

Exploring the mechanobiology of Cancer Associated Fibroblasts

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SUMMARY

Fibroblasts are a heterogeneous group of cells essential for the maintenance of the extracellular matrix (ECM). They occur in different phenotypic states, such as the 'inactive' quiescent fibroblast and the 'active' differentiated myofibroblast. The 'active' form, is characterised by α -smooth muscle actin (α SMA) and plays an important role in remodelling the ECM, for example in wound healing and fibrosis. A specialised subpopulation of myofibroblasts are cancer-associated fibroblasts (CAFs), which are abundant in the tumour environment and play a central role in tumour growth and metastasis. Differentiation of quiescent fibroblasts to CAFs is stimulated by factors such as TGF- β and mechanical stress from the ECM. This leads to a feedback loop where mechanical stresses enhance CAF activation, while CAFs remodel the ECM and exert mechanical forces via focal adhesions (FAs), with vinculin playing a crucial role as a mechanotransducer between cytoskeleton and ECM.

This study investigated the mechanobiology of CAFs using FRET (Förster Resonance Energy Transfer) tension sensors targeting vinculin in FAs. FRET measures mechanical stress via energy transfer between two fluorescent proteins. Increase in stress stretches the sensor protein, increases the distance between donor and acceptor and thus decreases FRET efficiency. So changes in the distance between donor and acceptor fluorophores affect FRET efficiency, allowing measurement of mechanical forces within vinculin (and so in the FA) and at high resolution. The thesis focused on two phenotypic conditions: untreated CAFs and TGF- β -activated myofibroblast-like CAFs. The first objective was to develop a reliable protocol to differentiate CAFs with TGF- β to the myofibroblast-like phenotype. We then used the FRET-based vinculin tension sensor to investigate how mechanical forces within FAs differ between differentiated and undifferentiated CAFs. Activated CAFs showed a higher mechanical stress in vinculin and an increase in the number of FAs per cell, indicating increased contractility and force transmission. Finally, a plasmid-based fluorescent reporter was developed to track differentiation in living cells via fluorescence. Although the ACTA2 promoter (promotor of α SMA) alone proved insufficient to effectively activate the reporter, this approach provides a basis for further optimisation with the addition of the ACTA2 enhancer and enhancement of fluorescence proteins.

In conclusion, this study shows that TGF- β induces rapid and efficient differentiation of CAFs to a contractile myofibroblastic phenotype, with increased mechanical tension within vinculin in FAs and more adhesion complexes. This confirms the important role of mechanotransduction in CAF activation and their interaction with the microenvironment. Future research could focus on validation of the differentiation protocol in multiple CAF lines, refinement of tension sensors and optimisation of the live-cell reporter.

LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
ABPs	Actin-Binding Proteins
ADC	Analogue-to-Digital Converter
APD	Avalanche Photodiode
BG	Background signal
CAF	Cancer-Associated Fibroblast
CFD	Constant Fraction Discriminator
CLSM	Confocal Laser Scanning Microscopy
DC	Dichroic Mirror
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMT	Epithelial-Mesenchymal Transition
Enh	ACTA2 enhancer
F-actin	Filamentous actin
FA	Focal Adhesion
FALCON	FASt Lifetime CONtrast
FCS	Fetal Calf Serum
FD-FLIM	Frequency Domain FLIM
FGF2	Fibroblast Growth Factor 2
FLIM	Fluorescence Lifetime Imaging Microscopy
FRET	Förster Resonance Energy Transfer
GPCRs	G-Protein-Coupled Receptors
HOMO	Highest Occupied Molecular Orbital
IC	Internal Conversion
IF	Immunofluorescence
IL-6	Interleukin-6
IL-8	Interleukin-8
IPF	Idiopathic Pulmonary Fibrosis
ISC	Intersystem Crossing
LAP	Latency-Associated Pro-peptide
LB	Luria-Bertani
LTBP1	Latent TGF- β -binding Protein 1
LUMO	Lowest Unoccupied Molecular Orbital
mG	mGarnet2

MMPs	Matrix Metalloproteinases
mT	mTagBFP2
NLS	Nuclear Localization Sequence
P1	ACTA2 promotor1
P2	ACTA2 promotor2
pBB	pcDNA3.1 backbone
PDGF	Platelet-Derived Growth Factor
PFA	Paraformaldehyde
PMT	Photomultiplier Tube
RT	Room Temperature
S	Singlet State
SCL	Stable Cell Line
SMAD	Suppressor of Mother Against Decapentaplegic
T	Triplet state
TAC	Time-to-Amplitude Converter
TC	Transfected Cells
TCSPC	Time-Correlated Single Photon Counting
TD-FLIM	Time Domain FLIM
TDC	Time-to-digital converter
TFM	Traction Force Microscopy
TGF- β	Transforming Growth Factor β
TGFBRI	TGF- β receptor type I
TGFBRII	TGF- β receptor type II
TIMPs	Tissue Inhibitors of Metalloproteinases
TNF- α	Tumour Necrosis Factor- α
TSMoD	Tension Sensor Module
VEGFA	Vascular Endothelial Growth Factor A
VinTL	Vinculin Tailless Sensor
VinTS	Vinculin Tension Sensor
VinTS-I997A	Vinculin Tension Sensor I997A
α SMA	α -Smooth Muscle Actin

INTRODUCTION

1.1 Fibroblasts: origins and types

The human body consists of a great diversity of cell types, each with specific functions that collectively contribute to the maintenance and optimal functioning of the organism. Certain cell types play a crucial role in maintaining tissue structure and function. This category includes fibroblasts, a specialized cell population that plays a key role in maintaining the extracellular matrix (ECM) and regulating tissue repair^{1,2}. The ECM is a complex network of structural and functional biomolecules, including proteins and polysaccharides, that not only provides physical support to cells but is also involved in the regulation of biological processes such as signal transmission, cell adhesion and mechanotransduction. Fibroblasts are primarily responsible for the synthesis and turnover of ECM components, including structural proteins such as collagen and elastin³. These components determine tissue strength and elasticity and thus contribute to the mechanical and functional properties of various tissue and organ system⁴.

Fibroblasts are cells of predominantly embryonic origin from the primitive mesenchyme⁵. Mesenchyme develops from the mesoderm, which arises during early gastrulation⁶. However, a small subpopulation of fibroblasts has a different origin and originates from the neural crest, which arises from the ectoderm⁷. Despite the extensive study of these cells, variation in origin and diversity within fibroblasts make it difficult to establish a unified definition of fibroblast cell type⁵. In healthy tissue, fibroblasts can be described as non-inflammatory, non-vascular and non-epithelial cells present as individual cells in connective tissue and surrounded by the extracellular matrix^{8,9}. Morphologically, fibroblasts are characterized by a slender, elongated shape with a spindle-shaped profile (Fig. 1). In addition, they may exhibit a degree of planar polarity^{8,9}.

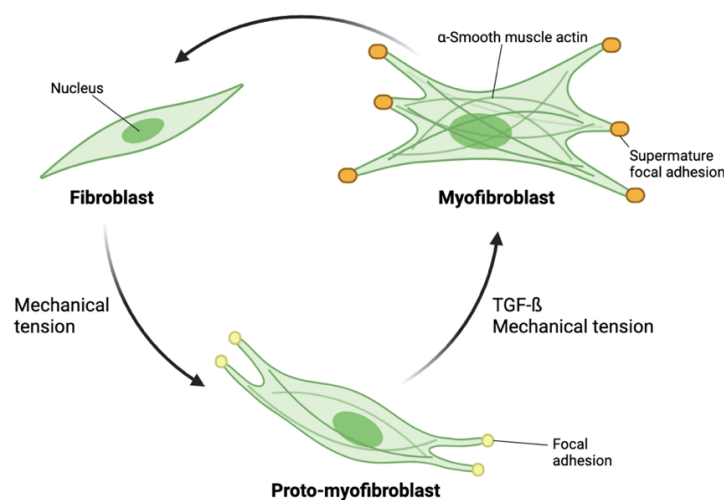


Figure 1. Schematic overview of fibroblast-to-myofibroblast differentiation. Fibroblasts exposed to mechanical stress and TGF- β differentiate into proto-myofibroblasts, which then progress to differentiated myofibroblasts. Differentiated myofibroblasts are mainly defined by their expression of α -smooth muscle actin and the development of supermature focal adhesions. Eventually, myofibroblasts can undergo apoptosis or return to the quiescent fibroblasts.

Fibroblasts can take different morphological and functional forms^{9,10}. *In vivo*, mainly the normal (quiescent) fibroblast is observed. This fibroblast responds to mechanical stress signals by producing cytoplasmic actin, which contributes to the formation of fibronexus-adhesion complexes¹⁰. These are specialised structures that connect intracellular stress fibres to extracellular fibronectin fibrils, thereby transferring forces between the myofibroblast and the ECM¹⁰. In addition, this fibroblast produces and organizes fibronectin and is referred to as a **proto-myofibroblast** (Fig. 1).

Upon the increase of mechanical stress signals, or by external stimuli such as transforming growth factor β (TGF- β), the proto-myofibroblast further differentiates into a **myofibroblast**^{9,10}. This form is distinguished by *de novo* expression of **α -smooth muscle actin (α SMA)** and the presence of advanced stress fibres. *In vivo*, this leads to the formation of larger fibronexus-adhesion complexes, while *in vitro*, on 2D culture surfaces, cells do not form fibronexus structures due to the lack of a well-defined 3D ECM environment. Instead, they develop supramature focal adhesions, which are enlarged and highly elongated adhesion sites. (Fig. 1)^{9,10}.

The function of ‘non-activated’ or quiescent fibroblasts can be described as their ability to respond to specific signals, such as growth factors, that trigger their differentiation into a myofibroblast or ‘activated’ fibroblast⁸. This differentiation plays a crucial role in several biological processes, including wound healing and fibrosis⁸. Fibroblasts and myofibroblasts also play an essential role in maintaining ECM homeostasis⁹. Myofibroblasts are responsible for the production of various fibrillar ECM components, such as fibronectin and collagen types I and III¹⁰. Furthermore, these cells produce matrix metalloproteinases (MMPs), which contribute to the dynamic balance and maintenance of ECM homeostasis¹¹. In addition to ECM components, fibroblasts and myofibroblasts produce cytokines and chemokines, which promote immune cell recruitment¹². Moreover, these cells exert physical forces essential for remodelling tissue architecture through their ability to generate and transmit mechanical stress¹⁰. This contractile ability is directly linked to the development of intracellular stress fibers, such as actin bundles, which enable myofibroblasts to exert tension on the ECM¹⁰. Mechanical stress is a key regulator of connective tissue remodelling and myofibroblasts play a crucial role in both physiological wound healing and pathological fibrosis¹³. The forces myofibroblasts exert on the ECM increase tissue stiffness, which in turn increases resistance to further contraction. This mechanical stress can then be sent directly to the cell nucleus or to nuclear-associated proteins such as YAP/TAZ¹⁴. However, the molecular pathways involved in the processes of wound healing and fibrosis are beyond the scope of this thesis.

1.1.1 Cancer-associated fibroblasts

The development of cancer cells triggers a response in the host, creating a microenvironment that includes various components of an otherwise healthy tissue¹⁵⁻¹⁷. This microenvironment, known as the tumour microenvironment, facilitates interactions between the components of the microenvironment and cancer cells. These interactions are key drivers of tumour formation and development¹⁷. The tumour microenvironment comprises several components, including immune cells, capillaries, the ECM and myofibroblasts¹⁸. These elements are located in the immediate vicinity of cancer cells and surround them, stimulating and regulating both cancer progression and metastasis¹⁷.

One of the most significant components within the tumour microenvironment are the myofibroblasts, which play a pivotal role in cancer progression and metastasis^{9,19}. These specialised myofibroblasts are referred to as cancer-associated fibroblasts (CAFs). CAFs are believed to influence various biological processes and molecular changes in the tumour environment across a wide range of tumour types²⁰.

In contrast to normal tissue, which contains only a small number of quiescent fibroblasts in the ECM¹⁸, the tumour stroma in cancer contains a significantly higher concentration of myofibroblasts and in this particular case CAFs²⁰, as characterized by the expression of α SMA. Cancer can, in fact, be conceptualised as a 'wound that never heals'^{18,29}. The accumulation and persistence of cancer cells create a sustained tissue injury that perpetuates a chronic wound healing response targeted at the cancer cells⁸. A similar theory to the wound healing process has been advanced in the context of cancer, suggesting that cancer cells recruit CAFs^{29,30}. This recruitment is primarily driven by growth factors such as TGF- β , platelet-derived growth factor (PDGF), and fibroblast growth factor 2 (FGF2). These growth factors are secreted by both cancer cells and infiltrated immune cells and play a crucial role in fibroblast differentiation²³. Of these growth factors, TGF- β plays the most prominent role in CAFs recruitment in numerous tumours. TGF- β stimulates both proliferation and invasion of CAFs⁸. Furthermore, these CAFs produce increased amounts of the ECM components, including fibrin and different types of collagens. This results in a stiffer ECM within the tumour microenvironment, which is an important factor in stimulating tumour growth and progression²⁴. The increased ECM stiffness can promote the transformation of normal epithelial cells into malignant cancer cells. In addition, this stiffness contributes to the proliferation, migration, and invasion of pre-existing cancer cells²⁴. The stiffer ECM induces intracellular contractions and increased stiffness of the actin cytoskeleton, which facilitates the mobility of cancer cells. Furthermore, cancer cells use the collagen bundles within the ECM as a kind of 'highway' for efficient migration. At the molecular level, the increased ECM stiffness activates the TGF- β signalling pathway, which stimulates the epithelial-mesenchymal transition (EMT) in cancer cells²⁴. This transition is a crucial driver for the invasiveness of cancer cells.

