modification, their known interacting proteins and target genes are at least partially distinct, indicating that these members of JMJD-family have their own specialized functions (Brauchle et al., 2013). The enzyme which has the opposing enzymatic activity to JMJD demethylases is H3K9 specific methyltransferase G9a (Tachibana et al., 2002). G9 induces the H3K9me2 levels at the target gene promotes leading to chromatin condensation and transcriptional inactivation.

The biological role of JMJD1a has been studied especially in metabolism and spermatogenesis, since the JMJD1a -/- mice are obese, have severe dysfunctions in their energy metabolism as well as the male mice are infertile and they have problems in the differentiation of sperm cells (Inagaki et al., 2009; Okada et al., 2007). JMJD1a has also a well-established role in the regulation of proliferation and other cancer-related processes such as migration. It has been found to be upregulated in several cancer types such as colon cancer and Ewing's sarcoma (Parrish, Sechler, Winn, & Jedlicka, 2015; Uemura et al., 2010) and it has a prognostic role in colon cancer (Uemura et al., 2010)

JMJD1a has been thought to participate in cancer development by transcriptional regulation of cancer promoting genes. JMJD1a is known to demethylate the promoter, and thus activate the transcription, of *MYC*

oncogene androgen receptor dependently in prostate cancer cells (Fan et al., 2015). The role of C-Myc as an oncogene is well established (Ott, 2014). In addition to *MYC*, JMJD1a has been shown to activate Homeobox A1 (HOXA1) expression in bladder cancer (Cho et al., 2012). Downregulation of HOXA1 leads to cell cycle arrest in several different cell types and it is overexpressed in carcinomas, which demonstrates its important role as a regulator of cell growth (Bitu et al., 2012).

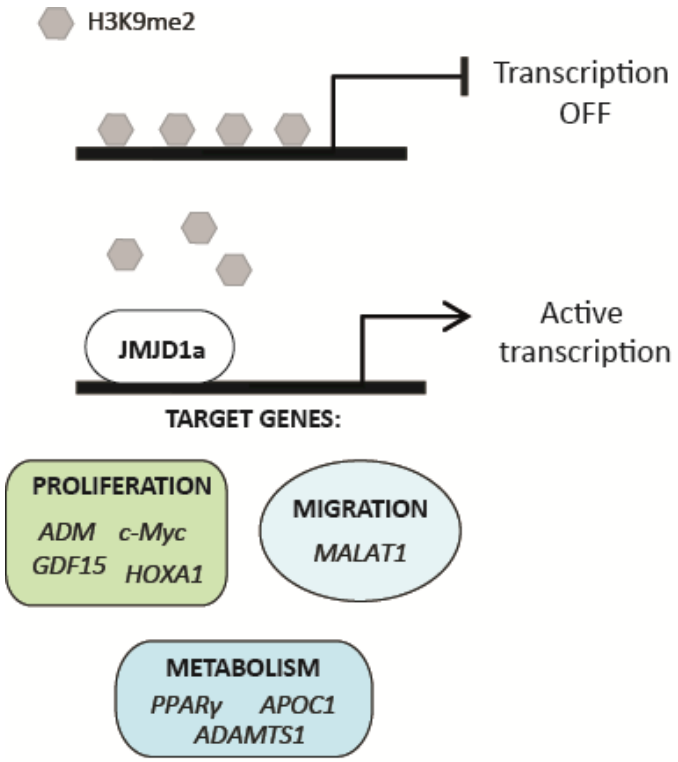

Figure 6. Epigenetic regulation of transcription by histone demethylase JMJD1a. JMJD1a, a histone 3 lysine 9 dimethyl specific demethylase enzyme. JMJD1a removes H3K9me2 marks from the target gene promoters. H3K9me2 is considered a transcription inhibiting epigenetic mark. When H3K9me2 is removed from the target gene promoter via JMJD1a, the transcription is activated. ADM and GDF15 are among the JMJD1a target genes that are known to contribute to the regulation of cancer cell proliferation.

While several JMJD1a target genes have been found, less is known how JMJD1a itself is regulated. However, JMJD1a is known to be transcriptionally regulated by Hypoxia inducing factor alpha (HIF1a) (Krieg et al., 2010a; Pollard et al., 2008). HIF1a is known to bind to JMJD1a promoter and activate its expression under hypoxic conditions. Additionally, transcription factor OCT-4 binds to JMJD1a promoter and positively regulate JMJD1a transcription in pluripotent stem cells (Loh, Zhang, Chen, George, & Ng, 2007). Loh et al. showed that the loss of JMJD1a leads to differentiation of the stem cells and they identified that JMJD1a regulated directly genes such as Tcl1, Tcfcp2l1, and Zfp57, which are important for maintenance of the pluripotent stem cells.

In addition to transcriptional regulation, JMJD1a is regulated post-translationally. Mitogen and stress activated protein 1 (MSK-1) phosphorylates JMJD1a at Serine 264 (S264) under heat shock which increases the JMJD1a activity and binding to the target gene promoters (M. B. Cheng et al., 2014). As an example, phosphorylated JMJD1a has shown to interact with Stat1 and regulate gene expression of known Stat1 target genes. In addition to MSK-1, Protein kinase A (PKA) has been identified as a regulator of JMJD1a activity. PKA phosphorylates JMJD1a S265 site and similarly to S264 this phosphorylation is needed to activate the target gene expression (Abe et al., 2015).

2.7. Filopodia in cancer cell motility

2.7.1. Filopodia

Filopodia are actin-rich protrusions which extend from the cell edges allowing them to probe the environment and move directionally. The role of the filopodia role has been extensively studied especially in the development and axon guidance of the neurons (Sagar, Prols, Wiegreffe, & Scaal, 2015). However, later on filopodia have been shown to be important also in pathological situation such as cancer. When cancer cells invade from the primary tumor to the stroma, they need to form contacts with stromal ECM and thus the filopodia are important regulators of cell motility especially in the context of cancer cell migration and invasion (Jacquemet et al., 2013; Machesky & Li, 2010).

In motile cells, filopodia outgrow from the lamellipodia, which are sheet-like membrane protrusions at the leading edge of a cell composed of a branched actin meshwork. Actin, actin remodeling proteins as well as plasma membrane composition are considered as important regulators of filopodia formation. Current model of the filopodia formation is presented in Figure 7.

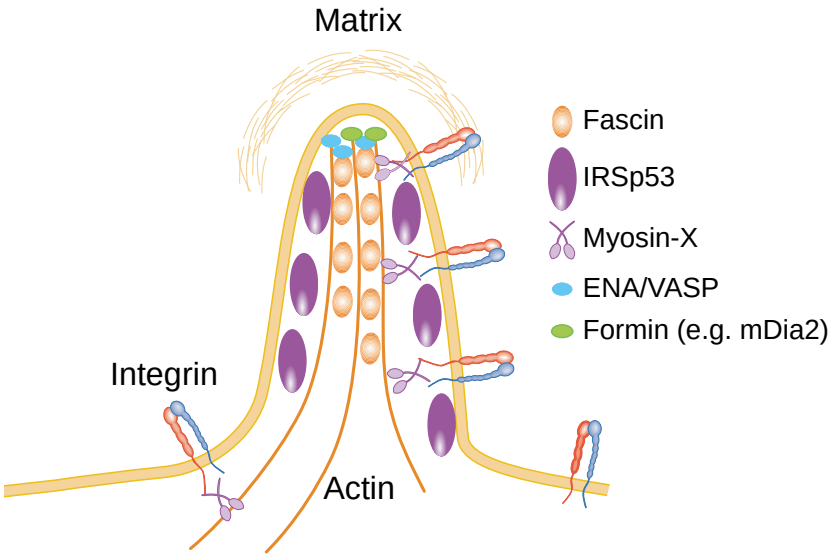

Figure 7. Filopodia formation. Filopodia are plasma membrane protrusions that form by the pushing force of bundled actin filaments against the membrane. Number of actin-associated proteins such as IRSp53, formins, actin crosslinking protein Fascin, and the motor protein Myo10 promote the formation and growth of filopodia (Mattila & Lappalainen, 2008).

2.7.1.1. Regulation of actin cytoskeleton within filopodia

The growth of filopodia is regulated by the actin cytoskeleton. Actin filament (F-actin) nucleation requires so called nucleation factors that facilitate the formation of actin nucleus, composed of at least three actin monomers. These filament precursor structures grow into actin filaments by the addition of ATP-bound globular (G)-actin monomers to the growing F-actin ends (+ ends, barbed ends), which are typically oriented towards the plasma membrane (Dominguez, 2009). The growth of the filament pushes and bends the plasma membrane, thereby facilitating the outgrowth of filopodia (Svitkina et al., 2003). Therefore the proteins which regulate the nucleation, such as Actin related protein 2/3 (Arp2/3) together with other nucleation promoting factors can be considered as promoters of filopodia formation.

Specialized functions of different types of filopodia are associated with distinct molecular composition and structure. Neurons for instance have dendritic filopodia which are able to develop into postsynaptic dendritic spines (Fiala, Feinberg, Popov, & Harris, 1998). Dendritic filopodia are composed of loose

actin network (Korobova & Svitkina, 2010), while commonly actin filaments are organized linearly forming parallel actin bundles (Small & Celis, 1978).

Arp2/3 is an actin nucleating protein, which promotes the formation of branched actin network (Padrick, Doolittle, Brautigam, King, & Rosen, 2011) together with its activator Wiskott–Aldrich syndrome protein (WASP) and WASP-family verprolin-homologous protein (WAVE). WASP/WAVE function as scaffolds that convert the signals from the small GTPase Cdc42 in order to promote polymerization and branching of actin. Indeed, Cdc42 and N-WASP together activate Arp2/3 and induce the efficient actin filament polymerization (Rohatgi et al., 1999). It has been shown that Arp2/3 localises in filopodia (Johnston, Bramble, Yeung, Mendes, & Machesky, 2008) and supports filopodia formation in neurons (Korobova & Svitkina, 2008)

Actin nucleating factors such as formins (e.g. mDia) nucleate and promote the growth of linear actin filaments. Depletion of mDia inhibits both lamellipodia and filopodia formation (Yang et al., 2007). In addition to formins, Ena/VASP proteins promote the growth of linear actin filaments, which are commonly found in filopodia. (Huber et al. 2008). This is known to be achieved by Ena/VASP binding to the growing ends of the actin filaments where it inhibits the filament capping by antagonizing the capping protein (CP) (Krause et al. 2003). In addition, ENA/VASP recruits Profilin to the site of newly forming filopodia. Profilin enhances ENA/VASP-mediated actin polymerization by delivering ATP-bound actin monomers to the growing filament ends (Barzik et al., 2005).