A key process within the cancer development is the angiogenesis, the supply of new capillaries that is crucial for tumour growth and survival. This process is regulated by several growth factors, including vascular endothelial growth factor A (VEGFA), epidermal growth factor (EGF), PDGF, interleukin-6 (IL-6), and interleukin-8 (IL-8)⁸. Although cancer cells themselves are responsible for the production of some of these growth factors, such as VEGFA, CAFs are also an important source of VEGFA²⁵. VEGFA plays a key role in inducing vascular leakage, leading to the formation of reactive perivascular areas²⁶. These areas contain, among other things, platelets and fibrin, which in turn promote the accumulation of immune cells, myofibroblasts, and proliferating endothelial cells.

1.2 Transforming Growth Factor Beta (TGF- β)

TGF- β is a key cytokine that stimulates quiescent fibroblasts to differentiate into myofibroblasts. The best-known TGF- β isoform involved in the differentiation of fibroblasts into myofibroblasts is TGF- β 1²⁷. Although TGF- β 2, TGF- β 3 and activin also contribute to this differentiation, they play a less prominent role²⁸. These signalling molecules are stored *in vivo* in an inactive form within the latency-associated pro-peptide (LAP) complex, which is located in the extracellular matrix (Fig. 2). When TGF- β is released from the LAP complex due to mechanical stress exerted by the ECM, it can bind to the transmembrane TGF- β receptor complex, which consists of a heterodimer of TGF- β receptor type I (TGFBRI) and type II (TGFBRII) (Fig. 2)²⁹. The classical TGF- β signalling pathway is regulated via serine/threonine phosphorylation of the receptors TGFBRI and TGFBRII. Specifically, the type II receptor (TGFBRII) phosphorylates the type I receptor (TGFBRI), which then activates the receptor-regulated suppressor of mother against decapentaplegic (SMAD) proteins, SMAD2 and SMAD3, by adding phosphate groups to them. Once phosphorylated, SMAD2 and SMAD3 form a complex with SMAD4. This complex then migrates to the nucleus, where it regulates the expression of target genes (Fig. 2)¹⁴. There, the complex binds to the promoter region of fibrotic genes, such as the gene encoding α SMA²⁹. Although this canonical SMAD pathway is central to the regulation of α SMA expression, it is only one part of a more extensive network of signalling pathways, which are beyond the scope of this thesis.

1.3 Mechanical stress causes myofibroblast activation

As previously discussed (§1.2), the LAP complex is localized within the ECM. Consequently, alterations in ECM properties can directly influence its function. Upon tissue damage, ECM stiffness increases, which impacts the LAP complex and may trigger the release of TGF- β . This release subsequently promotes the differentiation of quiescent fibroblasts into myofibroblasts. These myofibroblasts play then a critical role in tissue repair, as observed in skin wound healing³⁰.

In response to vascular injury, the body initiates haemostasis to prevent blood loss and promote tissue repair³¹. During this process, platelets aggregate and the clotting cascade is activated, ultimately resulting in the formation of a blood clot³¹. The resulting clot serves as an early provisional matrix and consists mainly of fibrin and fibronectin. Fibrin is generated from fibrinogen during clotting and forms a fibrous network that stabilises the clot and provides structural support at the wound site³¹. Fibronectin, a multifunctional glycoprotein, is also incorporated into the matrix and plays a crucial role in mediating cell adhesion and migration, both essential for the progression of wound healing. This early provisional matrix not only acts as a physical barrier against blood loss, but also temporarily retains activated platelets and growth factors (such as PDGF, VEGFA and TGF- β) at the site of injury, creating a bioactive scaffold that supports the recruitment and activation of various cell types involved in tissue regeneration.

As the healing process progresses, the ECM undergoes dynamic mechanical remodelling, driven by the activity of both infiltrating and resident cells. A key feature of this remodelling is strain-stiffening, where the ECM becomes progressively stiffer in response to tensile forces exerted by cells such as fibroblasts. This leads to the reorganisation and alignment of fibrillar ECM components, including fibrin and collagen, and marks a critical phase in tissue repair. The resulting increase in ECM stiffness increases mechanical stress in the tissue microenvironment. This stress is transmitted to cells via mechanosensitive surface receptors such as integrins and G protein-coupled receptors (GPCRs)³². Integrins, which physically connect the ECM to the cytoskeleton, play a central role in mechanotransduction. Another important downstream target is the LAP complex, as discussed in §1.2. Under mechanical stress, the LAP complex unfolds, releasing active TGF- β ²⁹. Once activated, TGF- β binds to its receptors on the surface of fibroblasts, triggering intracellular signalling cascades, such as the SMAD pathway, that drive the differentiation of fibroblasts into myofibroblasts (Fig. 2)¹⁴.

This process is part of a mechanobiological feedback loop, increased ECM stiffness enhances integrin-mediated activation of the LAP complex, resulting in a greater release of TGF- β , which in turn promotes additional fibroblast-to-myofibroblast differentiation and ECM firming. Mechanical signals, TGF- β signalling and cytoskeletal contractility are thus closely linked in regulating tissue repair via progressive ECM remodelling.

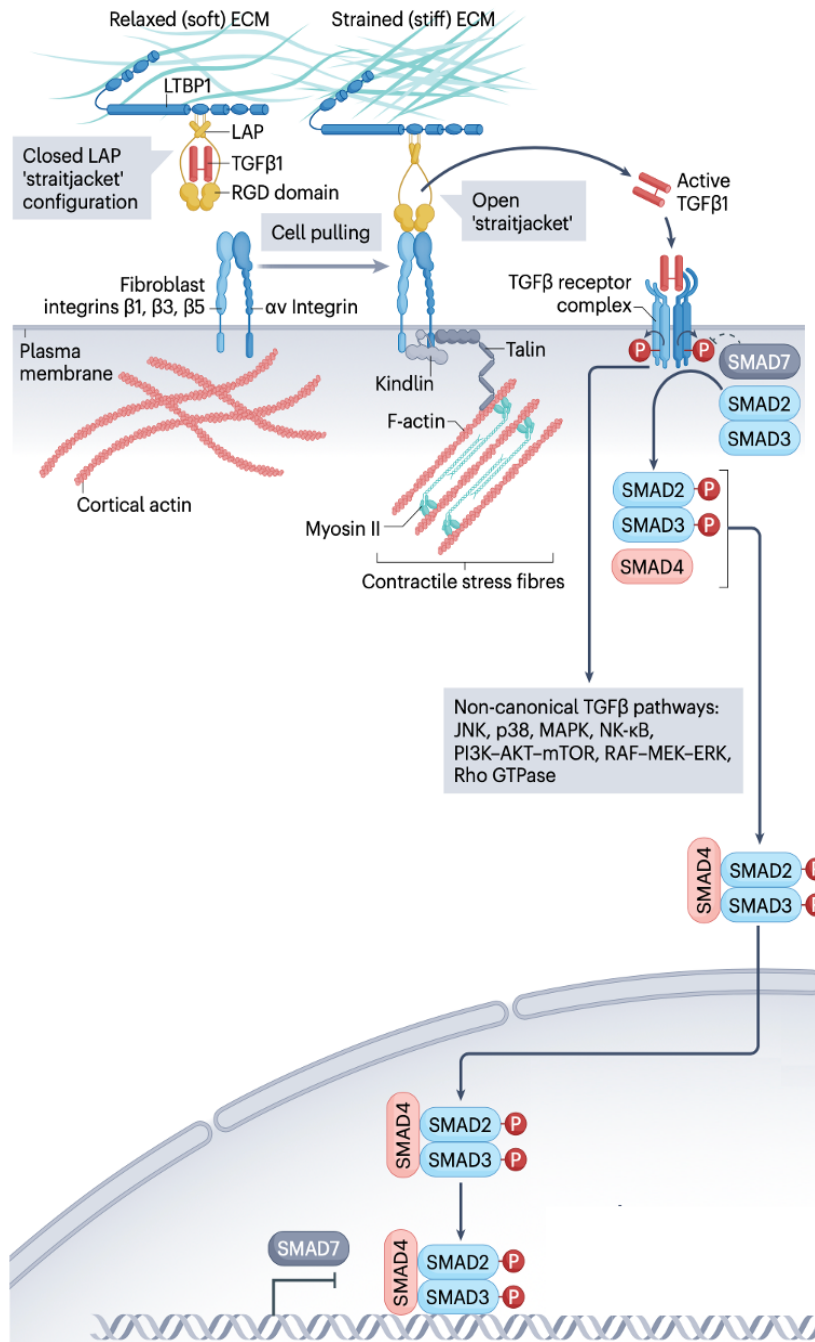


Figure 2. TGF- β signalling pathway for myofibroblast activation. Fibroblasts secrete TGF- β 1 in an inactive form, bound to the latency-associated peptide (LAP). This inactive complex is stored in the extracellular matrix (ECM) via binding to latent TGF- β -binding protein 1 (LTBP1). Mechanical stress, such as forces transmitted by stress fibres and integrins, can break the binding of the LAP complex in the ECM, releasing and activating TGF β 1. Once activated, TGF β 1 binds to the transmembrane TGF- β receptor complex, which consists of a heterodimer of TGF- β receptor type I (TGFBR1) and type II (TGFBR2). This activates the classical (canonical) SMAD signalling pathway. In this process, SMAD2 and SMAD3 are phosphorylated by the receptors, after which they form a complex with SMAD4. This complex migrates to the cell nucleus and binds to SMAD binding elements in the promoter region of fibrotic genes, such as ACTA2, which encodes α -smooth muscle actin (α SMA). SMAD7 acts as a negative regulator of this process¹⁴.

1.4 Focal Adhesions and Vinculin

Focal adhesions (FAs) represent the primary mechanical and biochemical interface between a cell and its extracellular microenvironment. As dynamic multimolecular complexes that mechanically couple the ECM to the intracellular actin cytoskeleton, they provide an essential platform for the detection and transduction of mechanical signals³³. This makes FAs particularly suitable for studying the mechanobiology of cells. The central components of FAs are integrins, a family of 24 $\alpha\beta$ -heterodimeric transmembrane receptors distinguished by their specific binding affinity for diverse ECM ligands and their ability to transduce bidirectional signals³⁴. Upon activation, integrins aggregate into FAs, where they are linked to the actin skeleton via a cascade of protein-protein interactions³⁴. Talin is an actin-binding proteins (ABPs) recruited to FAs and functions as a key adaptor between activated integrins and filamentous actin (F-actin). Talin consists of an N-terminal head domain, which recognizes and binds the cytoplasmic tail of β -integrins, and a C-terminal rod domain containing multiple binding sites for both actin and vinculin³⁵. In its auto inactive conformation, these binding motifs are obscured by intramolecular interactions. Mechanical tension, induced by actomyosin contraction, induces a structural stretch of talin, exposing cryptic binding sites and allowing further assembly of FA components such as vinculin³⁵.

Vinculin is an essential protein that plays a crucial role in the regulation of processes such as cell adhesion and migration^{36–38}. It is part of FAs and forms a bridge between the cytoskeleton protein actin and FA protein talin³⁸. The binding of vinculin to actin stimulates actin polymerisation, leading to the recruitment of remodelling proteins. Vinculin can be divided into two conformations: the active or 'open' form and the inactive or 'auto-inhibited' form. The active form is found in focal adhesion sites, while the inactive form is mainly found in the cell's cytoplasm^{38,39}.

In the auto-inactivated conformation, the binding sites for ligands, such as talin, are not accessible. This conformation is stabilised by intramolecular interactions between the N-terminal head domain and the C-terminal tail domain of vinculin³⁹. The active, 'open' conformation occurs when these interactions are broken. This activation proceeds through a strictly regulated two-step process, in which a 'semi-open' intermediate conformation is first formed before the fully open structure emerges⁴⁰. Studies have shown that vinculin in FAs occurs predominantly in the open conformation, while in the cytoplasm the auto-activated form is predominant⁴¹. The open conformation of vinculin in FAs plays a crucial role in cell-cell and cell-matrix interactions. In this context, vinculin functions as a scaffold protein that regulates the recruitment of other focal adhesion-associated proteins, including the Arp2/3 complex, which facilitates actin remodeling at FAs³⁸. Moreover, vinculin contributes to the stabilisation and maturation of FAs, partly by crosslinking actin bundles and promoting the formation of new filament structures. These interactions enhance the mechanical integrity of FAs and support sustained cell adhesion. However, it should be noted that vinculin adopts more complex structural conformations *in vivo* than *in vitro*. In particular, a subset of vinculin

molecules within FAs remains in the closed conformation, which appears to contribute to the dynamic turnover of FAs³⁸. This suggests that a finely tuned balance between the active and inactive forms of vinculin is essential for proper cellular functioning, particularly during processes such as cell migration.

Given its dynamic regulation, strategic location, and mechanosensitive nature, vinculin represents an ideal target for studying the mechanobiology of the cell. Its involvement in both cell–cell and cell–ECM interactions makes it especially relevant in contexts where force transduction, adhesion remodeling, and structural coordination are critical for cellular behaviour and tissue organisation.

1.5 Introduction to fluorescence microscopy

Fluorescence is a fundamental photophysical phenomenon that enables the visualization of biological structures with high sensitivity and spatial resolution. It is based on the ability of fluorophore molecules to absorb light at a specific excitation wavelength and subsequently emit light at a longer wavelength. This spectral shift, known as the Stokes shift, allows for the selective detection of fluorescent signals from target molecules and subcellular components^{42–44}.

1.5.1 Fluorescence

Fluorescence starts with the absorption of a photon by a fluorescent molecule, a fluorophore. The energy E possessed by the photon can be described by the relation:

$$E = \frac{h \cdot c}{\lambda} = h \cdot \nu \quad \text{Eq. 1}$$

Here, h is Planck's constant (6.626×10^{-34} J-s), c is the speed of light in vacuum (2.99×10^8 m/s), ν is the frequency, and λ is the wavelength of the photon.

When a fluorophore is in the ground state (S_0), it can absorb photons with sufficient energy. This results in electronic excitation to a higher singlet state (usually S_1 or S_2) (Fig. 3a)^{42–44}. At the molecular level, this means that an electron transitions from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO). This transition is extremely fast, on a time scale of femtoseconds. After excitation, the electron typically first relaxes to the lowest vibrational level of the S_1 state via non-radiative processes such as vibrational relaxation and solvent rearrangement, this process is called internal conversion (IC). Then the electron radiatively returns to the ground state (S_0) under emission of a photon. Since part of the absorbed energy is lost during the relaxation process, the emitted photon has a lower energy and thus a longer wavelength than the excitation photon. This emission is called fluorescence and occurs on a time scale of 1 to 10 nanoseconds. The difference

between the maximum excitation and emission wavelength is known as the Stokes shift (Fig. 3b). This shift is crucial in practice because it allows the emission signal to be spectrally distinguished from the excitation light. By using optical filters, the excitation light can be effectively suppressed, detecting only the fluorescence signal. This makes fluorescence a highly sensitive and specific analysis technique in complex biological systems.

Besides fluorescence, alternative relaxation pathways exist. For instance, via a spin forbidden transition (intersystem crossing (ISC)), the electron can transition to a triplet state (T) (Fig. 3a). This process leads to phosphorescence, a delayed emission that occurs on a much longer time scale, from milliseconds to seconds. Another possibility is quenching, in which the excited energy is not emitted as light but instead transferred to surrounding molecules via non-radiative transfer. This results in loss of fluorescence signal. In summary, fluorescence offers a powerful analytical technique for visualising molecules and biological structures^{42–44}.

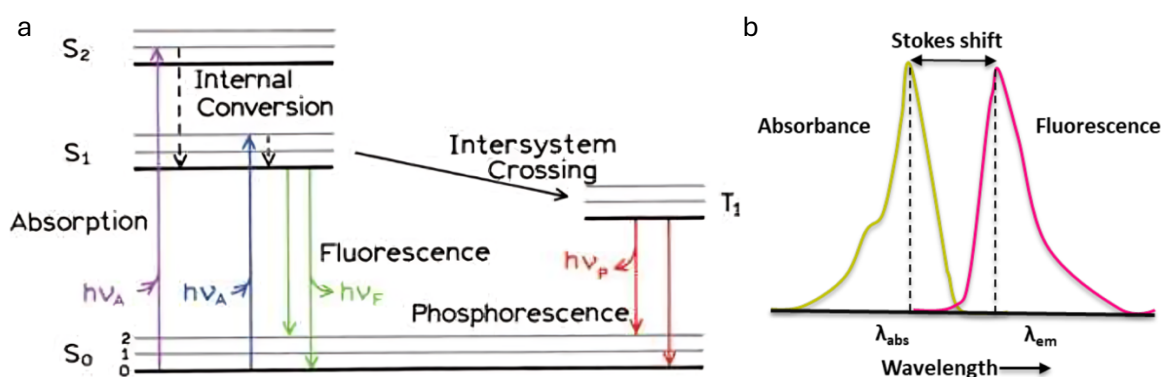


Figure 3. Jablonski diagram and Stokes shift. (a) Jablonski diagram illustrating electronic states and associated vibrational energy levels. S_0 , S_1 , and S_2 represent the ground and excited singlet states, while T_1 denotes the first excited triplet state. Absorption of a photon is indicated by the purple and blue arrows, fluorescence emission by the green arrow, and phosphorescence by the red arrow. The black dashed arrow represents internal conversion (IC) and the full black arrow represents intersystem crossing (ISC) from the singlet to the triplet state⁴². (b) Absorption and emission spectra illustrating the Stokes shift. The graph shows the relative intensity of light absorption and fluorescence emission as a function of wavelength. The yellow peak represents the absorption maximum at a shorter wavelength, while the purple peak indicates the emission maximum (fluorescence) at a longer wavelength. The Stokes shift, the spectral difference between absorption and emission maximum, is indicated by the double arrow⁴⁴.

1.5.2 Förster Resonance Energy Transfer

Building on the principles of fluorescence, more advanced techniques have been developed to study molecular proximity and interactions. One of the most prominent is Förster Resonance Energy Transfer (FRET). FRET is based on a radiation-free energy transfer process, in which the energy of an excited fluorophore (donor) is transferred to a second fluorophore (acceptor) via dipole-dipole coupling on a quantum scale (Fig. 4)⁴⁵. This energy transfer requires the

donor and acceptor to be within 1-10 nm of each other. After energy transfer, the acceptor enters an excited state, after which it returns to the ground state by energy loss via photon emission, manifested as fluorescence §1.5.1.

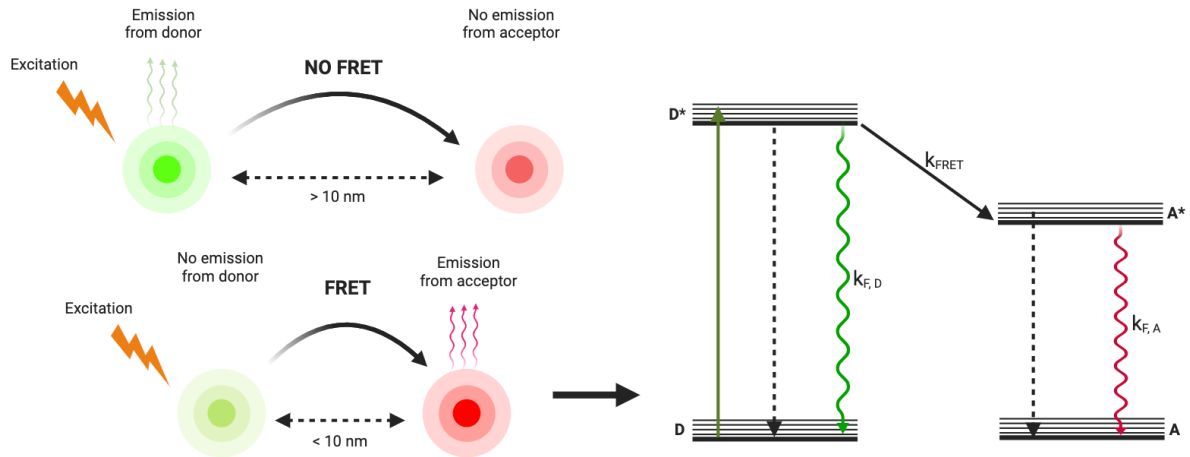


Figure 4. Principles of Förster Resonance Energy Transfer (FRET). When the distance between the donor and acceptor fluorophore is greater than 10 nm, no energy transfer takes place. However, if the distance is less than or equal to 10 nm, energy is transferred from the donor to the acceptor. This process can be represented in a simplified Jablonski diagram, in which energy is transferred from the excited donor via FRET to the acceptor, which then fluoresces. The rate of this energy transfer is determined by the rate constant, k_{FRET} . If there is no energy transfer (distance greater than 10 nm), the donor itself may fluoresce.

When considered at the mechanistic level, the process appears to involve a two-step process: (i) the excitation of the donor from the ground state by absorption of photons, and (ii) the energy transfer from the excited donor to the acceptor via dipole-dipole coupling⁴⁶. The energy transfer efficiency is expressed as the FRET efficiency (E_{FRET}), which indicates the probability of energy transfer from the donor to the acceptor. This efficiency is determined by the distance (R) between the two molecules and is related to the inverse sixth power of R , as shown in equation 2. Here R_0 , the Förster radius or critical distance, plays a crucial role. The Förster radius is the characteristic distance at which E_{FRET} is 50%. This relationship can be described by the following equation:

$$E_{FRET} = \frac{R_0^6}{R_0^6 + R^6} \quad \text{Eq. 2}$$

When R is smaller than R_0 , E_{FRET} approaches its maximum value, whereas E_{FRET} is close to its minimum when R is greater than R_0 . The Förster radius can be described using Equation 3, which defines its relationship when FRET is measured in an aqueous solution.

$$R_0 = [8.79 \times 10^{-5} (\kappa^2 \eta^{-4} Q_D J(\lambda))]^{\frac{1}{6}} \quad \text{Eq. 3}$$

In equation 3, κ^2 represents the orientation factor, which describes the angle between the dipole moments of the two fluorophores. For effective energy transfer, the separation vectors of the fluorophores must be in a favourable orientation to each other. The value of κ^2 can vary between 0 and 4, where $\kappa^2 = 4$ indicates parallel dipole moments in a head-to-tail configuration. A value of $\kappa^2 = 1$ describes a parallel 'side-to-side' orientation, while $\kappa^2 = 0$ corresponds to dipole moments forming a 90° angle. Since κ^2 is often unknown, it is usually assumed that κ^2 has an average value of 2/3. The relationship between the orientations of the fluorophores can be described by the following equation:

$$\kappa^2 = (\cos\theta_{DA} - 3\cos\theta_D\cos\theta_A)^2 \quad \text{Eq. 4}$$

In equation 3, η represents the refractive index of the medium, while Q_D denotes the quantum yield of the donor. $J(\lambda)$ represents the spectral overlap integral between the normalized donor fluorescence ($F_D(\lambda)$) and the acceptor absorption spectrum. The acceptor absorption spectrum is directly determined by the molar extinction coefficient $\epsilon_A(\lambda)$. The relationship for $J(\lambda)$ can be expressed as follows:

$$J(\lambda) = \frac{\int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda}{\int F_D(\lambda)d\lambda} \text{ mol}^{-1}\text{cm}^{-1}\text{nm}^4 \quad \text{Eq. 5}$$

The E_{FRET} can also be described using an alternative approach, namely through **the fluorescence intensity** or **the lifetime of the donor molecule**. In its most simplified form (Eq. 6, left), I_{DA} represents the intensity of the donor in the presence of the acceptor, and I_D represents the intensity of the donor in the absence of the acceptor. Similarly, τ_{DA} is the lifetime of the donor in the presence of the acceptor, while τ_D refers to the lifetime of the donor in the absence of the acceptor (Eq.6, right). The relationships between these parameters can be expressed as follows:

$$E_{\text{FRET}} = 1 - \left(\frac{I_{DA}}{I_D}\right) \text{ and } E_{\text{FRET}} = 1 - \left(\frac{\tau_{DA}}{\tau_D}\right) \quad \text{Eq. 6}$$

The FRET principle depends on various parameters. Overall, E_{FRET} is strongly related to the distance between the two fluorophores (R) and the Förster radius of the FRET pair (R_0). The Förster radius is in turn determined by the orientation factor (κ^2), the refractive index of the medium (η), the donor quantum yield (Q_D), and the spectral overlap integral ($J(\lambda)$). This makes it a complex system, but also a valuable principle for measuring the distance between the two fluorophores or the phenomena that alters this distance.

The FRET principle can be applied in various fields of research. For example, it is used as a powerful tool for studying biomolecular interactions, such as protein-protein interactions⁴⁷ or conformational changes⁴⁸. One of the applications of FRET, discussed further in §1.7.1, is their use in tension detection.

1.5.3 Confocal Microscopy

Two of the most commonly used fluorescent microscopy setups are widefield and confocal microscopy. The main difference between the two methods lies in the method of illuminating the sample: widefield microscopy involves simultaneous illumination of the entire specimen, whereas confocal microscopy selectively illuminates only a specific point within the sample⁴⁹. In widefield microscopy, collimated light that covers the entire field of view is usually used. Here, photons with a specific wavelength are absorbed by fluorophores in the sample, followed by emission with a longer wavelength. The most commonly used technique within widefield microscopy is epi-illumination, where a single lens acts as both condenser and objective lens. Since widefield microscopy was not applied as a detection mechanism in the experiments discussed in this thesis, this technique will be further disregarded.

Confocal laser scanning microscopy (CLSM) uses a monochromatic laser to illuminate a precisely defined area within a sample, the focal volume (Fig. 5)^{43,50}. The laser beam is sent pointwise across the sample, ultimately building a complete image with high resolution in both the xy- and z-plane. A key advantage of CLSM is the suppression of blurred, out-of-focus light, leading to increased contrast and images with higher resolution. This is made possible by the presence of a pinhole, a small opening just in front of the detector that only allows light from the focal plane to pass through⁵¹. Light from other planes is blocked, resulting in improved optical resolution.

The optical path starts at the laser, from which light is directed to the sample via a dichroic mirror (DC) (Fig. 5). This mirror reflects light of short wavelength (excitation light) and lets longer wavelength (emission light) through. The reflected light is then focused by an objective lens to the focal point in the sample, where excitation of the fluorophores takes place. The fluorescent light emitted by the fluorophores follows the same path back through the objective lens. Since it has a longer wavelength than the excitation light, it now passes the DC and reaches the pinhole. Only the focused signal from the focal plane is transmitted. This light is then captured by sensitive detectors such as an avalanche photodiode (APS) or a photomultiplier tube (PMT), which convert the photons into electrical signals⁴². These signals are used to digitally reconstruct the image pixel by pixel.

The laser beam is scanned across the sample using fast-moving galvanometric mirrors, allowing a detailed view of the entire sample. If necessary, the optical configuration can be modified with additional lenses to optimise the system for specific applications. Moreover, specific optical filters can be deployed to effectively block or let through unwanted wavelengths of light. This allows simultaneous detection of several fluorophores and further improves the signal-to-noise ratio.