In addition to being linearly organized, actin filaments are also cross-linked into tight actin bundles within filopodia (Esue, Harris, Higgs, & Wirtz, 2008; Jansen et al., 2011). Actin-bundling protein Fascin enhances this process specifically in filopodia. Indeed, it has been shown that fascin promotes filopodia formation (DeRosier & Edds, 1980) emphasizing the importance of actin crosslinking and generation of tight actin bundles in filopodia formation. Taken together, actin and actin regulation proteins are essential regulators of filopodia formation and depletion of actin regulatory proteins such as Arp2/3, Fascin or Ena/VASP, inhibits filopodia formation.

2.7.1.2. Plasma membrane structure and composition in filopodia

As discussed above, growing actin filaments bend the plasma membrane which facilitates the growth of filopodia. Filopodia have special membrane curvature, since membrane needs to bend outwards, and thin fingerlike filopodia have extremely high membrane curvature (Prevost et al., 2015). I-BAR domain proteins, such as IRSp53 promote for membrane bending and tubulation of the newly forming filopodia. I-BAR domain and IRSp53

overexpression is sufficient to induce filopodia formation (Krugmann et al., 2001; Yamagishi, Masuda, Ohki, Onishi, & Mochizuki, 2004). IRSp53 has high affinity to phosphatidylinositol lipids found at the plasma membrane and it interacts directly with small GTPases Cdc42 and Rac1 (Krugman et al 2001). In addition, IRSp53 interacts with Mena, which is a protein that facilitates filament elongation by competing with capping proteins on binding to growing filament ends (Mattila & Lappalainen, 2008). Considering of all these interactions, IRSp53 could promote filopodia formation by inducing the plasma membrane bending and functioning as a scaffold for filopodia inducing proteins.

In addition to plasma membrane curvature, the lipid and protein composition of the membrane contributes to filopodia formation. Cell front is accompanied with high levels of active phosphatidylinositide 3-kinase (PI3K) (Sasaki, Chun, Takeda, & Firtel, 2004). PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P_2] lipids and converts them to PI 3,4,5-triphosphate [(3,4,5)P_3] (Engelman, Luo, & Cantley, 2006). These different lipids may serve as docking sites for various proteins, some of which are important for filopodia growth. For example, different phosphoinositide lipids have differential binding affinity to proteins which have pleckstrin homology (PH) domain (Itoh & Takenawa, 2002). Moreover, WASP and N-WASP are known to be activated by PI(4,5)P2 binding (Rohatgi et al. Cell 1999). Membrane-associated receptors such as integrins and cadherins localize to the shaft and tips of the filopodia. Integrins are reported to be clustered and thus primed for activation and ligand binding by polymerizing actin at the filopodia tip (Galbraith CG, et al. 2007). A protein that transports integrins and VE-cadherin, among several other proteins, to filopodia tip is an unconventional myosin called Myosin-X (Myo10).

2.7.2. Myosin-X

Myo10 is an unconventional myosin, which is expressed especially in brain, endothelia, and many epithelial tissues (K. C. Liu, Jacobs, Dunn, Fanning, & Cheney, 2012; Plantman, Zelano, Novikova, Novikov, & Cullheim, 2013). Myo10 functions as a motor protein which localizes to the filopodia and its main function is to transport the cargo proteins to the filopodia tips (Berg, Derfler, Pennisi, Corey, & Cheney, 2000a; H. Zhang et al., 2004). Myo10 is important for filopodia formation since its overexpression induces massive induction of filopodia (Bohil et al. 2006). In addition to filopodia, Myo10 induces also the formation of invadopodia (Schoumacher, Goldman, Louvard, & Vignjevic, 2010), which are structures needed to ECM degradation and invasion.

Myo10 heavy chain consists of three regions: a myosin motor domain (head), a neck domain and tail domain (Figure 8). The motor domain is responsible for

binding to actin filaments as well hydrolyzing ATP, and generating force. The neck domain contains three IQ motifs which bind to calmodulin or calmodulin-like light chain (Homma, Saito, Ikebe, & Ikebe, 2001). The tail consists of an α-helical region that allows the Myo10 heavy chain to homodimerize, three PEST-motifs and three PH-domains. The PEST sequences are cleaved by calpain in a Ca^{2+} dependent manner (Berg, Derfler, Pennisi, Corey, & Cheney, 2000b). One of the three PH-domains binds to the key signalling lipid, phosphatidylinositol trisphosphate (PIP3). This binding is important for the activation and membrane localization of Myo10. It has been shown that Myo10 binds to PIP3 with high affinity. Inhibition of PIP3 by PI3K inhibitor leads to translocation of Myo10 from the filopodia tips to the cell body and further to decreased filopodia number (Plantard et al., 2010).

The C-terminal MyTH4 (myosin tail homology 4) domain binds to microtubules and FERM domain to cargo proteins such as VE-cadherin (Almagro et al., 2010), Deleted in colorectal cancer (DCC) (Y. Liu et al., 2012) and Integrin β1 (H. Zhang et al., 2004). The FERM domain of Myo10 is 28% similar to the FERM domain of Talin, and like Talin the Myo10 FERM is known to bind to NPXY-motifs of integrin β-subunits (H. Zhang et al., 2004).

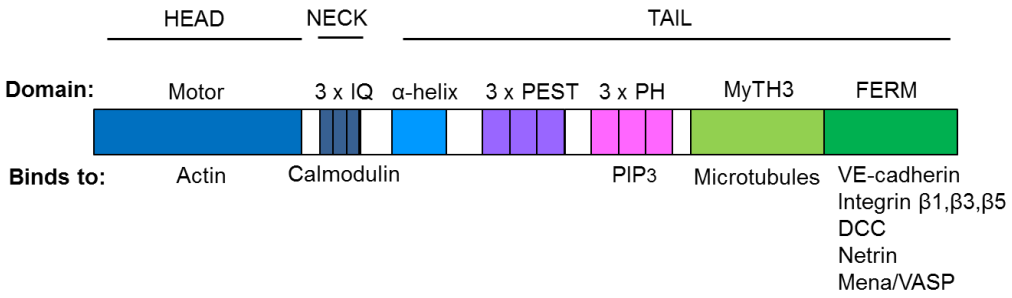

Figure 8. The structure of Myosin-X (Myo10). Myo10 contains motor domain which is responsible for actin binding as well as energy production via ATPase hydrolysis. Neck domain contains 3 IQ motifs which are binding sites for calmodulin. Tail contains domains for PIP3, microtubule and cargo binding. Several cargo proteins, such as VE cadherin, Integrin β1, β3 and β5 as well as Deleted in colorectal cancer (DCC) are known to bind the FERM domain of Myo10.

The main function of Myo10 is to act as a cargo transporter form the cell body to and from filopodia tips and actively transport cargo proteins which are needed for proper filopodia function (Galbraith, Yamada, & Galbraith, 2007). There is an increasing knowledge how Myo10 together with other filopodia-related proteins contribute to cancer progression, especially cancer cell

metastasis. There are several studies implying that filopodia promoting genes could have a prognostic role in cancer and potentially have a clinical relevance in several different cancer types (Cao et al., 2014a; S. J. Kim et al., 2012).

2.7.2.1. Myosin-X and invadopodia

Many filopodia-associated proteins, such as Myo10 and fascin, localize and induce the formation of invadopodia (Schoumacher et al., 2010). Similarly as filopodia, invadopodia are known to promote cell motility. Invadopodia are rich in proteases, such as MT1-MMP, and their main role as regulation of cancer related processes seems to lie on inducing ECM degradation (Kelly, Mueller, Yeh, & Chen, 1994). It has been proposed that Myo10 participates to invadopodia formation and ECM degradation by transporting molecules such as actin nucleation promotes VASP (Tokuo & Ikebe, 2004) and MT1-MMP to invadopodia. Indeed, Myo10 depletion reduced the cell's potential to degrade ECM approximately by half (Schoumacher et al., 2010).

2.7.3. The role of filopodia in cancer

Myo10 enriched structures such as filopodia and invadopodia has been connected to increased invasiveness, aggressiveness and decreased survival rates in various types of cancer (Leong et al., 2014; Machesky & Li, 2010). Altered gene expression of filopodia promoting genes increases the number and stability of the filopodia and these changes could have a prognostic role in cancer. Indeed, it has been shown that Myo10 promotes breast cancer cell invasion and metastasis and correlates with reduced survival of breast cancer patients (In this thesis, (Cao et al., 2014b).

Fascin and profilin participate to the actin crosslinking and dynamic turnover of actin filaments in filopodia. Fascin, is usually expressed at low levels in normal epithelium, but is up regulated in several types of carcinomas (Zhang et al. 2006). Its function has been studied particularly in Esophageal Squamous Cell Carcinoma (ESCC), where high *fascin* expression was shown to be associated with increased risk of invasion (Takikita et al., 2011). In addition, Fascin silenced breast cancer cells are more sensitive to chemotherapy compared to fascin expressing cells, mainly due to reduced PI3K signalling and thus reduced FAK and Akt activity (Ghebeh et al., 2014).

Profilin 1 on the other hand, has shown to exhibit more variable impact on cancer progression. It has shown to be upregulated in renal cell carcinoma (RCC) (Minamida et al. 2011) and downregulated in pancreatic cancer (Grønborg et al. 2006) and inhibit the aggressivity of breast carcinoma (Zo et al. 2007). In conclusion, since the association between the filopodia-related

proteins and cancer progression has been shown in a context of various different cancer types, filopodia genes could be considered to have oncogenic properties when dysregulated.