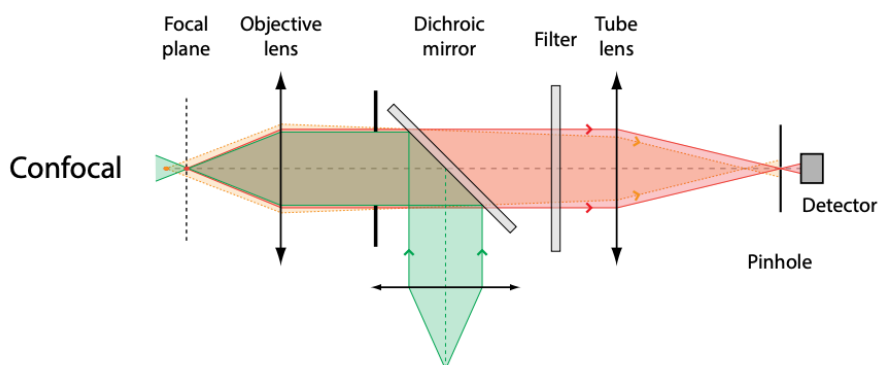


Figure 5. Schematic representation of a confocal microscope setup. The green beam represents the excitation light used to illuminate the sample, while the red beam illustrates the emitted fluorescence signal collected for imaging⁵².

1.5.4 Fluorescence lifetime imaging microscopy

Now that the fundamental principles and tools have been introduced, we can proceed to visualisation at the microscopic level. Since this thesis makes use of FRET-based interactions, the relevant methods will be briefly outlined here. FRET signals are primarily visualised in two ways: intensity-based FRET and fluorescence lifetime imaging microscopy (FLIM).

In intensity-based FRET, the energy transfer between the donor and acceptor fluorophores is assessed by monitoring changes in their fluorescence intensities⁵³. This can involve measuring the intensity of the donor, the acceptor, or both. The fluorescence intensity of a fluorophore refers to the amount of emitted light following excitation and is influenced by several factors, including fluorophore concentration, quantum yield, excitation power, and detection efficiency (e.g. the sensitivity and settings of the detector). When the donor and acceptor fluorophores are in close proximity (<10 nm), the donor can non-radiatively transfer energy to the acceptor. This process results in a reduction of donor fluorescence, commonly referred to as donor quenching, while simultaneously causing an increase in acceptor fluorescence due to the received energy, commonly referred to as acceptor sensitization⁵³. By comparing the fluorescence intensity of the donor in the presence versus absence of the acceptor, E_{FRET} can be quantitatively determined (Eq. 6, left).

An alternative approach to intensity-based FRET is FLIM. FLIM is a technique used to measure the **fluorescence lifetime** of fluorescent molecules, defined as the average time a fluorophore remains in its excited state before returning to the ground state by emitting a photon. Unlike intensity-based fluorescence imaging, FLIM provides information that is independent of fluorophore concentration, photobleaching, or optical path length, making it a powerful tool for probing the molecular environment, interactions, and dynamics^{54,55}.

The lifetime is an intrinsic property of each fluorophore and indicates how long the fluorophore remains in the excited state before returning to the ground state. When a FRET pair is present and an acceptor fluorophore is in close proximity to a donor fluorophore, the fluorescence lifetime of the donor will decrease. This is due to the fact that the excited donor has an additional de-excitation pathway available via energy transfer to the acceptor. The result is a shortening of the donor's fluorescence lifetime.

FLIM is a time-dependent detection method that can be performed in two different ways: (i) through the frequency domain (FD-FLIM) or (ii) through the time domain (TD-FLIM). The latter is also called the 'pulse' method because the sample is excited with a short light pulse that contains a higher photon density than a continuous laser^{54,56}. FD-FLIM is based on the phase shift and demodulation of a signal caused by the fluorescence of the fluorophore, properties that are then converted into the fluorescence lifetime^{54,57,58}. TD-FLIM measures the time it takes an emitted photon of the fluorophore to reach the detector^{56,59}. Since only TD-FLIM is used in this thesis, FD-FLIM will not be discussed further.

The decay curve is constructed by measuring the time difference between when a laser emits an excitation pulse and when a single fluorescence photon is detected. These time differences are collected in a histogram, in which the number of detected photons (counts) is plotted against time (Fig. 6). The resulting curve reflects the exponential decay of fluorescence intensity, from which the fluorescence lifetime of the fluorophore can be calculated. In a traditional TD-FLIM approach, often Time-Correlated Single Photon Counting (TCSPC) is used to accurately record the photon arrival times^{77,78}. In this technique, a Constant Fraction Discriminator (CFD) detects the exact arrival time of each photon and provides it with a so-called time tag. Then, a Time-to-Amplitude Converter (TAC) measures the time interval between the excitation pulse and the detected photon. This analogue voltage is converted into a digital time interval by an Analogue-to-Digital Converter (ADC). A limitation of this system is the dead time, the period during which the system cannot detect new photons because it is busy processing the previous event. To still time-stamp each photon correctly, even when not every excitation pulse leads to detection, the system is often used in reverse mode. In this case, the photon acts as the start signal and the subsequent laser pulse as the stop signal⁶⁰. To further improve the efficiency and time resolution of the system, the TAC and ADC can be combined in a digital circuit, Time-to-Digital Converter (TDC)⁶². This approach reduces dead time and increases measurement accuracy. An important artefact that can undermine the reliability of FLIM measurements is the pile-up effect. This occurs when the detector is overloaded due to an excessively high flux of incoming photons (e.g. more than 10^6 photons per second). In such cases, early photons are counted disproportionately, while later photons are no longer registered. This distorts the histogram and leads to an inaccurate determination of fluorescence lifetime. To avoid this effect, TCSPC is typically performed under conditions of low excitation intensity. This minimises the chance of simultaneous photon detection and keeps the measurement signal reliable.

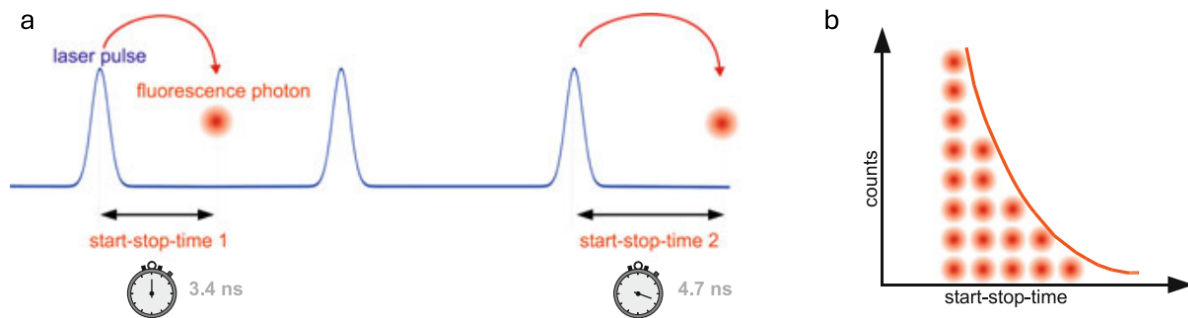


Figure 6. Principle of Time-Domain FLIM using Time-Correlated Single Photon Counting (TCSPC). (a) The blue peak represents the laser excitation pulse, while the red dot indicates the arrival time of an individual emitted photon. (b) After collecting a sufficient number of photon events, a histogram of photon arrival times is generated, typically exhibiting an exponential decay curve corresponding to the fluorescence lifetime⁶¹.

More recently, alternative approaches to traditional TCSPC have been developed⁶³. One such method is the bitstream approach, in which photon arrival times are continuously recorded. This allows for flexible post-acquisition selection of photon subsets for analysis, offering greater adaptability in complex experimental settings⁶³. The fluorescence decay curve indicates the temporal probability distribution of photon emission after excitation by a light pulse. In the case of single fluorescent species, this decay curve can easily be analysed using a mono-exponential fitting approach (Fig. 7a). When multiple emitting components contribute to the photon decay, a multiexponential fitting model can be applied (Fig. 7b). This is usually the case in FRET experiments, as each pixel usually contains a mixture of donors. This mixture consists of donors participating in FRET and donors not participating in FRET. Furthermore, among the donors participating in FRET, the extent of energy transfer can vary. In most biological systems, FRET is not a simple binary event of 'FRET or no FRET'. Rather, it can occur at varying levels, such as low, medium, or high FRET. Several elements affect the degree of FRET, including the distance between the donor and acceptor, how the fluorophores are orientated, and the nature of their molecular environment. As a result, the range of possible FRET outcomes is virtually infinite, making the fitting approach troublesome.

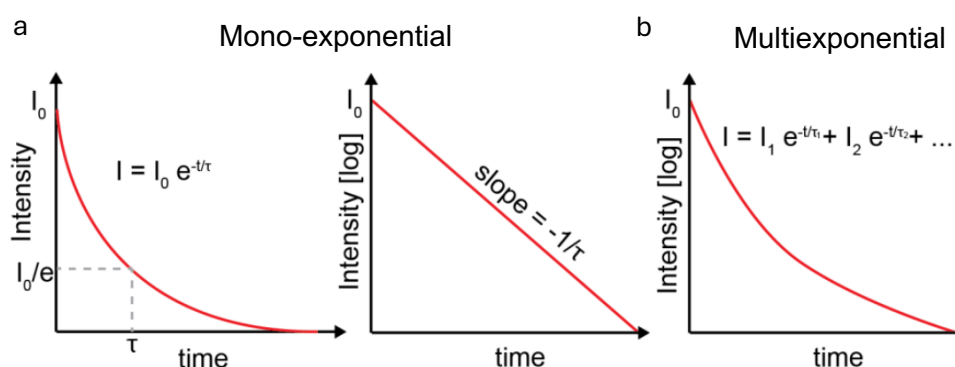


Figure 8: Visualisation of TD-FLIM with the universal circle diagram: relationship between mono-, bi- and tri-exponential decreases. (a) The universal circle diagram in TD-FLIM. TD-FLIM uses a pulse laser and TCSPC to convert photon arrival times to frequency domain space. The universal circle diagram, with a centre at (0,0) and a radius of 1, visualises photon arrival times, with the circumference representing a full period of laser pulses. (b) Relationship between bi-exponential and tri-exponential decreases and their mono-exponential components. Bi-exponential and tri-exponential decreases are analysed by the contribution of different mono-exponential components, which are visually presented as positions on the universal circle⁶⁴.

Decay curve of each pixel is transformed into a single point in the phasor plot⁶⁴. This means that the pixels are classified based on information about their fluorescence lifetime and visualized as a two-dimensional histogram, known as the phasor plot⁶⁴. In this plot, the cosine-Fourier transform (g) of the fluorescence signal is shown on the x-axis, while the sine-Fourier transform (s) is shown on the y-axis. The phasor semicircle has a centre at (0.5), a radius of 0.5 and extends from (0.0) to (1.0) (Fig. 8a). All mono-exponential decay curves, fluorescence lifetimes characterised by a single exponential decay factor, lie on this semicircle. The point (0,0) represents an infinite fluorescence lifetime, meaning that at this point, the fluorescence does not decay measurably over time. On the other side, the point (1,0) is where the fluorescence lifetime is zero, meaning that all emission occurs immediately after excitation⁶⁴.

This concept can be extended to systems with two exponential decay components⁶⁴. When a pixel is characterized by two exponential decays, the mono-exponential positions of the individual decay components are located on the semicircle (Fig. 8b). The combined phasor location of these components is determined by a line connecting both mono-exponential positions, the combined phasor location lies somewhere along this line. From this information, the photon intensity fraction (f_n) of each individual mono-exponential component can be calculated. A larger photon fraction of one of the components results in a phasor location closer to that component's position on the semicircle⁶⁴.

This principle can be further extended to systems with three or more exponential decay components. In such cases, the combined phasor location becomes a weighted combination of the positions of the individual components on the semicircle. However, it is important to note that, in situations with multiple mono-exponential components, the resulting phasor location is not unique. This is because the same phasor location can result from different combinations of decay components.

A final important point not yet discussed is that the unique locations on the semicircle are reserved for mono-exponential decay components, while the inner side of the semicircle is used for the combined phasor locations of multi-exponential systems⁶⁴. In contrast, the outer side of the semicircle is reserved for non-exponential contributions. These non-exponential contributions can arise, for example, from reflection peaks or artefacts created by the detection system and can thus influence the final phasor location⁶⁴.

Now that we understand the structure of a phasor plot, we can apply phasor analysis to a FRET sample. This allows us to derive FRET efficiencies directly from the phasor plot, without the need to know the absolute lifetimes of the fluorophores⁶⁴. In phasor analysis of FRET samples, the pixels contain a mixture of quenched and unquenched donors, resulting in a bi-exponential decay. The combined phasor location is determined by connecting the individual mono-exponential locations of the quenched and unquenched donors⁶⁴.

For FRET analysis, the lifetime of the unquenched donor, τ_D , is essential as a reference point, as it indicates the state in which no FRET occurs (Fig. 9a)⁶⁴. Ideally, when all donor fluorophores are perfectly quenched, the phasor location shifts along the semicircle to the position (1,0), where the lifetime is zero (Fig. 9b). This shift reflects the change in donor lifetime due to FRET, denoted as τ_{DA} . The path following the donor lifetime is called the FRET trajectory and includes all possible phasor locations along the semicircle corresponding to a FRET efficiency range from 0% to 100%. However, this is a simplified approach that we adopt here for clarity. Since the phasor points are located exactly on the semicircle, we can easily determine the lifetime using equation 6. The E_{FRET} value can then be derived directly from the FRET trajectory⁶⁴.