2.7.4. Mutant p53 and its role in cancer cell motility

One of the most frequently mutated genes in various different cancers is tumor suppressor *TP53*. It is mutated in approximately 50 % of all cancers (Vogelstein et al. 2000). Wild type p53 functions as transcription factor which induces apoptosis, senescence and preserves genome integrity together with other tumor suppressor proteins such as p63 and p73 (Muller et al., 2009). The tumor suppressor functions of p53 is mainly mediated by activation of cyclin dependent kinase inhibitor p21, which induces cell cycle arrest and senescence and thus protects from tumor formation when activated (Gartel & Tyner, 2002). Mutations in TP53 are commonly missense mutations which lead to expression of point-mutated p53. P53 mutants, which are commonly found in human cancers, are R273H (R270H in mice) and R175H (R172H in mice), R273H inhibits the DNA binding of p53 and disturbs the normal conformation of p53 (Cho et al., 1994).

Mutations do not only impair the tumor suppressive functions of WT p53 but these gain-of–function mutations actively enhance the tumor progression. More than 80% of p53 mutations occur at the zinc finger, a DNA binding domain, which leads to the synthesis of highly expressed and extremely stable protein incapable of carrying its normal tumor-suppressive function. Part of the tumor promoting capacity of mutant p53 depends on its inability to function together with its tumor suppressor homologs p63 and p73. WT p53 binds to p63 or p73 and the protein heterodimer regulates the expression of tumor suppressor genes such as genes which are important for DNA damage response and cell death (Hams et al. 2004).

Mutant p53 has a well-established role as a promoter of tumor growth. However, there is emerging evidence that mutant p53 has an important role also in tumor migration and metastasis. Interestingly, p53 knock-out (KO) mice develop tumors in high frequency, but these tumors rarely spread to distal organs (Attardi and Jacks, 1999). This indicates that the gain-of-function attributes of mutated p53 could be responsible for increased motility of the cancer cells. However, it has been established that loss of wt p53 changes the morphology and polarity of the cells and induces the migration and invasion through 3D matrix. (Alexandrova et al., 2000; Gadéa et al., 2002; Guo et al., 2003; Guo and Zheng, 2004; Gadea et al., 2007.

A possible mechanism how mutant p53 could regulate cell motility was described recently (Adorno et al., 2009). Adorno et al. showed that TGF-β

induced phosphorylation of SMAD2 transcription factor binds to mutant p53-p63 heterodimer and the complex together is needed for TGF-β and mutant p53 induced cancer cell invasion. An additional mechanism, where mutant p53 enhances the cancer cell invasion and migration via p63, is by increased trafficking of integrin adhesion receptors and epidermal growth factor receptor (EGFR). Active recycling of these receptors towards the leading edge of the migrating cell is essential for the motility of the cell. It was found that p53 promotes cell invasion via the inhibition of p63, since the loss of wt p53 and p63 led to increased recycling of the receptors (Muller et al. 2009). Mutant p53 participates to EMT-programming via activating the expression of Twist, slug and snail transcription actor (Shiota et al. 2008). It has also been shown that wt p53 promotes Slug degradation by MDM2 (Wang et al., 2009b). Additionally, mutant p53 inhibits let-7i, which is a tumor suppressor miRNA that inhibits cancer cell migration, invasion and metastasis (Subramanian et al 2015).

In addition, mutant p53 regulates RhoA GTPase, which is important regulator of contractility and cell motility. Enhanced amoeboid migration of p53-null fibroblasts can be opposed by pharmacological inhibition of ROCK (Gadea et al., 2007). This indicates that RhoA has an important role of cancer cell motility downstream of mutant p53. Indeed, mutant p53 was shown to induce the expression of the guanine nucleotide dissociation inhibitor RhoGDI (Bossi et al., 2008) and RhoGEF GEF-H1 (Mizuarai et al., 2006), both of which are known activators of RhoA activity.

P53 has an established role in regulation of invadopodia structures, which cells use to degrade the ECM-proteins and thus are important for the invasion through the ECM network. Invadopodia are composed of actin and actin associated proteins such as fascin and Myo10. P53 mutation has shown to correlate with high levels of fascin in colorectal cancer. It was shown that mutant p53 regulates fascin expression, and thus invadopodia formation, via mechanism that is mediated by NF-kB (Sui et al. 2015). Its association in the context of cancer cell invasion and metastasis was also associated to filopodia formation (in this PhD thesis, (Arjonen et al., 2014). As a conclusion, the emerging role of mutant p53 as regulator of cancer cell invasion can be, at least partially, explained by its role as activator of filopodia and invadopodia associated genes, such as Myo10 and fascin.

3. AIMS OF THE STUDY

The aim of this thesis was to identify the mechanisms how tumor stroma influences tumor growth and to identify the role of filopodia in breast cancer metastasis.

Tumor stroma, also known as the tumor microenvironment, regulates cancer cell growth and invasion and this is at least partially mediated by activated fibroblasts, known as CAFs, within the stroma. However, the impact of reduced number of normal fibroblasts in the stoma, rather than increased number of CAFs, is not well known.

While increased growth of cancer cells is an important hallmark of cancer, the main cause of death among cancer patients is the metastatic spreading of the cancer cells. Metastasis is accompanied by increased migration and invasion capacity. The number of filopodia increases in cancer compared to normal cells and the role of filopodia has been linked to increased motility. Myosin-X induces the formation of filopodia but its role in cancer, especially in cancer cell metastasis, has not been studied earlier.

The specific aims of this study were:

1. To study if CAF produced ECM is different from the matrix produced by normal fibroblasts

2. To identify the mechanism how normal stroma could regulate tumorigenesis

3. To study if Myo10 and filopodia are important for breast cancer invasion and migration

4. MATERIALS AND METHODS

More specific description of the methods can be found from the original articles (I-III).

DNA constructs

EGFP-Myo10 and EGFP-Myo10ΔFERM2 constructs have been described in (Zhang et al. 2004) and the p53-R273H construct is described in (Bullock et al. 1997). shp53 pSUPERretro-puro, RNAi resistant R175H (hp53 R175H in pRRLsin.PPTs.hCMV.GFPpre) and w tp53 (mp53ASwt pBABEpuro) constructs are described in Adorno et al. (Adorno et al. 2009). JMJD1a-GFP DNA constructs were ordered from Genecopoeia.

Experimental procedures

Method	**Used in:**
Adhesion assay	(II)
Cell culture	(I,II)
Cell derived matrices	(I)
Chromatin immunoprecipitation	(I,II)
Chick Chorioallantoic Membrane (CAM) –assay	(I)
Fluorescent associated cell sorting (FACS)	(II)
Immunofluorescence	(I,II)
Immunohistochemistry	(I,II)
Invasion assay	(II)
Lung extravasation assay	(II)
Microscopy:	
Atomic force microscopy	(I)
Confocal microscopy	(I,II)
Scanning electron microscopy	(I)
Live-cell imaging	(II)
Migration assay	(II)
Nuclear fractionation	(I)
Orthotopic breast tumor model	(I,II)
Proliferation assays	(I,II)
Statistical analyses	(I,II,III)
Taqman RT-PCR	(I,II)
Transfections	(I,II)
Western blotting	(I,II)

Cell lines

Cell line	Description	Used in
MDA-MB-231	Human breast cancer	(I,II)
MFC-7	Human breast cancer	(II)
MCF10a	Normal human breast	(II)
BT-474	Human breast cancer	(II)
MDA-MB-468	Human breast cancer	(II)
HeLa	Human cervical cancer	(I)
UT-SCC (#67, #54 and #76)	Human Squamous cell carcinoma	(I)
Normal fibroblasts*	Fibroblasts isolated from non-cancerous regions of HNSCC patients	(I)
CAFs *	Fibroblasts isolated from tumor stroma of HNSCC patients	(I)
TIFF	TERT immortalized human foreskin fibroblasts	(I)
HCT-116	Human colorectal carcinoma	(II)
MiaPaca	Human pancreatic carcinoma	(II)
Panc1	Human pancreatic carcinoma	(II)
AsPC1	Human pancreatic carcinoma	(II)

* Corresponding to UT-SCC cell lines

RT-PCR primers and Universal probe library (UPL) probes

Gene	Forward primer	Reverse primer	UPL probe
Myo10	AGGACTTTCCACCTGATTGC	CGTGGACCTGACCTAGCA	#20
TP53	TCCTCCATGGCAGTGACC	CTTTCCACGACGGTGACA	#71
EGR-1	AGCCCTACGAGCACCTGAC	GGTTTGGCTGGGGTAACTG	#22
JMJD1a	CCAGCCTCAAAGGAAGACCT	ACTGCACCAAGAGTGGTTT	#71
YAP	TGGATTTTGAGTCCCACCAT	ATCCCAGCACAGCAAATTCT	#47
TAZ	ATTCGAATGCGCCAAGAG	AACTGGGGCAAGAGTCTCAG	#4
CTGF	CTCCTGCAGGCTAGAGAAGC	GATGCACTTTTTGCCCTTCTT	#56
THBS1	TGGAGACCAGCCATCGTC	CAATGCCACAGTTCCTGATG	#85
GAPDH	GCCCAATACGACCAAATCC	AGCCACATCGCTCAGACA	#60

Antibodies

Antibodies	Description	Application	Used in
Actin	A1978, Sigma	WB	(I,II)
Collagen I	NB600-408, Novus	IF	(I)
pERK1/2	9102, Cell Signaling	WB	(II)
EGR-1	sc-110, Santa Cruz	WB, ChIP	(II)
ERK1/2	L34F12, Cell Signaling	WB	(II)
Fibronectin	F3648, Sigma	IF	(I)
GAPDH	5G4,HyB test	WB	(I)
H3K4me3	ab8580, Abcam	ChIP	(II)
H3K9me2	7658, Cell Signaling	WB	(I)
Histone 3	4499, Cell Signaling	WB	(I)
HSC-70/hsp73	ADI-SPA-815-F, ENZO	WB	(II)
Integrin β1	610468, BD Biosciences	WB	(I,II)
IgG control (rbt)	sc-2027, Santa Cruz	ChIP	(II)
IgG control (ms)	Santa Cruz	ChIP	(I)
JMJD1a	12835-1-AP, Proteintech	WB,IF	(I)
JMJD1a	sc-376608, Santa Cruz	WB,IF,ChIP	(I)
Lamin A/C	sc-7292, Santa Cruz	WB	(I)
Myo10	845-944,Strategic Diagnostics	WB	(II)
Myo10	Sigma	IHC, WB	(II)
p53	DO-7, Santa Cruz	WB	(II)
SMA-α	A2547, Sigma	WB	(I)
Talin	T3287, Sigma	WB	(I,II)
Tubulin	12G10, Hybridoma Bank	WB	(I,II)
Vimentin	HPA001762, Sigma	IHC	(II)
YAP/TAZ	sc-101199, Santa Cruz	WB,IF	(I)