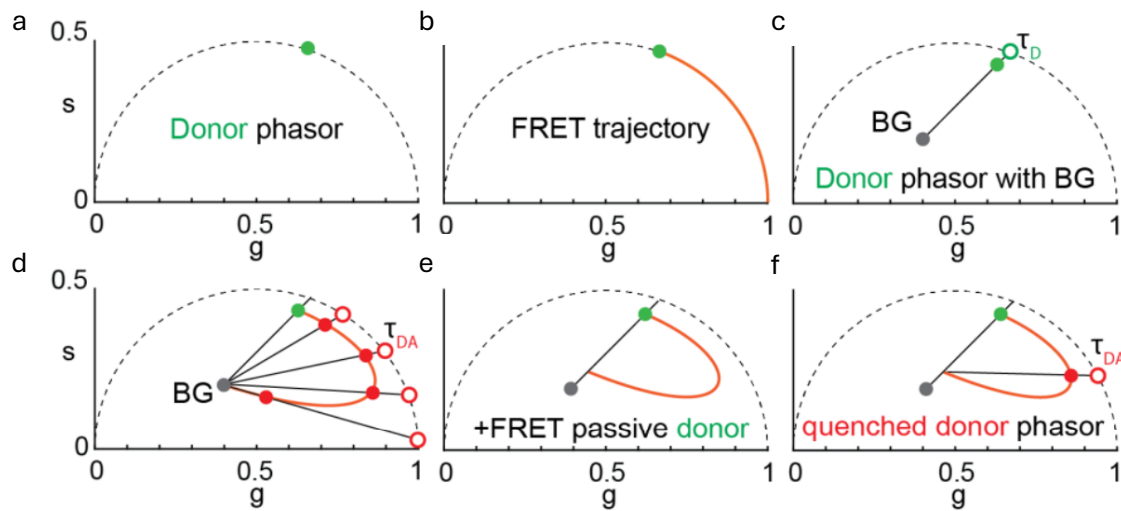


Figure 9. Overview of the FRET trajectory theory construction in the phasor diagram. Iterations for constructing the FRET trajectory theory in the phasor diagram. (a) We see the phasor of the donor alone, without FRET. (b) The FRET trajectory is shown, showing the change in donor lifetime as FRET occurs. (c) Autofluorescence (BG) is introduced, which disrupts the FRET signals and shifts the donor phasor location inward, away from the semicircle. (d) Autofluorescence shifts the measured donor-phasor location inwards from its original position on the semicircle. (e) In the presence of passive donors, who do not participate in FRET, the FRET trajectory can also be affected, as seen in the addition of passive donors to the FRET trajectory. (f) The quenched donor phasor is shown, indicating high FRET efficiency. The phasor diagram reflects the change in donor lifetime due to FRET interaction⁶⁴.

Although the above approach assumes the most ideal conditions, we need to consider additional complications in biological samples. In such samples, autofluorescence, both from the sample itself and from the background, often occurs (Fig. 9c)⁶⁴. This autofluorescence distorts the FRET signal, leading to the measured phasor location of the donor without quenching no longer being exactly on the semicircle, but shifting inwards (Fig. 9d). This affects the FRET trajectory. In situations of almost complete quenching of the donor (at high E_{FRET} values), the fluorescent output/quantum yield of the donor will decrease significantly. This means that the photons originating from the background signal (BG) will contribute relatively more to the measured signal, resulting in the FRET trajectory at high E_{FRET} values bends inwards, towards the phasor location of the background signal. As a result, the FRET trajectory is affected by a combination of (i) the fixed phasor location of the background signal and (ii) the phasor location of the quenched donor, τ_{DA} , which varies depending on the donor's lifetime. Moreover, passive donors, who do not participate in FRET can also affect the FRET pathway, as they remain unquenched and will interfere with the detected FRET signal (Fig. 9e)⁶⁴.

We now compare the ideal situation, where the FRET trajectory lies perfectly along the semicircle and the lifetime of each point can be determined directly, with the reality, where the FRET trajectory shifts inwards and it is impossible to determine the specific lifetime of a point on the trajectory⁶⁴. The FRET trajectory remains a valuable reference with which we can estimate E_{FRET} . E_{FRET} can be derived by graphically unmixing the signal. Indeed, phasor analysis can be considered as a linear combination of the quenched donor τ_{DA} and the combined signal from the BG and passive donors (Fig. 9f)⁶⁴. By drawing a fractional line between the detected phasor location and the BG phasor location, the fraction of τ_{DA} can be determined.

1.6 Detection of myofibroblasts *in vitro*

1.6.1 α -Smooth Muscle Actin

α SMA is a contractile actin isoform characteristic of myofibroblasts, which are crucial for wound healing and fibro contractive disorders⁶⁶. In these cells, α SMA is integrated into stress fibres, enhancing intracellular tension and facilitating the formation of supermature FAs that are larger and more stable than those in regular fibroblasts. The expression of α SMA correlates with increased cell adhesion and the presence of focal adhesion components such as vinculin. Mechanically, α -SMA increases tension on FAs, contributing to their stability⁶⁶. Induction of α SMA by stimuli like TGF- β further enhances these adhesive properties.

1.6.2 Immunostaining

The most commonly used technique for myofibroblast detection is immunostaining of α SMA, a major marker of fibroblast activation. Immunostaining is based on the principle of antigen-antibody interaction, using antibodies that specifically bind to antigens⁶⁷. To visualize the

antigen-antibody complexes formed, a detectable probe, such as a fluorescent dye or an enzyme, is coupled to the antibody (Fig. 10)⁶⁸. When using a fluorescent dye, the signals are observed and analysed with a fluorescence microscope. There are two main methods of immunostaining: direct and indirect immunostaining (Fig. 10)⁶⁹. In direct immunostaining, the primary antibody is conjugated to a detectable marker, which binds directly to the specific antigen (Fig. 10a)⁶⁸. In indirect immunostaining, both a primary and a secondary antibody are used (Fig. 10b)⁶⁷. The primary antibody, which is unlabelled, binds specifically to the antigen of interest. These primary antibodies are usually produced in animals such as rabbits, mice and goats. The secondary antibody, which is conjugated to a fluorescent dye, binds specifically to the primary antibody. To avoid cross-reactivity, secondary antibody is produced in a different animal from the one in which the primary antibody was generated⁶⁸. Moreover, multiple secondary antibodies can bind to one primary antibody, resulting in an amplification of the detectable signal. The reverse is also possible, where a specific secondary antibody can bind to multiple primary antibodies⁷⁰.

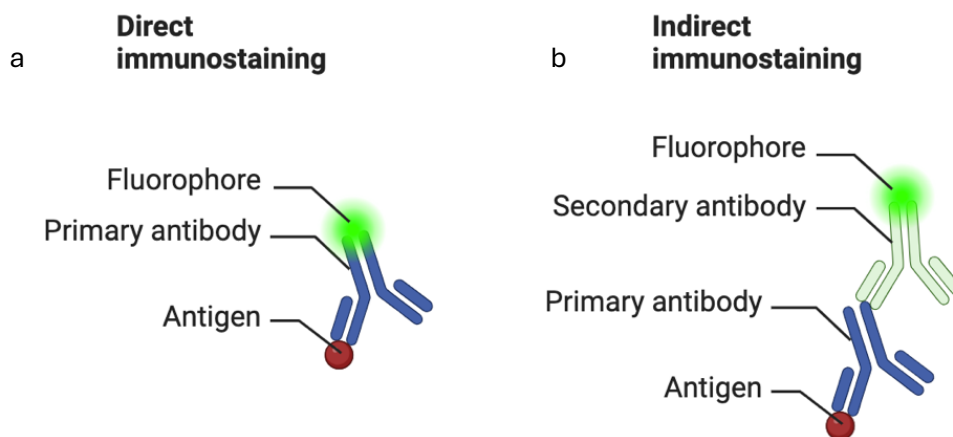


Figure 10. Overview of direct and indirect immunostaining methods. (a) Direct immunostaining, the primary antibody is coupled to a detectable marker and binds directly to the antigen. (b) Indirect immunostaining, an unlabelled primary antibody binds to the antigen, and a secondary antibody coupled to a detectable marker binds to the primary antibody, amplifying the signal.

Prior to immunostaining, cells are fixed with paraformaldehyde (PFA) to preserve the morphological integrity of the cells⁷¹. A major limitation of this fixation step is that it makes the technique unsuitable for live-cell imaging, meaning that immunofluorescence (IF) provides only snapshots of cellular states. This makes it impossible to study dynamic processes with this method. In addition, IF has some technical limitations, including quenching, nonspecific binding (leading to false-positive signals), photobleaching, autofluorescence and bleed-through. These factors can complicate the interpretation of results and require careful optimisation of the experimental setup.

To circumvent the limitations associated with cell fixation required for immunostaining, this thesis aims to develop a reporter plasmid for live-cell analysis. This approach makes it possible to detect the presence of myofibroblasts under specific experimental conditions without using immunostaining. The application of this reporter plasmid opens the door to the study of dynamic processes in CAFs, which can provide important insights into their behaviour and function in real time.

1.7 Measuring mechanical tension in CAFs

To better understand the role of fibroblasts in cancer development and progression, it is essential to examine their mechanical properties and how they respond to forces in their microenvironment. Fibroblasts, particularly CAFs, generate contractile forces and remodel the ECM, which significantly affects tumour growth, invasion, and metastasis. Various techniques have been developed to study the mechanical properties of fibroblasts.

One widely used method is traction force microscopy (TFM), which measures the forces that cells exert on soft substrates by tracking the displacement of fluorescent beads embedded within them⁷². While TFM offers valuable insight into the forces generated by cells at the cellular and tissue levels, it lacks the resolution to measure mechanical forces at the molecular scale.

To address the limitations of conventional force measurement techniques, molecular tension sensors based on the principle of FRET have been developed^{73,74}. These nanoscale biosensors enable the quantification of piconewton-range forces acting on individual proteins within living cells. FRET-based tension probes typically comprise a donor and an acceptor fluorophore connected by an elastic, flexible linker. The efficiency of energy transfer between these fluorophores is inversely related to their spatial separation, which is altered upon mechanical loading. Consequently, changes in FRET efficiency serve as a direct readout of molecular tension, allowing real-time visualization of force transmission at the single-molecule level with high spatial and temporal resolution.

These molecular tension sensors have emerged as powerful tools for probing the mechanobiology of cells, offering novel insights into how mechanical forces are sensed and transduced at the molecular scale. Elucidating these mechanotransduction mechanisms is essential, as mechanical cues are known to regulate a broad range of fundamental cellular processes, including migration⁷⁵, proliferation⁷⁶, differentiation⁷⁷, and tissue morphogenesis⁷⁸.

1.7.1 FRET-based molecular sensors

During mechanosensing and force transmission, mechanical forces are often conveyed through the deformation of load-bearing proteins within the cellular architecture⁷⁶. The tension on these proteins induces conformational changes, leading to structural rearrangements at

the molecular level. Given that FRET efficiency is highly sensitive to the distance between the donor and acceptor fluorophores, this property can be exploited to monitor such structural alterations. Specifically, mechanical loading results in a measurable change in the distance between the fluorophores embedded within or linked to the protein of interest, thereby modulating the FRET signal. This enables real-time, non-invasive quantification of force-induced molecular deformations with high precision⁷⁶.

A genetically encoded molecular tension sensor module (TSMoD) is a specialized FRET-based construct designed to measure piconewton-scale mechanical forces in living cells (Fig. 11a)⁷³. In the absence of mechanical force, the flexible linker ensures that the fluorophores remain in close proximity, resulting in high FRET efficiency (Fig. 11c). However, when the TSMoD is introduced into a load-bearing protein experiencing mechanical tension, the linker stretches, increasing the distance between the fluorophores and decreasing FRET efficiency (Fig. 11d). This force-dependent modulation of FRET enables TSMoDs to serve as real-time, molecular tension sensors. Importantly, TSMoDs are modular in design: they can be genetically fused to different target proteins to probe mechanical forces in specific cellular structures or pathways. Their sensitivity to force can be fine-tuned by adjusting the mechanical properties (e.g. stiffness and length) of the linker domain, as well as the Förster radius of the chosen FRET pair.

In this thesis, TSMoDs based on the Clover-mScarlet-I FRET pair were used to investigate mechanical stress in vinculin in CAFs. These constructs use a flexible GGSGGS₇ linker as the extendable domain, optimized to detect physiologically relevant force ranges⁷³. When freely expressed in the cytoplasm, these sensors exhibit high FRET efficiency due to the relaxed state of the linker. Upon insertion into a load-bearing protein, such as vinculin or talin, mechanical loading results in linker extension and measurable changes in FRET signal, providing insight into the intracellular force landscape (Fig. 11b).