Reagents

Reagent	Application	Used in
Hiperfect	Transfection of siRNA	(II)
RNAi max	Transfection of siRNA	(I)
Lipofectamine 2000	Transfection of plasmid DNA	(II)
Lipofectamine 3000	Transfection of plasmid DNA	(I)
Optimem	Transfection	(I,II)
Collagen	Coating	(I)
Fibronectin	Coating	(I)
WST-1	Proliferation assay	(II)
Mowiol	Mounting medium	(I,II)
Dapi	Nuclear staining	(I,II)
Phalloidin	Staining of the actin filaments	(I,II)
DMSO	Solvent	(II)
UO126	MEK inhibitor	(II)
ROX mix	RT-PCR	(I,II)
CellTracker green	Live cell stain	(II)
CellTRracker red	Live cell stain	(II)
Matrigel	3D matrix	(II)
Hydrogels of various stiffness	Mechanotransduction studies	(I)
Paraformaldehyde	Fixation	(I,II)
Gelatin	Preparation of CDMs	(I)
Glutaraldehyde	Fixative, Preparation of CDMs	(I)
Ascorbic acid (Sigma)	Preparation of CDMs	(I)
Ammonium hydroxide (Sigma)	Preparation of CDMs	(I)
Dnase I (Roche)	Preparation of CDMs	(I)
Horse serum (HRS)	Blocking regent	(I,II)
RNA isolation kit	mRNA isolation	(I,II)
Triton-X (Fisher Scientific)	permeabilization	(I,II)

5. RESULTS

5.1. Stromal ECM regulates cancer cell proliferation

5.1.1. CAFs have altered matrix architecture (I)

Fibroblasts are the main cell type to produce and organize ECM in the tissue. We were interested to study the role of activated fibroblasts, called CAFs, on ECM organization. We used CAFs isolated from HNSCC-patient stroma as well as normal fibroblasts (NF) from the skin of the same patients. We found that CAFs can be identified by the SMA-α expression, which is commonly used as a CAF-marker (I, Figure 3A). In addition, CAFs had increased expression of YAP/TAZ and integrin β1 expression compared to NFs (I, Figure 3B-D, Supplementary Figure 2G-H). Furthermore, based on our RNA sequencing results, all three CAFs and all three NFs clustered together (I, Supplementary Figure. 2C-D), which indicated that all NFs resembled each other and importantly CAFs, derived from different SCC patients, were similar to each other (I, Supplementary Figure 2C-D). However, all NF and CAF still expressed genes, such as vimentin and fibronectin, which are typical for the fibroblasts (I, Figure 2E).

Several different fibroblast cell lines have been successively used to produce cell derived matrix (CDM) *in vitro* by ascorbic acid induction (Cukierman et al. 2001). We analyzed the organization of normal fibroblast and CAF CDMs by immunofluorescence and by scanning electron microscopy (SEM). Based on the immunofluorescence analysis of the CDMs, CAF CDM was denser compared to NF-derived CDM, represented by the collagen I and fibronectin immunostaining (I, Figure 3E, Supplementary Figure 3A). SEM analysis, the surface structure of NF CDM was more organized compared to CAF-derived CDM (I, Figure 3F). We decided to use telomerase immortalized foreskin fibroblasts (TIFF) to model normal ECM (I, Supplementary Figure 2B). Normal human telomerase immortalized foreskin fibroblasts (TIFFs) lacked the SMA-α expression and the analysis of the CDM organization revealed that TIFF-derived CDM resembled that of normal fibroblast (I, Figure 1A). Since high collagen density has been connected to increased stiffness, we measured the elastic modulus of the CDMs by atomic force microscope (AFM). We found that CAF derived CDMs were approximately two times stiffer than their corresponding NF CDMs (I, Figure 4C). In addition the stiffness of TIFF CDM resembled that of normal fibroblasts (I, Figure 4D).

5.1.2. Normal ECM is tumor restrictive and induces sustained growth inhibition (I)

Stiffness is known to regulate many characteristics which are important for cancer cells, one of them being cell growth. We were interested to investigate whether NF- and CAF-derived matrices have distinct impact on carcinoma cell proliferation. We chose to study two different and commonly used cancer cell lines MDA-MB-231 breast carcinoma and HeLa cervical carcinoma cells. The proliferation of both cell lines was inhibited by NF CDM compared to the CAF-derived CDM (I, Figure 3G).

Given that the TIFF CDM mechanically resembled the NF CDM, we chose to continue the studies using TIFF CDMs as a model system. Similarly to NF CDM TIFF CDM was sufficient to reduce the proliferation of MDA-MB-231 cells compared to tissue culture plastic (I, Figure 1B) and compared to CAF derived CDM (I, Supplementary Figure 3C). ECM proteins such as fibronectin or collagen (I, Supplementary Figure 1A) I or solubilized CDM did not have any effect on MDA-MB-231 cell proliferation (I, Supplementary Figure 1B). In order to study if the growth inhibition was induced by soluble factors, we co-cultured MDA-MB-231 cells with different fibroblast cell lines. We could not see any significant difference in MDA-MB-231 cell proliferation when cells were cultured together with the normal fibroblast, with CAFs or if cancer cells were cultured in conditioned fibroblast medium (I, Supplementary Figure 1C and 3E-F). Thus, the growth inhibitory effects were inherently related to the intact normal CDM matrix.

In order to test whether normal CDM could induce sustained, possibly epigenetic, changes on cancer cell proliferation, cells were detached from the matrix by trypsinazation and re-plated on plastic (CDM to plastic). Interestingly, the growth inhibition of both cell lines was sustained even when cells were detached from the matrix and plated again on plastic (I, Figure 1C). Illumina whole genome transcription analysis was performed in order to investigate which genes are affected by direct exposure to CDM and which genes are differentially expressed in a sustained manner after CDM detachment (Figure 8). MDA-MB-231 and in HeLa cells were cultured 6 days on CDM on day 6 and 5 days after CDM detachment (I, Figure 1E).

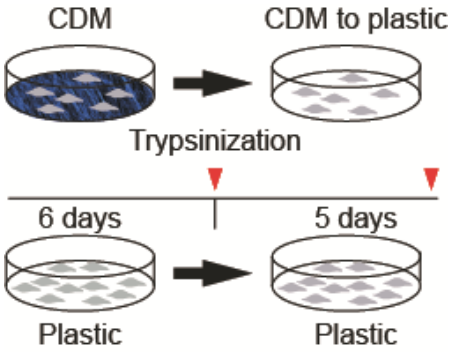

Figure 8. Schematic representation of the experimental setup. MDA-MB-231 and HeLa cells were plated on normal TIFF-derived CDM and on plastic. Their growth was monitored over 6 days after which they were trypsinized and re-plated on tissue culture plastic. Red arrows indicate the time of RNA-sample collection for Illumina microarray analysis.

Table 1. Common differentially expressed genes (FDR < 0.05) genes in the MDA-MB-231 and HeLa cell lines on CDM, CDM to plastic (PL.) and both conditions [CDM and CDM to PL). Values represent signal log-ratio (SLR).

CDM			**Both conditions**		
Symbol	**MDA-MB-231**	**HeLa**		**CDM**	
MMP3	3.32	1.43	**Symbol**	**MDA-MB-231**	**HeLa**
SERPINB2	2.09	1.35	PDE7B	-1.08	**-1.62**
ANGPTL4	1.49	3.06	SORBS2	-0.77	-2.15
COL1A2	1.43	1.37		**CDM to PL.**	
COLEC12	1.36	1.20	**Symbol**	**MDA-MB-231**	**HeLa**
COL1A2	1.20	1.91	PDE7B	-1.36	-1.05
PLAUR	1.12	1.37	SORBS2	-0.72	-1.08
CTSK	1.11	2.11	**CDM to PL.**		
PLAUR	1.09	1.72	**Symbol**	**MDA-MB-231**	**HeLa**
SERPINE1	0.90	1.41	FABP5	0.85	0.74
TMEM79	0.81	1.31	NME1	0.84	0.63
SERPINE2	0.75	1.82	GDF15	-1.91	-0.67
HMGCS1	-1.72	-1.51	DDIT4	-3.66	-0.61
ACAT2	-1.28	-1.89	MUC1	-1.78	-0.90
SCNN1A	-1.12	-1.26	ALDOC	-1.49	-0.97
PTGS1	-0.89	-1.64	FBXO32	-1.25	-1.22
FHL1	-0.86	-1.68	ANG	-1.21	-0.91
SORBS2	-0.81	-1.78	JMJD1A	-0.61	-0.64

The expression of 27 genes was changed on CDM and 151 genes after CDM detachment were changed in the same direction in both cell lines (MDA-MB-231 and HeLa) (Table I; I, Figure 1F, Supplementary table 1). Many matrix modifying proteins such as *MMP3*, *PLAUR1* and *SERPINE 1* and *SERPINE 2* were upregulated on matrix in both cell lines while many genes which are regulating cholesterol synthesis pathway (*IDI1*, *ACAT1*, *HMGCS1*) were downregulated on CDM. After the CDM detachment several hypoxia related genes were downregulated (*JMJD1a*, *ALDOC*, *DDIT4*, *GDF15*, *ANG* and *MUC1*) and MYC regulated genes, such as *FAPB5*, *NME1* were upregulated

5.1.3. JMJD1a is regulated by extracellular stiffness (I)

Since we were interested to study the mechanism of sustained -possibly epigenetic- growth inhibition, we searched for genes that are known to mediate epigenetic processes. Histone demethylase enzyme JMJD1a was one of the genes whose expression was altered after the CDM detachment. JMJD1awas validated to be downregulated on a protein level on CDM together with known mechanosensitive proteins YAP/TAZ (I, Figure 3D-E), while JMJD1a transcription was not changed (I, Figure 3F). After CDM detachment, JMJD1a downregulation was detected both on protein level and on mRNA level (I, Figure 3A-C). In addition to the expression changes, JMJD1a localization was found to be regulated by CDMs since we found that the cytoplasmic pool of JMJD1a increased when cells were cultured on CDMs (I, Figure 5G-H). This regulation was similar to the well characterized mechanosensitive proteins called YAP and TAZ (I, Figure 5I).

We tested whether CDM induced downregulation of JMJD1a was mediated by stiffness. We used hydrogels of different stiffness (0.5 kPa, 4 kPa and 50 kPa) to answer the question if extracellular stiffness is dependent on inhibition of JMJD1a expression and cytoplasmic translocation. We found that JMJD1a expression was decreased on hydrogels of low stiffness (I, Figure 5F). Furthermore, we found that JMJD1a localization was mediated by tension such that JMJD1a cytoplasmic pool increased when cells were exposed to low stiffness, similarly to on CDMs (I, Figure 5G-I). The regulation resembled the tension induced regulation of YAP and TAZ.

However, we decided to investigate whether JMJD1a is regulated by intracellular tension such as actomyosin network, which is known to regulate YAP/TAZ localization. JMJD1a was prominently nuclear even when the cellular actomyosin network was disrupted (data not shown). In addition, we used dominant negative Nesprin-2 construct to disconnect the nucleus from the actin cytoskeleton. However, we could not detect any changes in JMJD1a localization when endogenous Nesprin-2 was replaced with the dominant

negative (data not shown) indicating that intracellular tension is not a significant regulator of JMJD1a.

5.1.4. JMJD1a regulates proliferation and YAP /TAZ expression (I)

JMJD1a has been reported to regulate proliferation of several different cancer types, but its contribution on breast cancer growth had not been studied. We were interested to test whether JMJD1a regulated growth of breast cancer cells and if the JMJD1a expression is related to the decreased proliferation on soft matrices. We found that JMJD1a silencing reduced the proliferation of MDA-MB-231 breast cancer as well as patient derived SCC cells *in vitro* (I, Figure 2G and Supplementary Figure 3H). In line with the JMJD1a silencing results, we found that the high exogenous JMJD1a expression was sufficient to increase the MDA-MB-231 cell proliferation as well as rescue the matrix induced growth inhibition (I, Figure 2H-I). The *in vivo* tumor growth of MDA-MB-231 was studied in chick chorioallantoic membrane (CAM) model. Silencing of JMJD1a reduced the tumor growth significantly (I, Figure 2J).

We were interested to study the connection of JMJD1a and mechanosignalling in detail. According to the immunofluorescence analysis JMJD1a and YAP/TAZ levels correlated in cells (I, Figure 6C). Interestingly, JMJD1a silencing induced the downregulation of YAP/TAZ expression both on protein (I, Figure 6D and E) and on mRNA (I, Figure 6G) level as well as reduced the expression of classical YAP/TAZ target genes Thrombospondin 1 (THBS1) and Connective tissue growth factor (CTGF) (I, Figure 6F). Forced expression of JMJD1a was sufficient to increase the expression of YAP/TAZ, THBS1 and CTGF (I, Figure 6H-J). In addition, we found that overexpression of JMJD1a is sufficient to sustain the YAP/TAZ expression on soft substrates such as on CDMs and soft hydrogels (I, Figure 6K and 6M). However, we could not detect any changes on YAP phosphorylation (pYAP-S127) when JMJD1a was depleted from the cells (data not shown) indicating that JMJD1a regulates YAP/TAZ on transcriptional, not on post-translational level. Furthermore, pYAP levels were not significantly altered by JMJD1a overexpression when cells were cultured on soft CDMs (I, Figure 6K-L).

In line with the correlated expression of JMJD1a and YAP/TAZ *in vitro*, JMJD1a and YAP/TAZ levels correlated also in clinical tumors. JMJD1a and TAZ (WWTR1) mRNA levels were found to correlate both in breast cancer samples (720 samples) and in SCC (47 samples) (Supplementary Fig. 7). In addition to total levels, nuclear localization of YAP/TAZ was accompanied with nuclear JMJD1a in breast tumors (I, Figure 7A) indicating that JMJD1a could be an important regulator of YAP/TAZ in human cancers.

Taken together, we found that ECM organization and elastic modulus are altered in CAF derived CDMs compared to normal fibroblast derived CDM. In addition, we found that soft tumor stroma regulates tumor growth by inducing whole genome wide epigenetic changes via histone demethylase JMJD1a. JMJD1a was identified as an important regulator of breast cancer cell growth *in vitro* and *in vivo* as well as previously unknown regulator of YAP/TAZ transcription.

5.2. Filopodia are essential for cancer cell motility

5.2.1. Myo10 regulates adhesion, migration and invasion of breast cancer cells (II)

The number of filopodia is increased in malignant cells and the expression of filopodia-related genes, such as Fascin, is known to correlate with poor prognosis. However, the role of Myo10 in breast cancer was not known. In order to study the biological function of Myo10 in breast cancer cells, stable Myo10 silenced clones (shMyo10) were created. We found that Myo10 depleted MDA-MB-231 cells adhered more slowly on collagen I (II, Figure 2F) and spreading cells had less –if any- filopodia (II, Figure 2H). Thus we concluded that Myo10 and filopodia are needed for the effective cell adhesion. We also analyzed the migration and invasion in 3D using Matrigel gels. We found that cell migration was less directional (II, Figure 2C) and the invasion of Myo10 silenced cells was reduced (II, Figure 2D).

Myo10 binds β-integrins in order to transport them to the filopodia tips. We could see that the intrafilopodial movement of Myo10 is highly dynamic and integrin β1 moves to the filopodia tip together with Myo10 (II, Figure 3A). Even though, integrins are seen in the filopodia tip, it is not known whether the integrin activity is important for the filopodia formation. Thus we treated the cells with integrin function blocking antibody (mab13) or with antibody that stabilizes the active conformation of integrin β1 heterodimers (12G10). Cells were able to form filopodia when integrins were inactivated, but the filopodia were shorter and their structure was different (II, Figure 3C). In addition, filopodia were more dorsally localized and cells had less integrin α5 positive filopodia when filopodia formation was induced by Myo10 construct that lacks the FERM-domain (EGFP-Myo10ΔFERM2) (II, Figure 3B and 3D). This implies the interaction between Myo10 and integrins is important for the proper filopodia formation and localization. We also tested whether integrin-Myo10 interaction has a functional role in cancer cell invasion. Cells which were transfected with wild type Myo10 were able to invade into the Matrigel plugs while EGFP-Myo10ΔFERM2 cells were not. This implies that correct localization of integrins is needed for Myo10 induced invasion of cancer cells.

Since Myo10 regulated the invasion and migration *in vitro*, we were interested to see whether it is needed for the cancer cell spreading *in vivo* as well. Control and Myo10 silenced cells were injected into perivitelline cavity zebrafish embryos. While the control cells were capable of invading to the tail of the developing fish, shMyo10 cells were invading only locally around the injection site (II, Figure 4A). Similar results were obtained using a mouse model. Differentially labelled Control and Myo10 silenced cells representing three different breast cancer cell lines were injected into the blood stream of nude mice and their extravasation to the lungs was examined after 48 h. We found that Myo10 depleted cells extravasated significantly less to lungs (II, Figure 4C-D). We also tested the ability of these cells to colonize and to metastasize to the lungs. 4 weeks after the tail vein injection, mice injected with control cells had extensive lung metastases while shMyo10 injected mice had less and smaller metastases (II, Figure 4E). These cells were also incapable of metastasizing from the primary tumor to the lymph nodes as shown using the orthotopic breast cancer model (II, Figure 4G). All these data indicate that Myo10 is important for the breast cancer invasion and metastasis *in vivo*.

5.2.2 Myo10 expression correlates with patient survival and p53 mutation (II,III)

The expression of several filopodia-related genes was analyzed in a large breast cancer data-set (Miller et al. 2005). Cancers were divided into five main subtypes: Her2-positive, basal-type, Luminal A and Luminal B and normal-like. Genes which are known to drive filopodia formation were included in to the analysis. We found the expression of *MYO10*, *FSCN1* and *FNML2* genes was upregulated in the basal type breast cancer (III, Figure 3). We also compared the expression of these genes to the well-established cancer-related mutations such as p53, proliferation marker Ki67 as well as estrogen-, progesterone and Her2-receptor status and found that filopodia-related genes were upregulated especially together with p53 mutation. Since the basal-type breast cancer is considered as the most aggressive and the most invasive breast cancer subtype, we wondered whether filopodia, and especially Myo10, could have a prognostic role in the survival of breast cancer patients. In addition, we were interested to study if the p53 mutation could be related to high Myo10 expression in basal-type breast cancer patients.

We used tissue microarray (TMA) to analyze the Myo10 expression in 649 breast cancer patient samples. Myo10 was expressed especially on the tumor edges and on the borders of lymph node metastases (II, Figure 5B). We found that high Myo10 expression indeed correlated with the decreased survival of breast cancer patients (II, Figure 5E). The prognostic role of Myo10 expression was strong especially among patients with lymph node metastasis (II, Figure 5F). According to our earlier in silico analysis (III, Figure 3), we found that

Myo10 expression was highest in basal type-breast cancer and it correlated with mutated p53.

Myo10 expression was also studied in pancreatic ductal adenocarcinoma (PDAC) mouse model. Myo10 expression was approximately two times higher in the pancreas of mutant p53 (R172H) mice compared to wt mice (II, Figure 6C). TP53 mutation is known to occur in the late stages of PDAC advancement. In the early stages of PDAC development, called pancreatic intraepithelial neoplasia (PanIN), p53 mutation as well as Myo10 expression were both low or absent (II, Figure 6D), However, in late PanIN, when p53 mutation typically occurs, also Myo10 levels were found to be increased. Further, when PDAC was fully developed, both Myo10 and p53 were highly expressed.