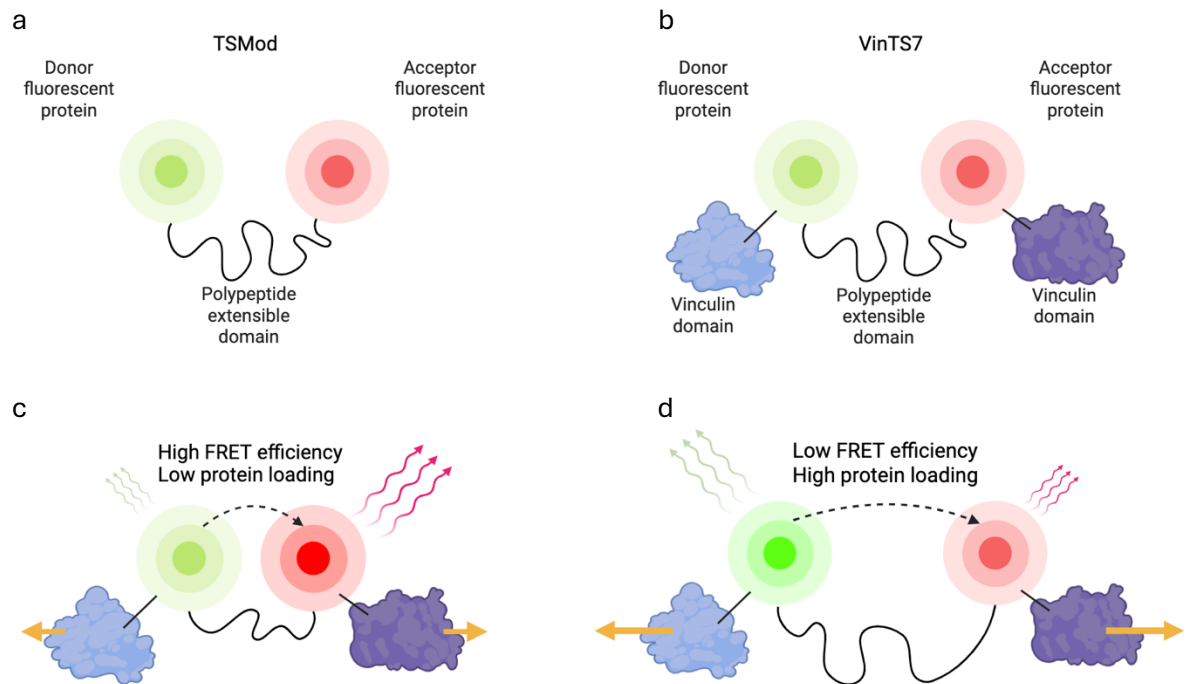


Figure 11. Overview of FRET tension sensors. (a) Structure of TSMod, a cytosolic tension sensor composed of a donor–acceptor fluorophore pair separated by an elastic linker. (b) Structure of VinTS7, a vinculin-based tension sensor in which the TSMod module is inserted between the head and tail domains of vinculin. (c) Conformation of VinTS7 under low mechanical tension, resulting in a short fluorophore distance and thus high FRET efficiency. (d) Conformation of VinTS7 under high mechanical tension, where stretching of the sensor module increases the distance between donor and acceptor, leading to reduced FRET efficiency.

2 OBJECTIVES

This thesis consists of three related objectives aimed at elucidating the mechanobiology of cancer-associated fibroblasts (CAFs) using molecular FRET tension sensors. The primary aim is to investigate the mechanotransduction processes within vinculin, a key component of focal adhesions (FAs), under different phenotypic states of CAFs. To this end, two experimental conditions were established: in the first, CAFs were treated with transforming growth factor beta (TGF- β) to induce differentiation towards a more contractile, 'activated' phenotype known as the myofibroblast. In the second, untreated CAFs served as an undifferentiated control group. These two conditions allowed a comparative analysis of the mechanobiological behaviour in different CAF phenotypes.

2.1 Objective I: Activation assay

The first objective was to develop a robust and reproducible protocol for inducing differentiation from CAF to myofibroblast using TGF- β . This step is crucial for the entire study, as the phenotypic shift must be clearly defined to allow meaningful comparison in subsequent mechanobiological analyses. Successful differentiation was verified by the expression of α -smooth muscle actin (α SMA), a widely recognised marker specifically upregulated in myofibroblasts.

2.2 Objective II: Investigation Mechanobiology in CAFs

The second and central objective was to assess the mechanical forces exerted within focal adhesions of CAFs using FRET-based vinculin tension sensors. These measurements were performed under both experimental conditions, differentiated (+TGF- β) and undifferentiated (-TGF- β) CAFs, using the protocol from objective 1. The aim was to determine how the phenotypic condition affects intracellular force transfer, particularly in adhesion complexes.

2.3 Objective III: Development of a fluorescent reporter for live-cell imaging of CAF differentiation

The third objective involved designing a plasmid-based fluorescent reporter system capable of indicating successful CAF differentiation in live-time. Unlike the fixed-cell approach required for FRET imaging, this reporter system would enable live-cell imaging by emitting a nuclear fluorescent signal upon α SMA expression. Such a tool would enable the investigation of dynamic cellular responses to environmental stimuli during the transition to the myofibroblast phenotype.

3 MATERIAL & METHODS

3.1 Cell line and cell culture

The CAF cell line used, CT5.3hTERT, was derived from human lung tissue. These cells were cultured in Gibco™ Dulbecco's Modified Eagle Medium (DMEM) with a glucose concentration of 4.5 g/L, supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich), 1% (v/v) GlutaMax™ (Thermo Scientific™), and 0.1% gentamycin (Carl Roth). For the starvation medium, the fetal bovine concentration was reduced to 0.1% (v/v) (Sigma-Aldrich), while all other components remained the same. Cultures were incubated at 37°C with 5% O₂ and 5% CO₂. Cells were split once the confluency reached 80-90%.

3.2 OBJECTIVE I: Activation assay

3.2.1 Cell stimulation

The CAFs were stimulated with different concentrations (1 ng/mL, 3 ng/mL and 5 ng/mL) of recombinant human TGF-β1 protein (R&D Systems, Cat# 240-B-010). Stimulation was maintained for 24 hours before the cells were fixed. In one of the experimental conditions, cells were subjected to an overnight starvation environment prior to TGF-β1 treatment by incubation in serum-free DMEM.

3.2.2 Immunostaining

Cells were fixed with a 4% (v/v) paraformaldehyde (PFA) solution in PBS for 15 minutes at room temperature (RT), followed by rinsing three times with PBS. The cells were then permeabilised with a solution consisting of 0.1% (v/v) Triton X-100 and 0.1% Tween-20 in PBS. This incubation was performed for 15 min at RT. After permeabilization, the solution was replaced with a blocking solution containing 10% (v/v) FBS and 0.1% (v/v) Tween-20 in PBS. The cells were washed three times with the blocking solution and incubated for 15 minutes at RT.

Indirect immunostaining was used for the staining of αSMA. The primary antibody, Anti-Actin α-Smooth Muscle Actin (mouse monoclonal, Sigma-Aldrich, A5228), was added at a dilution of 1:400 (6.25 µg/mL) in blocking solution and then incubated overnight at 4°C. Cells were then washed three times with blocking solution, each rinsing step being performed with a 5-minute incubation time under constant shaking. A secondary antibody, Goat anti-Mouse IgG (H+L) Cross-adsorbed Secondary Antibody, Alexa Fluor™ 633 (Invitrogen™, A21070), was used at a dilution of 1:1000 (2 µg/mL) in blocking solution. The cells were incubated for two hours at RT. After incubation, cells were washed three times with blocking solution, with each rinsing step performed with an incubation time of 7 minutes under shaking.

The nucleus and actin of the CAFs were labelled by direct immunostaining. For nuclear staining, DAPI and Hoeschst Nucleic Acid Stains (Invitrogen™, D1306) was used at a dilution of 1:2000 (2.5µg/mL) in blocking solution, followed by a 5-minute incubation at RT. The cells were then rinsed three times with blocking solution. For actin staining, Alexa Fluor™ 488 Phalloidin (Invitrogen™, A12379) was used at a dilution of 1:2000 in blocking solution, with an incubation time of one hour at RT. After this incubation, the cells were washed three times with blocking solution.

3.2.3 Fluorescence Microscopy Imaging

All fluorescence images were acquired using a Leica TCS SP8 confocal microscope equipped with a 63× HC PL APO 1.2 NA objective. Images were recorded at a resolution of 512×512 pixels with variable zoom. Emission signals were detected using a Leica HyD SMD hybrid photomultiplier tube, operating within a spectral detection range of 400–800 nm. Fluorophore excitation was achieved using lasers at 638 nm for αSMA, 405 nm for nuclear staining, and 488 nm for actin visualization.

3.3 OBJECTIVE II: Investigation Mechanobiology in CAFs

3.3.1 Seeding

In preparation for the stimulation assay, CAFs were seeded four days before fixation in an 8-well plate to allow sufficient adhesion and growth. A seeding density of 10,000 cells per well was used to ensure that the cells would reach 60-70% confluence by the time of fixation. This confluence level was chosen to maintain optimal cell viability and response to stimulation while avoiding overgrowth.

3.3.2 Stimulation Assay

The day after seeding, CAFs were stimulated with 1 ng/mL of transforming growth factor-beta 1 (TGF-β1) (R&D Systems, Cat# 240-B-010). The stimulation was maintained for 48 hours to allow sufficient activation of signalling pathways associated with myofibroblast differentiation and αSMA expression.

3.3.3 Transient transfection with FRET-Tension Sensor

Two days after seeding, CAFs were transiently transfected using JetOPTIMUS® transfection reagent (Polyplus-transfection®). For each reaction, 500 ng of plasmid DNA was diluted in 100 µL of DMEM (without any supplements), followed by the addition of 1 µL JetOPTIMUS® reagent. The transient transfection complex was then incubated for 10 minutes at RT to allow for the efficient formation of DNA-lipid complexes. After the incubation period, the transfection mixture was gently added dropwise to the cells to ensure even distribution. The cells were

then incubated under standard culture conditions (37°C, 5% CO₂) to facilitate uptake of the DNA-lipid complexes. After 3 hours of incubation, the medium containing the transfection complexes was removed and replaced with fresh supplemented DMEM medium. The following day, four days after seeding, the cells were fixed using the fixation protocol described in §3.2.2.

The primary construct used in this study was VinTS7 (Clover-GGSGGS7-mScarlet-I), shortly called VinTS7 in the text, was developed by Samet Aytekin, adapted from the plasmid originally described by LaCroix et al (2018)⁷³ and obtained from Addgene (plasmid # 111764). This construct is a genetically encoded vinculin tension sensor that comprises a FRET donor–acceptor pair, Clover and mScarlet-I, separated by an elastic, synthetic polypeptide linker consisting of seven repeats of the sequence (GGSGGS), denoted as (GGSGGS)₇. This modular design enables the detection of mechanical tension across vinculin by translating force-induced conformational changes into measurable shifts in FRET efficiency, allowing for quantitative analysis of intracellular tension. In addition to VinTS7, control constructs were also utilized: VinTL7, which lacks the actin-binding tail of vinculin, and VinTS-I1197A, a point mutant that disrupts vinculin linkage at the tail domain. Both controls were also designed by Samet Aytekin using the same backbone plasmid (Addgene plasmid #111764)⁷³. Furthermore, the TSMo7 construct, consisting solely of the FRET module and the elastic linker, without vinculin fusion, was employed as a control to assess FRET changes independent of vinculin-mediated force transmission. Due to the absence of a targeting domain, TSMo7 is diffusely localized in the cytosol, where it remains untethered to any specific cellular structures. This allows for the evaluation of baseline FRET efficiency under tension-free conditions. TSMo7 was likewise designed by Samet Aytekin and is based on the TSMo plasmid obtained from Addgene (plasmid #111761), originally described by LaCroix et al. (2018)⁷³.

3.3.4 Viral Transfection with FRET-Tension Sensor

To enable the production of lentiviral particles encoding the FRET-based tension sensor constructs TSMo7 and VinTS7, HEK293T cells are used as a packaging system due to their high transfection efficiency and viral yield. This protocol, as well as the cloning of VinTS7- and TSMo7-containing pHR viral plasmids, were mainly performed by Sarah Vorselmans. The desired culture plates are coated with collagen solution (100µg/mL in dH₂O) and incubated for 30 minutes at 37°C to promote attachment of HEK293T cells. After incubation, the coating solution is removed, and the plates are thoroughly rinsed once with PBS. HEK293T cells (passage number < P30) are then seeded onto the collagen-coated plates. Plates are incubated for 24 h at 37°C in a humidified incubator with 5% CO₂. For viral production, HEK293T cells are transfected with three plasmids: the transfer plasmid (pHR), the packaging plasmid (pCMV8.74) and the envelope plasmid (pMD2.G). The DNA concentrations used depend on the size of the culture plate. In a standard 6-well plate, 476 ng pHR, 952 ng pCMV8.74 and 572 ng pMD2.G are dissolved in 400 µL Opti-MEM™ I Reduced Serum

Medium (Thermo Scientific™) 8 µL Turbofect Transfection Reagent (Thermo Scientific™) is then added to this mixture. The solution is mixed briefly and then incubated for 15–30 minutes at RT to allow complex formation. In the meantime, the HEK293T cells are gently washed with PBS and provided with fresh supplemented DMEM without antibiotics (2 mL per 6-well). The transfection solution is added dropwise to the cells, starting the production of the virus containing the target plasmid. After 4–6 hours, the transfection medium is removed and replaced with fresh supplemented DMEM. Transfection efficiency was monitored by fluorescence of the HEK293T cells. Upon successful expression, the viral supernatant was collected in a falcon tube. The virus-containing supernatant is then filtered with a 0.45µm syringe filter to remove any HEK293T cells present. To increase the efficiency of viral transduction, polybrene (a cationic polymer that reduces charge repulsion between the virus and the cell membrane) can be added to a final concentration of 5–10µg/mL. The virus solution is then added dropwise to CAFs (target cell) and mixed thoroughly to ensure homogeneous distribution. On the day after infection, CAFs were washed 3 times with PBS to remove any remaining virus particles.

3.3.5 Fluorescence Lifetime Microscopy Imaging

All FRET imaging was performed using a Leica TCS SP8 confocal fluorescence microscope equipped with the FALCON (FAst Lifetime CONtrast) module. FRET images were acquired using a 63X HC PL APO 1.2 NA water immersion objective, with a fixed 1.0X zoom and a resolution of 512×512 pixels (pixel size = 180 nm). Excitation was performed at 488 nm (donor channel) using a pulsed laser operating at a pulsed laser operating at 20 MHz, with a laser power of 6 µW and a unidirectional scan speed of 50 Hz. For detection, a hybrid photon counting detector (Leica Microsystems GmbH) was set at 495–540 nm for the donor channel. Photon arrival times and photon counts were processed using the Leica FALCON module.