5.2.3 Mutant p53 regulates Myo10 expression via transcription factor EGR-1 (II)

These findings that Myo10 expression positively correlated with p53 mutation, encouraged us to study whether mutated p53 regulates Myo10 expression. We found that Myo10 and mutant p53 are co-expressed in basal type breast cancer cells (MDA-MB-231), while the level of Myo10 was low or absent normal breast cell line MCF10a and MCF7 which represent luminal type breast cancer and which both have wild-type p53 (II, Figure 6A). We could also reproduce these findings of PDAC mouse model in PDAC cell lines, since Myo10 was highly expressed in cell lines which possess mutant p53 (MiaPaca, Panc1) while it was almost absent in p53-null AsPC1 cell line (II, Figure 6E).

Besides positive correlation, we found direct evidence that mutant p53 could regulate Myo10 expression. Re-expression of mutant p53 (R175H and R273H) induced Myo10 expression in MCF7 breast cancer cells. In addition, the overexpression of mutant p53 (R273H) in HCT-116 cells led to increased Myo10 expression compared to the parental HCT-116 which have wtp53 or full p53 knock-out (KO) cells (II, Figure 6B). Vice versa, silencing of mutant p53 reduced the expression of Myo10 both on mRNA and protein levels. In addition, forced expression of mutant p53, but not wt p53, was sufficient to rescue the p53 silencing induced Myo10 downregulation (II, Figure 6F-G)

Promoter analysis revealed that Myo10 promoter does not contain classical p53 consensus sequence. However, we could find predictive binding site for early growth responsive protein 1 (EGR-1). EGR-1 was interesting since it has shown to regulate the expression of filopodia related genes (Cermak et al., 2010). Silencing of mutated p53 in MDA-MB-231 cells decreased EGR-1 transcription (II, Figure 7B) and further, depletion of EGR-1 further reduced

Myo10 expression (II, Figure 7C). Importantly, EGR-1 was shown to bind directly to Myo10 promoter (II, Figure 7D).

Mutant p53 was shown to have a functional role in regulating cancer cell invasion since the silencing of mutant p53 reduced the invasion of highly invasive MDA-MB-231 cells (II, Figure). However, mutant p53 depletion could not further reduce the impaired invasive capability of shMyo10 cells (II, Figure 7E, Supplementary figure 8B). Furthermore, overexpression of Myo10 in mutant p53 depleted cells increased the invasion significantly, while it failed to further increase the invasion potential of control silenced cells (II, Figure 7H) indicating that p53 induced invasion is partially mediated by Myo10. To conclude, we found that Myo10 is essential for breast cancer cell motility. We identified mutant p53 as promoter of Myo10 transcription together with transcription factor EGR-1 and thus found a new role for mutant p53 in the context of cancer cell motility.

6. DISCUSSION

6.1. Modelling of tumor stroma using CDMs (I)

Stromal cells, especially fibroblast, are active contributors of carcinogenesis. Tumor progression involves active crosstalk between stromal and carcinoma cells. They may interact via direct cell-cell contact or indirectly via paracrine signaling such as producing growth factors and cytokines or secreting exosomes (Paggetti et al., 2015; Werth et al., 2008). It has been shown that mutations in tumor suppressor genes are as frequent in stroma as in carcinoma cells, thus is may be likely that stromal cells may be as important contributors of cancer initiation and progression as cancer cells.

The main role of normal tissue-resident fibroblasts is to produce ECM and maintain normal homeostasis. Thus one of the mechanisms how activated fibroblasts, CAFs, participate in cancer progression is via altering the normal architecture of the ECM. The impact of abnormal ECM organization has been studied particularly in the context of cancer cell invasion. Several studies have shown the importance of CAFs as leader cells for invading carcinomas (Calvo et al., 2013). In addition, changes in ECM structure, especially in the collagen organization, in cancer patients have been acknowledged as a contributor of cancer progression. However, how CAFs could remodel their ECM and how that could affect the cancer cell growth has not been intensively studied. In addition, the impact of normal, not cancer, matrix on cancer evolution is not well-established.

We used primary fibroblast cell lines isolated from the HNSCC patients. By using NF and CAF pairs isolated from the same patient, we were able to ask the question whether there is difference in their ability to organize the ECM structure. We first decided to look whether we could find a difference in the gene expression between NFs and CAFs which could indicate that their contractility or ability to remodel the matrix could be different. In line with earlier published observations, we found increased YAP and TAZ expression in CAFs compared to normal fibroblast. It has been shown that increased stiffness induces RhoA activation in CAFs which leads to upregulation of YAP/TAZ expression. Increased YAP/TAZ expression has shown to be important for CAF maintenance, contractility, angiogenesis as well as enhance cancer cell invasion (Calvo et al., 2013). Indeed, we could see that CAFs maintained their phenotype in culture for several passages. However, it was not rare that NFs transformed into CAFs. Constant monitoring of the gene expression, in our case the expression of SMA-α, of these cell lines was required. In line with this notion, some researchers have suggested that NFs should be maintained specifically on low-stiffness supports to prevent their conversion to CAFs by

continued exposure to the stiff environment of tissue culture plastic (D. Vignjevic, personal communication).

Part of the CAFs ability to influence cancer progression can be explained by their ability to affect the biochemical composition of the ECM and to modify the ECM structure (Levental et al., 2009; Provenzano et al., 2008). The role of integrin receptors, especially integrin β1, has been recognized by several research groups to participate in the generation of traction forces and tension induced signaling (Paszek et al., 2005; Roca-Cusachs et al., 2013; X. Zhang et al., 2008) and thus could have role in the ECM organization and remodeling. We found that CAFs are accompanied with increased integrin β1 expression, which could partly facilitate the different ECM organization capacity. Encouraged by these findings, we decided to look whether we could identify possible differences between the matrices derived by NFs and CAFs.

Biochemical and –physical properties of ECM, can be studied by producing fibroblast derived CDMs (Cukierman et al., 2001). The main interested was to study if and how the stromal ECM, produced by NFs and CAF, are distinct. We found that the collagen and fibronectin fiber organization CAF derived CDMs were denser compared to NF CDMs. Accumulation of collagen is frequently found in tumors (Provenzano et al., 2008). The role of excess collagen on tumor development has been studied especially using a mouse model, where the mice have a mutation in the α1 chain of type I collagen making it resistant to collagenase digestion. Collagen accumulation results in fibrosis in various tissues such as skin, uterus and mammary gland (Provenzano et al., 2006a). However, the tumor susceptibility of the mice remains to be investigated.

High collagen density, especially in the breast tissue, is a prognostic marker for tumorigenesis (Huo et al., 2015) and it increases the potential of breast cancer cells to invade and metastasize (Provenzano et al., 2008). Collagen signatures, which are classified into different subgroups, called TACS 1-3, are connected to cancer developmental status (Provenzano et al., 2006b). It has been shown that the highest collagen signature, TACS-3, is associated with poor survival and, importantly, serves as an independent prognostic marker regardless of tumor grade, size or hormone receptor and Her2 status (Conklin et al., 2011). Indeed, high collagen density in the breast increased both tumor formation and cancer cell invasion by approximately three-fold (Provenzano et al., 2008). These earlier studies connecting the tumor stoma with increase collagen density are in line with our results since we found that CAF matrices are associated with higher density of collagen and fibronectin fibrils.

The changes in ECM organization, especially collagen density, have been connected to increased tension within the tumor stroma. We found increased YAP/TAZ and integrin β1 expression in CAFs compared to NFs. Both YAP/TAZ and integrin β1 have earlier been connected to increased tension. Matrix

stiffness induces the RhoA and ROCK mediated contractility and cytoskeletal tension via integrin clustering (Paszek et al., 2005). Similarly, YAP/TAZ in CAFs is known to induce intracellular and ECM stiffening (Figure 1, (Calvo et al., 2013). The changes on Integrin β1 and YAP/TAZ expression implied that CAF and NF derived CDMs could differ by their elastic modulus. We decided to analyze the CDM stiffness by AFM. Indeed we found that CAF CDMs were approximately half stiffer that NF derived CDMs. It has been found that enzymatic collagen crosslinking by LOX induces that stiffening of the stromal collagen (Cox et al., 2013) and this could be among the underlying causes of the increased stiffness of the CAF matrix observed by us.

In conclusion, it has been shown that tumor stroma is stiffer and differs by its organization compared to the normal stroma. Our results imply that we could mimic the organization normal vs. tumor stromal ECM *in vitro*. Importantly, by exploiting the CDMs, we could study the effect of fibroblasts on matrix structure without the effect of cancer cells and other stromal components (Cukierman et al., 2001). Secondly, we could recapitulate the findings which were generated from different model systems such as *in vivo* models in mice and findings from cancer patients by exploiting CDMs (Levental et al., 2009; Provenzano et al., 2006a). Our results imply that stromal ECM produced by normal fibroblast could function as an anti-tumorigenic barrier, since it is sufficiently soft and possibly exhibit other growth inhibitory features which are absent from CAF derived ECM.

6.2. Stiffness mediated regulation of cancer cell growth (I)

Since we found that NF and CAF CDMs differ in their stiffness, we decided to test whether stiffness is an important regulator of carcinoma cell growth in our system. We first tested whether TIFF-cell derived CDM, representing NF CDM, is capable of inhibiting cancer cells growth. We decided to use TIFF cells to model normal fibroblast and NF CDMs, since it has been shown that immortalization of the normal fibroblasts by hTERT does not lead to malignancy (Lee, Choi, & Ouellette, 2004). Indeed we found that TIFFs do not express CAF marker genes, such as SMA-α. In addition, TIFF-derived CDM resembled patient fibroblast derived NF CDM by its organization and stiffness. According to our results the growth of both cell lines tested, MDA-MB-231 and HeLa, was inhibited by CDM. Furthermore, we found that CAF derived CDMs were less potent inducing growth suppression compared to NF derived CDMs. These data implied that growth inhibition could be directed by elastic modulus of CDMs.