3.3.6 FLIM-FRET analysis

For FRET analysis, the fluorescence decay curves of each pixel are transformed into a phasor space using the integrated Phasor module of the SP8 FALCON microscope. In short, the complete decay curve $I(t)$ is converted into two values per pixel using a Fourier transform: a real part (G) and an imaginary part (S), according to the following equations:

$$G(\omega) = \frac{\int_0^\infty I(t) \sin(n\omega t) dt}{\int_0^\infty I(t) dt} \quad \text{Eq. 7}$$

$$S(\omega) = \frac{\int_0^\infty I(t) \cos(n\omega t) dt}{\int_0^\infty I(t) dt} \quad \text{Eq. 8}$$

Here $\omega=2\pi f$, with f the repetition rate of the pulse laser (20 MHz in this case). Each pixel is thus assigned a coordinate (G , S) in phasor space, which together form a 2D plot of all lifetime

information in the image. The G and S values are then exported for further analysis in a custom MATLAB script to calculate FRET efficiency. The position of the donor fluorophore Clover (with an average lifetime of ~3.0 ns) defines the start point of the FRET trajectory line. The end point of this line is determined by the signal from background fluorescence (estimated at G = 0.5, S = 0.5 from control cells). Each pixel is then mapped to the nearest value on the FRET trajectory line, resulting in a FRET efficiency image. To reduce noise and refine the data, a 9×9 median filter is applied to the image, as described by Ranjit et al. (2018)⁷⁹. The FRET efficiencies and acceptor intensity data were analysed and integrated using a MATLAB script, as made available by Aytekin et al. (2025)⁸⁰.

3.4 OBJECTIVE III: Development of a fluorescent reporter for live-cell imaging of CAF differentiation

The reporter plasmid consists of four/five functional elements; i) vector backbone of pcDNA3.1, ii) ACTA2 promoter, responsible for the expression of α SMA, iii) ACTA2-enhancer, iv) fluorescence protein mTagBFP2, localised to the cell membrane, v) fluorescence protein mGarnet2, localised to the nucleus

The aim of this part of the thesis was to construct two different reporter plasmids: one with the ACTA2-enhancer and one without ACTA-2 enhancer, both containing the ACTA-2 promoter (Fig. 12). This allows us to compare the plasmids and determine whether the presence of the ACTA2-enhancer improves the detection efficiency of α SMA production, and hence the CAF activation.

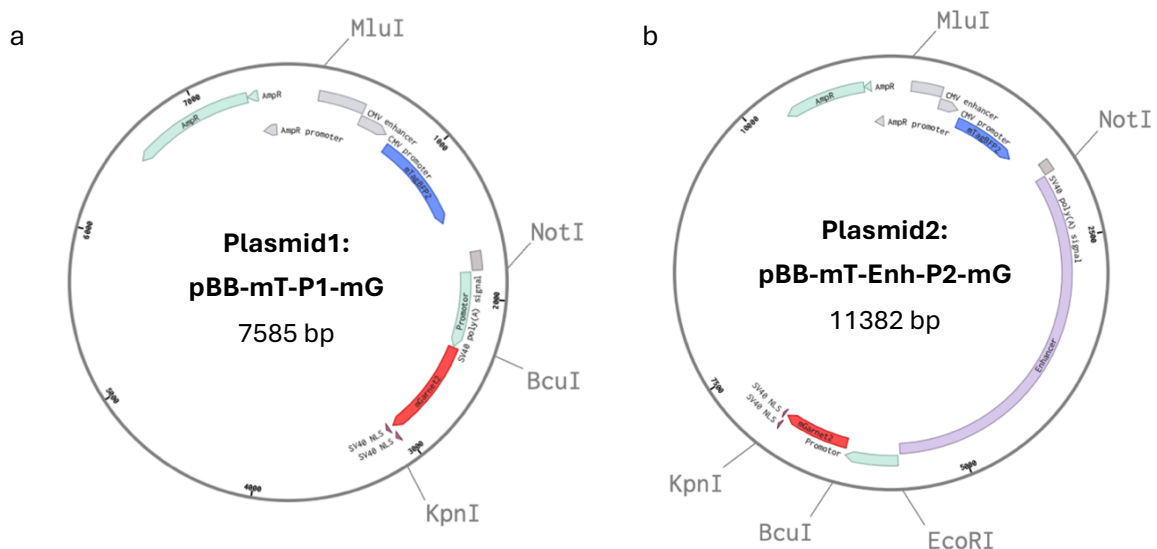


Figure 12. Schematic representation of the reporter plasmids used to study ACTA2 promoter activity. (a) Plasmid 1: pBB-mT-P1-mG (without ACTA2 enhancer). (b) Plasmid 2: pBB-mT-Enh-P2-mG (with ACTA2 enhancer) share a common backbone derived from pcDNA3.1. pBB = pcDNA3.1 backbone, mT = mTagBFP2, Enh = ACTA2 enhancer, P1 = ACTA2 promoter 1, P2 = ACTA2 promoter 2, mG = mGarnet2

3.4.1 PCR DNA Fragments

The backbone fragment was obtained from the plasmid pcDNA3.1-Clover. pcDNA3.1-Clover was a gift from Michael Lin (Addgene plasmid #40259)⁸¹. The backbone of the plasmid is amplified via PCR (Suppl. Table 1 and 2), using self-designed primers (Suppl. Table 3). Primers 1, 2, 3 and 4 were used to introduce the MluI, NotI, EcoRI, BclI and KpnI cutsites. The ACTA2 promoter and ACTA2 enhancer were isolated from genomic DNA extracted from CT5.3hTERT-CAF cells using the QIAamp® DNA Mini Kit (Cat. No. 51304). Both sequences were then amplified by PCR with primer 5 and 6 for the ACTA2 enhancer primer and 7 or 8 and 9 for the ACTA2 promoter. The fluorescent protein mTagBFP2 was isolated from the plasmid mTagBFP2-Farnesyl-5 using primers 10 and 11. mTagBFP2-Farnesyl-5 was a gift from Michael Davidson (Addgene plasmid #55295)⁸³. Similarly, mGarnet2 was isolated from the plasmid LifeAct-mGarnet2 using primers 12 and 13. LifeAct-mGarnet2 was a gift from Ulrich Nienhaus (Addgene plasmid #104308)⁷⁷. All primers contained specific restriction enzyme-overhang sequences, which facilitated the stepwise assembly of the different fragments into the construct. All custom primers are designed by Guillermo Solis Fernandez and Samet Aytekin. The PCR reactions were performed using Phusion High-Fidelity Polymerase (Thermo Scientific™, F530S). After amplification, the integrity of the constructs was verified by gel electrophoresis. The correct band was then excised and purified using the GeneJET Gel Extraction Kit (Thermo Scientific™, K0691). With all fragments successfully amplified, the following sections were carried out stepwise to insert each fragment into the pcDNA3.1 backbone. For the plasmid containing the ACTA2 enhancer, the fragments were added in the following order: first the ACTA2 promoter, followed by the ACTA2 enhancer, then mTagBFP2, and finally mGarnet2. The same construction strategy was followed for the plasmid without the ACTA2 enhancer, with the only difference being the omission of the step in which the ACTA2 enhancer was inserted.

3.4.2 Restriction Digestion

DNA fragments were digested with the corresponding restriction enzymes (Fig. 13) using FastDigest Restriction Enzymes (Thermo Scientific™) (Fig. 13(1)). The restriction enzyme assay was performed for 1h at 37°C. After the digestion, the fragments were separated on through agarose gel electrophoresis. The correct bands were then excised and purified using the GeneJET Gel Extraction Kit (Thermo Scientific™, K0691).

3.4.3 Ligation

The DNA fragments, which now contained the correct sticky ends, were ligated using T4 DNA ligase (Thermo Scientific™, EL0014) (Fig. 13(2)). A molar insert:backbone ratio of 3:1 was applied, using a backbone concentration of 50-100 ng each time. The ligation reaction was performed overnight at 16°C to ensure efficient assembly of the fragments.

3.4.4 Bacterial Transformation

The DH5 α *E. coli* bacterial strain was used for the transformation procedure (Fig. 13(3)). 50 μ L of this bacterial suspension was mixed with the ligation product and placed on ice for 15-30 minutes in preparation for transformation. A heat shock was then performed at 42°C for 45 seconds without shaking. Immediately afterwards, the cells were placed back on ice and cooled for 2 minutes to promote the recovery process. After this cooling phase, 1 mL of sterile Luria-Bertani (LB) medium was added. The suspension was then incubated for 1h at 37°C with shaking. The cells were then centrifuged at 4500 RPM for 4 minutes, forming a bacterial pellet. Most of the supernatant was carefully removed, leaving about 150 μ L of liquid in the tube. This remaining volume was gently pipetted up and down with a low pipette volume setting to evenly resuspend the bacteria and spread over the antibiotics-containing plates. Finally, the plates were incubated overnight for colonies to grow. Selected colonies were transferred into 10 mL of ampicillin-containing LB medium and incubated overnight at 37°C under continuous shaking to promote bacterial growth (Fig. 13(4)). The next day, amplified plasmid DNA was isolated from the bacterial cultures using the GeneJET Plasmid Miniprep Kit (Thermo Scientific™, K0502) (Fig. 13(5)) and sent for Sanger sequencing to verify the accuracy of the inserts (Fig. 13(6)).

3.4.5 Fluorescence Lifetime Microscopy Imaging

All imaging was performed using a Leica TCS SP8 confocal fluorescence microscope equipped with the FALCON module. FRET images were acquired using a 63X HC PL APO 1.2 NA water immersion objective, with a fixed 1.0X zoom and a resolution of 512 $\times$ 512 pixels (pixel size = 180 nm). For TagBFP2, excitation was performed at 405 nm using a pulsed laser operating at a pulse frequency of 20 MHz, with a laser power of 6 μ W and a unidirectional scan rate of 50 Hz. For mGarnet2, excitation was performed at 630 nm using the pulsed laser. Photon arrival times and photon counts were processed using the Leica FALCON module.

3.5 Statistics

For comparisons between two conditions, an unpaired t-test was performed. In cases involving more than two conditions, statistical analysis was conducted using an ordinary one-way ANOVA. Statistical significance was defined as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. All graphs and statistical analyses were generated using GraphPad Prism version 10.4.1 (GraphPad Software).

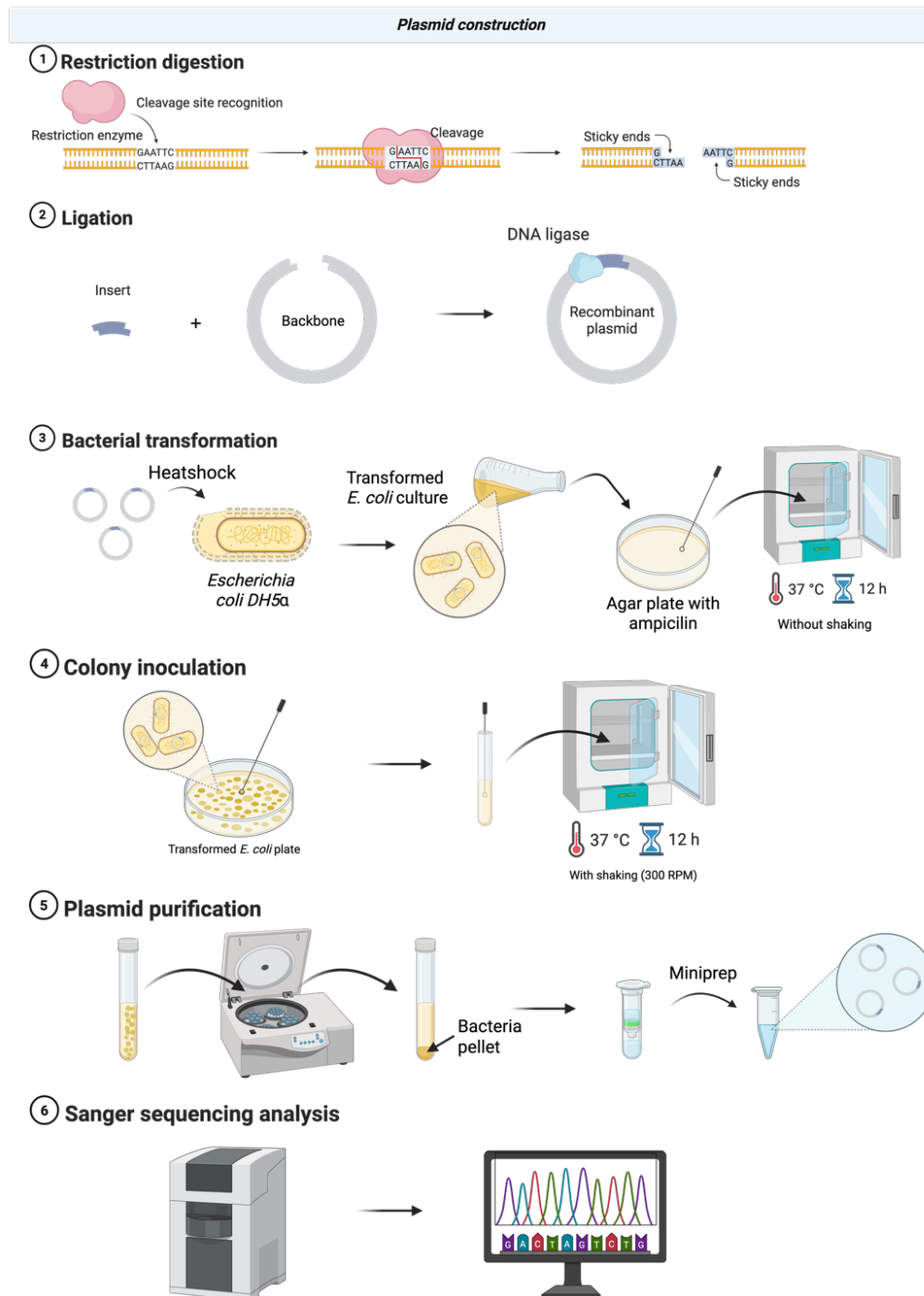


Figure 13. Cloning strategy for the construction of the reporter plasmid. (1) DNA fragments were digested using the appropriate restriction enzymes to generate compatible sticky ends. (2) The digested insert and backbone were ligated using DNA ligase in a 3:1 molar ratio and incubated overnight. (3) The ligation products were transformed into *E. coli* DH5α competent cells via heat shock. After recovery in LB medium, the transformed bacteria were plated on ampicillin-containing agar plates and incubated overnight at 37°C to allow colony formation. (4) Individual bacterial colonies obtained after transformation were screened by colony PCR using insert-specific primers. Positive colonies were inoculated in LB medium containing ampicillin for overnight growth. (5) Plasmid DNA was isolated using a miniprep kit (GeneJET, Thermo Scientific™). (6) Plasmids were submitted for Sanger sequencing to verify the integrity and sequence accuracy of the cloned DNA.