Indeed, it has been shown that pharmacological depletion of tumor stroma in pancreatic cancer led to softening of the tumor, which further reduced the tumor growth and improved the survival of cancer patients (Von Hoff et al.,

2011). Furthermore, enzymatic collagen crosslinking by LOX supported the tumor growth and metastasis *in vivo*. It was further shown that LOX treatment was sufficient to induce the stiffening of the collagen. Increased tissue stiffening of the mammary glands was associated with integrin clustering and further PI3 kinase activation upon EGF-stimulation (Cox et al., 2013; Levental et al., 2009) indicating that collagen crosslinking is an important regulator of breast, probably also other, cancer cell growth and invasion.

However, there are several other parameters, in addition to stiffness, which can be different between NF and CAF CDMs. Mass spec analysis of normal and tumor tissue revealed that normal ECM and ECM extracted from tumors differ by their biochemical content (Naba et al., 2012). Indeed, it would be highly interesting to analyze different NF and CAF matrices via mass spectrometry- based approaches in future. However, we did analyze the transcriptomes of different NF and CAF cell lines and could see that their transcriptomes are partially distinct. However, detailed analysis and possible functional role of these differences still remained to be studied.

Since we were interested to analyze specifically the role of mechanosignalling on CDM induced regulation of JMJD1a, we decided to use mechanical supports of different stiffness. By using commercial hydrogels of different stiffness, we could see that these cell lines are highly sensitive to stiffness and their proliferation is affected by extracellular tension. We mainly used 0.5 kPa hydrogels which are considered to correspond to normal breast tissue and 4 kPa gels which correspond to stiffness similar to cancerous breast tissue (Lopez, Kang, You, McDonald, & Weaver, 2011). In addition, as a comparison we used the stiffest 50 kPa gels which were the closest to tissue culture plastic.

Interestingly, the growth inhibition was sustainable since the carcinoma cells continued to grow slower when they were detached from the CDM and re-plated on tissue culture plastic. It has been shown that high collagen content, accompanied with increased stiffness, induces the tumor formation and lung metastasis of breast cancer cells (Provenzano et al., 2008). Provenzano et al. found that increased invasion was sustained even when tumor explants were transferred into 3D collagen gels *in vitro*. They could show that tumor explants from collagen-dense tumors had increased colony formation and invasive potential into collagen gels in vitro even after 10 days of extraction. The mechanism of the sustained phenotype was not described, but it could be that there is also an epigenetic mechanism mediating the sustained invasive phenotype, similar what we see in the context of proliferation.

We and others have shown that stiffening of the tumor stroma contributes to tumor development and therefore it could be considered a target for the cancer therapy. Interestingly, kinase inhibitors, such as B-Raf inhibitor PLX4720,

which have been used to treat melanoma patients, are known to be effective initially but long term use induces drug resistance and eventually, even promotes melanoma progression. The mechanism of drug-resistance was discovered recently to be linked to increased activation of melanoma associated fibroblast (MAFs). PLX4720 was found to increase the number and contractility of MAFs and lead to stiffening of the tumor stroma. This further induced the integrin mediated activation of MAPK-pathway and thus induced proliferation and survival of melanoma cells (Hirata et al., 2015). This study suggests a possible mechanism why cancer drugs, such as kinase inhibitors, have not succeeded in the clinics and frequently cancer cells acquire resistance to the drug. The study of Hirata et al. implies that the resistance could arise due to the unexpected effects on stromal cells, indicating that the impact of stroma, and especially stromal stiffness, has an important role also on therapy response.

6.3. Mechanotransductional regulation of epigenomics (I)

My findings in this thesis describe a model of how the epigenomics could be regulated by the matrix stiffness via mechanotransductional regulation of histone demethylase JMJD1a. There are studies showing that the epigenetic landscape changes when cells are exposed to different extracellular stiffness (Downing et al., 2013; Y. Li et al., 2011). However, how stiffness regulates JMJD1a, or histone demethylases in general, has not been studied earlier. However, it has been shown that inhibition of H3K9 specific methyltransferases G9a or SUV39h1 are regulated in stiffness dependent manner. It was found that stiffness dependent regulation of G9a/SUV39h1 affects the self-renewal capability of melanoma cells, particularly highly tumorigenic subpopulation of melanoma cells called tumor-repopulating cells (TRCs) (Tan et al., 2014). They showed that the expression of histone methyltransferases increase on soft which facilitates the colony growth of TRCs. We found that the expression of histone demethylase JMJD1a expression is downregulated on soft substrates and downregulation inhibits growth of carcinoma cells. Tan et al. hypothesize that stiff matrix could be softened via degradation and remodelling of the matrix, which further induces the proliferation of TRCs. However, we hypothesize that matrix stiffening, which has been shown to occur during cancer development, reactivates the cancer cell proliferation via JMJD1a expression.

In addition to reduced expression, we found that the subcellular localization of JMJD1a is regulated by stiffness. JMJD1a is translocated from the nucleus to the cytoplasm on normal CDM as well as on soft hydrogels. We found this interesting since the mechanosensitive regulation of localization of epigenetic enzymes, has not studied previously. It is likely that the localization of several other histone modifying enzymes, such as G9a or SUV3h1, could be regulated

by stiffness and mechanical forces could contribute to the regulation of genome-wide gene expression of cells. This would be an interesting avenue for investigation in the future.

We were interested to identify the mechanism of mechanosensitive regulation of JMJD1a and thus decided to tests whether known JMJD1a regulators or established mechanotransducers could be responsible of the mechanosensitive regulation of JMJD1a. HIF1a and PKA are known regulators of JMJD1a expression and activity. HIF1a binds to JMJD1a promoter upon hypoxic condition and induce the transcription of JMJD1a. The expression of several hypoxia related genes were downregulated on CDM in the microarray analysis. In addition, there is some evidence that HIF1α could possibly be regulated by stiffness. However, we could not detect any downregulation of HIF1a on lover stiffness (data not shown). Based on these results we concluded that HIF1a is not a likely tension mediated regulator of JMJD1a. PKA regulates JMJD1a activity via phosphorylation. Phosphorylated JMJD1a was show to associate to the regulatory complex and activate its target gene transcription (Abe et al., 2015). PKA has also been connected to mechanotransduction and it is known to be regulated by tension. Tension, induced by stretching with magnetic beads, induced the PKA phosphorylation (T197) (Collins et al., 2014). The phosphorylation of this site is known to be needed for full activation of PKA (X. Cheng, Ma, Moore, Hemmings, & Taylor, 1998) indicating that increased tension activates PKA. Since we did not see any change on JMJD1a localization after PKA activation, we concluded that PKA activity is not important regulator of JMJD1a localization on stiff environment. Whether PKA activation participates on JMJD1a regulation on soft environment, still remains to be investigated.

Known mechanosensitive proteins, transcription factors YAP/TAZ are known be regulated similarly as JMJD1a: they are transported from the nucleus to the cytoplasm and their expression is downregulated on soft (Dupont et al., 2011b). The mechanosensitive regulation of YAP/TAZ is regulated by intracellular stiffness and actomyosin network, since pharmacological disruption of actin cytoskeleton organization induces the accumulation of YAP/TAZ to the cytoplasm (Dupont et al., 2011b). Dupont et al. could show that mechanosenstive regulation of YAP/TAZ is dependent on ROCK since inhibition of its activity leads to cytoplasmic translocation even on stiff substrates. In addition, stiffness induced regulation of other epigenetic enzyme, methyltrasferase G9a, is mediated by GTPase Cdc42 (Tan et al., 2014). Tan et al. showed that extracellular tension induced intracellular stiffening of the cells and this was induced by Cdc42. We were interested to test whether JMJD1a could be regulated by similar manner. However, the inhibition of actomyosin network nor the RhoA effector, ROCK-kinase, did not have any effect on JMJD1a localization.

We also hypothesized that intracellular or nuclear stiffness could participate in JMJD1a regulation, since it has been shown that inhibition of LINC complex, insensitizes the nucleus to substrate rigidity and impairs the intracellular force transmission (Lombardi & Lammerding, 2011; Lovett, Shekhar, Nickerson, Roux, & Lele, 2013). However, in our system not JMJD1a or YAP/TAZ were affected when nucleus was disconnected from the actin cytoskeleton using dominant negative Nesprin-2. All these results implied that JMJD1a is regulated entirely by extracellular, rather than intracellular, tension. In addition, it the regulation is similar to known mechanosensitive transcription factors YAP/TAZ but the mechanism of regulation is likely to be distinct. However, the exact mechanism of JMJD1a mechanosensitive regulation was not discovered.

6.4. Transcriptional regulation of YAP/TAZ (I)

We found that JMJD1a participates in mechanotransduction by inducing YAP/TAZ transcription. This was interesting since the regulation of YAP/TAZ transcription has not been previously studied. YAP/TAZ regulation occurs mainly on the post-translational level, via phosphorylation and phosphorylation induced degradation (Zhao et al., 2010). YAP/TAZ are known regulators of growth, differentiation and they are known to be activated during cancer development (Jerhammar et al., 2014b; N. G. Kim et al., 2011; F. Liu et al., 2015).

We found that JMJD1a depletion decreased the YAP/TAZ expression on a transcriptional level. In addition, we could not detect any changes on YAP/TAZ phosphorylation (YAP-S127) nor changed protein stability upon JMJD1a silencing and YAP/TAZ localization was not changed when JMJD1a was silenced. Phosphorylation of YAP/TAZ is leads to cytoplasmic localization, degradation and thus decreased stability of YAP/TAZ. Our results implied that the JMJD1a mediated regulation of YAP/TAZ is caused by changes at the transcriptional level. We hypothesize that the transcriptional regulation could be mediated by JMJD1a catalytic activity, by removal of transcription suppressing histone mark H3K9me2 on YAP/TAZ promoter. However, this hypothesis still remains to be confirmed. This could be done by measuring the H3K9me2 levels of YAP/TAZ promoter upon JMJD1a silencing by chromatin immunoprecipitation (ChIP).

JMJD1a is known to regulate proliferation of several different cancer cell types and some of the known target genes, which contribute to the proliferation control, are C-Myc, HOXA1 and GDF-15 (Cho et al., 2012; Fan et al., 2015; Krieg et al., 2010b). Since we identified YAP/TAZ transcription factors to be regulated by JMJD1a in several different cancer cell lines (breast cancer, cervical cancer and SCC), they are probable contributors of JMJD1a induced proliferation control among other known target genes. In conclusion, our results

indicate that not only JMJD1a is regulated by tension and mechanosignalling, but it also functions as previously unknown mechanoregulator upstream of YAP/TAZ.

6.5. Filopodia as promoters of cancer cell motility (II)

In order to invade from the primary tumor and metastasize, cells need to go through the basement membrane, invade through the stromal ECM network and intravasate into blood or lymph vessels. The colonization of the spreading cancer cells is dependent on cells ability to adhere and extravasate into the secondary organs such as lymph nodes or lungs. Many of the filopodia related genes are found to be upregulated in cancer and the function of some, for example *FSCN1*, has been link to increased invasion of carcinoma cells (Takikita et al., 2011). The role or unconventional myosin, called Myo10 as a promoter of filopodia formation has been well established, but its role in cancer had not been acknowledged earlier.

Myo10 is an important regulator filopodia and invadopodia formation (Schoumacher et al., 2010). Filopodia allow cells to probe the environment in order to move directionally. This is indeed important, especially during development and cancer progression when cancer cells invade from the primary tumor. Invadopodia, on the other hand are important devices for ECM degradation and thus invasion through the ECM. We found that Myo10, an important regulator and promoter of filopodia formation, induces motility of breast and pancreatic cancer cells. Silencing of Myo10 reduced the migration and invasion of breast and pancreatic cancer cells into 3D Matrigel matrix. In addition, we found that depletion of Myo10 and filopodia reduced the adhesion capacity of MDA-MB-231 breast cancer cells to collagen and fibronectin ligands. The role of Myo10 in breast cancer cell invasion has been recently acknowledged by other research groups (Cao et al., 2014b). Myo10 was shown to direct cancer cell invasion and metastasis via invadopodia induction (Schoumacher et al., 2010). We concluded that Myo10 regulates cancer cell invasion and migration through filopodia. However, we cannot exclude the possibility that some of the effect that we see could be mediated by invadopodia.

The main function of Myo10 is to transport cargo proteins, such as Integrin β1, to the filopodia tips. Integrin has been found to be activated at the filopodia tips but its role as a regulator of filopodia formation had not been established. We found that integrin activity on the filopodia tips is important regulator of filopodia structure and cancer cell invasion. Integrin inactivation by function blocking antibody, was sufficient to reduce the filopodia growth. In addition, we found that Myo10, which lacked the FERM-domain and thus is not able to transport the integrins to the filopodia tips, was unable to induce cancer cell invasion

through Matrigel matrix. These results indicated that Myo10 mediated transport and activation of integrins at the filopodia tips is important for the Myo10 induced invasion of cancer cells. Why is the integrin signalling important for filopodia function? It might be due to proper polarization and increased ability to sense the environmental signals in order to move directionally. Polarization includes integrin activation at the cell front as well as inactivation and endocytic trafficking of the integrins on the cell rear (De Franceschi, Hamidi, Alanko, Sahgal, & Ivaska, 2015). It is likely that integrins, as environmental sensors, induce the correct localization of filopodia and only correctly localized filopodia are able to mediate the invasion.

In line with these findings, silencing of Myo10 reduced the extravasation of several different breast cancer cell lines to the lungs when injected to the blood stream via tail vein. In addition, these cells were less potent to form colonies to the lungs within 4 weeks of time. The results implied that Myo10 silenced cells were less able to adhere (as also shown *in vitro)* and extravasate though the blood veins into the lung tissue. Myo10 silenced MDA-MB-231 cell not only has less lung colonies but the colonies were also smaller, which implied that Myo10 could also have a role in cancer cell growth after metastasis. Indeed, Myo10 has been reported to regulate cell division. Myo10 regulates mitotic spindle formation and pole localization in *Xenopus* embryo (Woolner, O'Brien, Wiese, & Bement, 2008). We also tested whether Myo10 silencing affected the proliferation of breast cancer cell. We could not detect any significant changes on cell growth upon Myo10 depletion. However, we did these experiments on 2D, on tissue culture plastic and it is possible that the proliferation of cancer cells in regulated by Myo10 *in vivo.*

6.6. p53 mutation drives Myo10 expression in basal-type breast cancer (II, III)

We produced meta-analysis from the earlier published data related to transcriptional profiles of primary breast tumors (L. D. Miller et al., 2005) and found that Myo10, together with several other filopodia related genes, is upregulated especially in the most aggressive basal type breast cancer. Since the breast cancer is a heterogeneous disease, it has been divided to five different subgroups: luminal A, luminal B, normal breast-like, Her2, and basal-like (Sorlie et al., 2001). These different groups differ in their gene expression as well as in their aggressiveness and predicted prognosis. The patients with Lumina A and B as well as normal like breast cancer are considered to have better prognosis and relatively good therapy response (Schnitt, 2010). Her2-type is accompanied with the upregulation of Her2-receptor while the basal-type breast cancer is typically triple negative, indicating the low or absent expression of hormone receptors (Estrogen and progesterone receptors) as

well as Her2. Her2 and basal type cancers are more aggressive, they have generally poor prognosis (Schnitt, 2010).

High Myo10 correlated with decreased survival especially among patients with regional lymph node metastasis. The correlation was significant also when the whole population was analysed and was especially pronounced among patients with regional lymph node metastasis. This could indicate the important role of Myo10 as regulator of cancer cell motility. The patients with metastasized cancer are most likely to be accompanied with highest Myo10 expression and the worst prognosis. Our results indicate that Myo10 expression could be possibly used as a prognostic marker especially to predict the survival and especially how likely the cancer will metastasize.

According to the meta-analysis related to the expression of filopodia-related genes in breast tumors, Myo10 was found to be upregulated upon p53 mutation. Mutated p53 has a well-established and studied role as a regulator of tumor growth and cancer cell invasion. The loss of p53 may function in several levels. Firstly, the mutation leads to loss of wt p53 and thus inhibition of normal p53 responses. In addition, mutant p53 possess gain-of-function capacities, which means that the mutated protein has entirely new or enhanced activity over the wt protein. However, since p53 is known to form functional tetramers, the mutation in only one allele or alternatively forced expression of mutant p53, may function as a dominant negative, inhibiting the normal tumor suppressor function of wt p53. Since we could not detect any difference in Myo10 expression between the wt p53 and full KO p53 cell lines we concluded, that the Myo10 upregulation in mutant p53 cells and tumors was due to gain-of-function, rather than resulting from the loss of wt p53. We used two common mutants of p53, R175H and R273H, which both results to accumulation of extremely stable mutant p53 in cells. Both mutants induced Myo10 expression in a similar manner, indicating that the impact was not specific to any particular mutation.

EGR-1 levels and mutant p53 activity has shown to correlate in several different cancer cell lines (Sauer, Gitenay, Vo, & Baron, 2010; Weisz et al., 2004). Sauer et al could not detect the direct binding of p53 to EGR-1 promoter but they found that mutant p53 drives EGR-1 expression via ERK-pathway activation (Sauer et al., 2010). However, the binding has been shown to occur in another study (Weisz et al., 2004). Since we found that Myo10 levels correlate with mutant p53, and revealed that EGR-1 directly binds to Myo10 promoter we concluded that mutant p53 regulates expression of Myo10 via EGR-1 and this pathway is needed for efficient invasion and metastatic spreading of basal type breast cancer cell.

Taken together, we identified a mechanism by which mutant p53 regulates Myo10 transcription via transcription factor EGR-1. We propose that p53 regulates EGR-1 expression rather than binding directly to Myo10 promoter. Increased Myo10 levels in mutant p53 expressing cells leads to increased filopodia number promoting invasion, migration and metastasis of breast cancer cells and reduces the survival of breast cancer patients.

7. SUMMARY AND CONCLUSIONS

The importance of tumor stroma on tumor evolution has been acknowledged by several studies. It has been shown that tumor stroma contributes to tumor growth, cancer cell metastasis, influences the drug response as well as serves as an independent prognostic factor. All these parameters imply that tumor stroma is probably as important contributor for cancer patient survival as are the malignant cells and therefore it would be important to know how the microenvironment influences cancer progression. Tumor stroma contains activated fibroblast, CAFs, and their role as promoters of cancer cell growth and invasion has been elucidated. Since the main role of fibroblasts is to produce and assemble ECM we were interested to investigate whether the ECM produced by CAFs differs from normal. Indeed, we found that collagen and fibronectin organization and elastic modulus were altered in CAF-derived CDMs, and these different matrices have distinct impact on cancer cell growth. The aim of this study was to identify the mechanism how normal stroma could regulate carcinogenesis. We found that soft tumor stroma regulates tumor growth by inducing whole genome wide epigenetic changes via histone demethylase JMJD1a. We found that JMJD1a in an important regulator of breast cancer cell growth *in vitro* and *in vivo* and identified YAP/TAZ to be regulated by JMJD1a.

Even though the dysregulated cell growth is important hallmark of malignancy, the main cause of death among cancer patients is the not the growth of the primary tumor but the metastatic spreading of the cancer cells. For example, in the case of breast cancer patient, metastasis the only cause of death and thus it is important to reveal the molecular mechanisms of cancer cell motility in order to improve cancer therapy and prolong the life of cancer patients. The role of filopodia and especially the impact of filopodia inducing protein, Myo10, were investigated in the context of breast cancer metastasis. We found that Myo10 and filopodia are important for breast cancer invasion and migration. In addition, we found that Myo10 is, when dysregulated and upregulated by mutant p53, a potent promoter of breast cancer metastasis.

To summarize, we were able to add new details and identify mechanisms how tumor stroma, particularly stromal ECM, as well as filopodia participate to tumor development by regulating cancer cell invasion and growth.

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