4 RESULTS

4.1 Objective I: Activation assay

4.1.1 Activation assay

CAF cells were seeded at a density of 10,000 cells per well in an 8-well polystyrene plate (125 cells/mm²) and incubated for 24h at 37°C in an environment with 5% O₂ and 5% CO₂. Following incubation, cells were stimulated with recombinant TGF- β at concentrations of 1 ng/mL, 3 ng/mL, and 5 ng/mL. Control cells were cultured without TGF- β to assess baseline α SMA expression. After 24 hours of incubation, cells were fixed with 4% (v/v) PFA and subsequently processed for immunostaining. In total, the complete protocol spanned four days, including cell seeding, stimulation, fixation, and immunostaining.

4.1.2 Effect of TGF- β concentration

Confocal microscopy imaging (Fig. 14) confirmed successful differentiation of fibroblasts into myofibroblasts in all TGF- β -treated conditions, as evidenced by the presence of strong α SMA expression (red fluorescence). In contrast, untreated CAFs exhibited no detectable α SMA expression (Fig. 14e), indicating that TGF- β stimulation is essential for the start of myofibroblast differentiation. Among the TGF- β -treated groups (1 ng/mL, 3 ng/mL, and 5 ng/mL), α SMA fluorescence was consistently observed across nearly all cells, confirming a robust activation response (Fig. 14b-d). However, the fluorescence intensity of α SMA appeared comparable across these conditions, suggesting that increasing TGF- β concentration beyond 1 ng/mL does not necessarily enhance differentiation. This finding implies that even at the lowest tested concentration, TGF- β is sufficient to drive fibroblast activation, and higher doses do not confer an additional benefit in promoting myofibroblast differentiation.

4.1.3 Effect of preconditioning in starvation medium

To investigate whether exposure to a stress environment enhances differentiation efficiency, a subset of CAFs was preconditioned in starvation medium (0.1% FCS) prior to TGF- β stimulation with 1 ng/mL (Fig. 14a). Confocal microscopy analysis revealed that preconditioned CAFs exhibited slightly higher α SMA fluorescence intensity compared to the non-preconditioned cells treated with the same concentration of TGF- β . This suggests that metabolic stress may have a minor effect on α SMA expression levels. However, the observed increase in fluorescence intensity was minimal and did not indicate a biologically enhancement of differentiation efficiency. The overall percentage of CAFs expressing α SMA remained comparable between the preconditioned and non-preconditioned groups, reinforcing the conclusion that starvation medium preconditioning does not substantially impact myofibroblast

differentiation. These findings suggest that TGF- β alone is the primary driver of differentiation, independent of prior metabolic stress conditions.

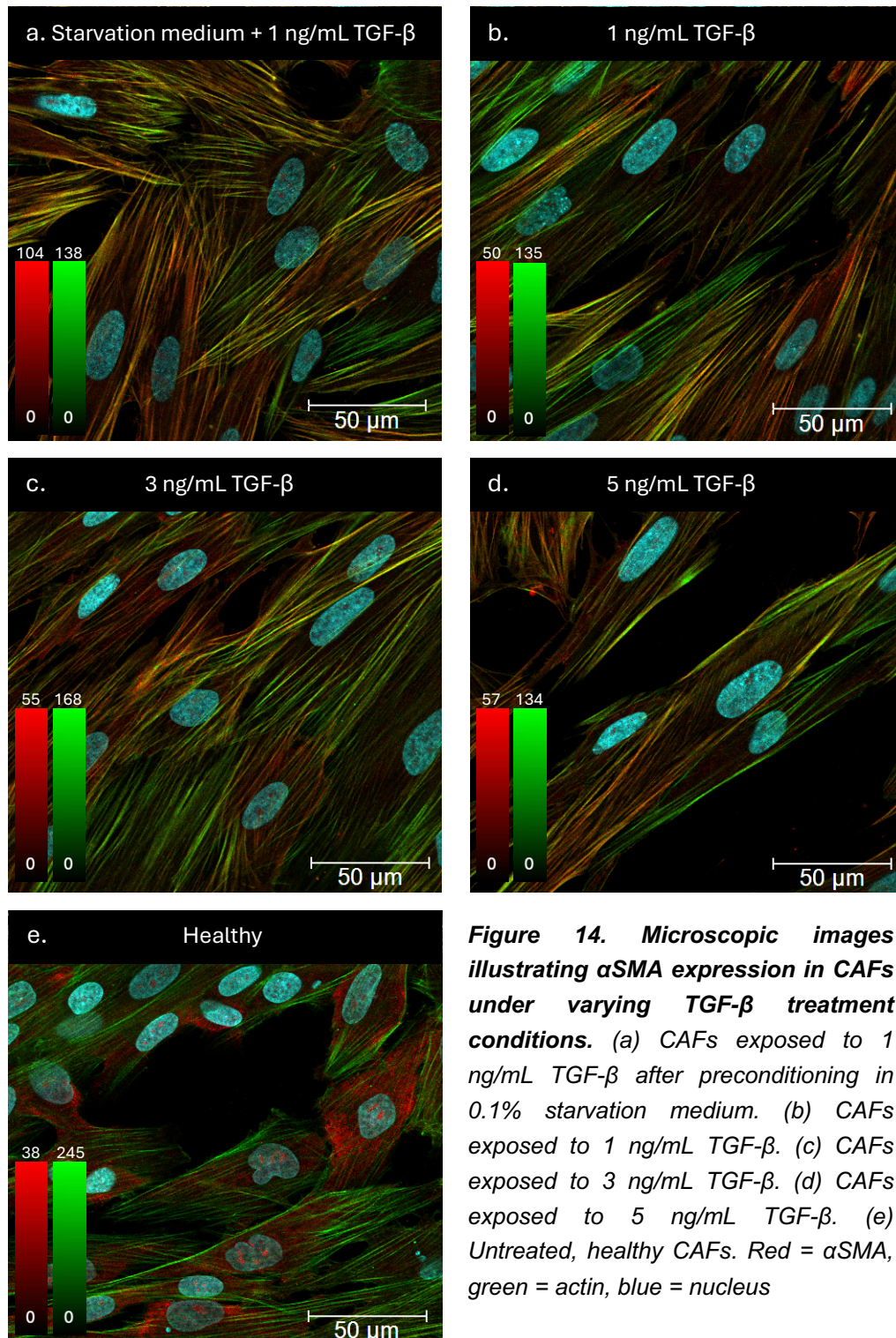


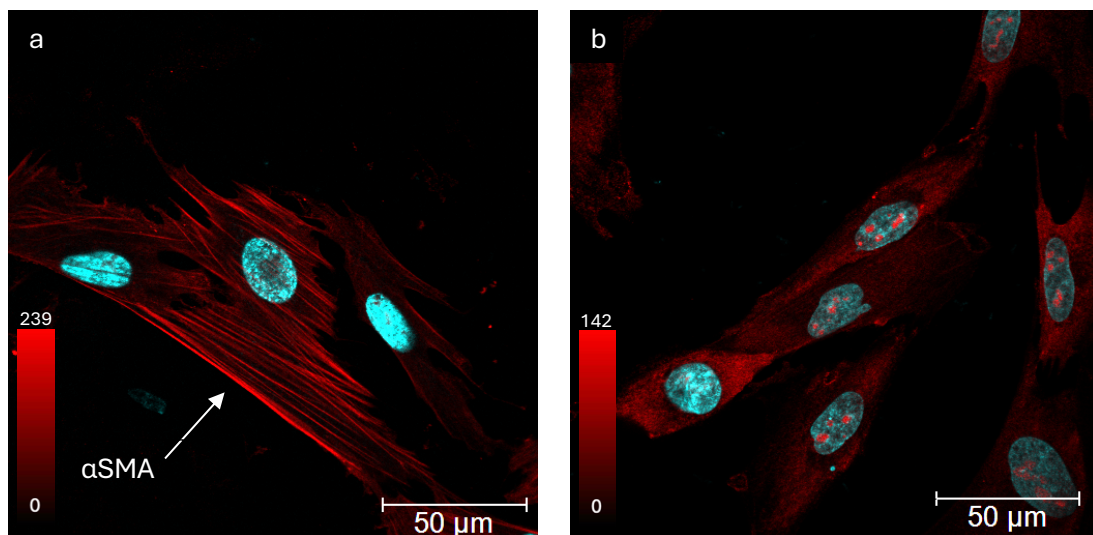
Figure 14. Microscopic images illustrating α SMA expression in CAFs under varying TGF- β treatment conditions. (a) CAFs exposed to 1 ng/mL TGF- β after preconditioning in 0.1% starvation medium. (b) CAFs exposed to 1 ng/mL TGF- β . (c) CAFs exposed to 3 ng/mL TGF- β . (d) CAFs exposed to 5 ng/mL TGF- β . (e) Untreated, healthy CAFs. Red = α SMA, green = actin, blue = nucleus

4.1.4 Quantitative validation of activation assay efficiency

To quantitatively evaluate the efficiency of the activation assay, cells were seeded on day 1. On day 2 (i.e. 24 hours after seeding), the standard culture medium was replaced with starvation medium and TGF- β was added at a final concentration of 1 ng/mL. On day 4, cells were fixed with 4% PFA. For the control condition (no TGF- β treatment), the same protocol was followed with the exception that the medium was not replaced and no TGF- β was added. Immunostaining was then performed for α SMA and nuclei. For both conditions, ten representative images were randomly selected for analysis. Only cells whose nuclei were completely within the field of view were included in the analysis. For each cell, the presence of α SMA expression was assessed. Cells with detectable α SMA expression were classified as 'activated', while cells without detectable expression were considered 'non-activated' (Fig. 15a-b). Activation efficiency was calculated as the proportion of α SMA-positive cells relative to the total number of analysed cells per condition (Eq. 9).

$$\text{Activation efficiency} = \frac{\alpha\text{SMA-positive cells}}{\text{total cells}} \quad \text{Eq. 9}$$

In the TGF- β -treated condition, a total of 57 cells were counted over the ten images, of which 50 were identified as α SMA-positive, corresponding to an activation efficiency of 87.719%. In the untreated condition, 47 cells were counted over the ten images, of which 4 were α SMA-positive, resulting in an activation efficiency of 0.085% (Fig. 15c)



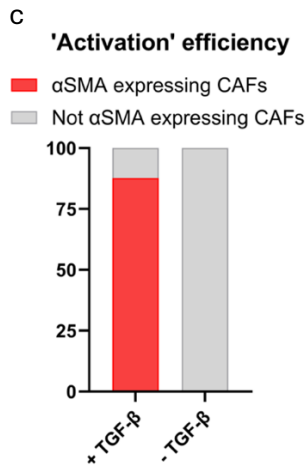


Figure 15. Microscopic images and percentage graph bar showing αSMA expression in CAFs with and without TGF-β treatment. (a) Three visible CAFs (full nucleus in frame) stimulated with 1 ng/mL TGF-β following preconditioning in 0.1% starvation medium. αSMA expression is indicated by white arrow. (b) Six visible CAFs (full nucleus in frame) without TGF-β treatment, showing minimal or absent αSMA expression. (c) Percentage bar graph of 'activation' efficiency.

4.2 Objective II: Investigation Mechanobiology in CAFs

4.2.1 VinTS7

The first experiment investigated the difference in FRET efficiency between CAFs treated with TGF-β and non-treated CAFs. At the beginning of the experiments within Objective II, CAFs were transiently transfected with the VinTS7 plasmid (TC-VinTS7), a genetically encoded vinculin tension sensor. This construct consists of the Clover-mScarlet-I FRET pair, separated by a synthetic, elastic polypeptide linker composed of seven repeats of the (GGSGGS) motif. At a later stage, a stable cell line was generated that constitutively expressed VinTS7 (SCL-VinTS7), thereby eliminating the need for repeated transient transfection, which could potentially interfere with normal cellular physiology. Since a difference in mean FRET efficiency per FA per cell was observed between the initially transiently transfected CAFs and the stable VinTS7-expressing cell line, these two conditions were analysed separately in subsequent experiments.

For the TC-VinTS7 CAFs, a total of 59 TGF-β-treated cells and 51 untreated cells were imaged. Figure 16 shows representative images of three TGF-β-treated (a-c) and three untreated CAFs (d-f), demonstrating the localization of VinTS7 within FAs. No statistically significant difference in mean FRET efficiency per focal adhesion per cell was observed between the TGF-β-treated (+ TGF- β) and untreated (- TGF- β) groups ($p = 0.9099$) (Fig. 17a). The mean FRET efficiencies were comparable between both conditions, with averages of $19.25 \pm 2.51\%$ for the TGF-β-treated CAFs and $19.31 \pm 2.67\%$ for the untreated CAFs. In the SCL-VinTS7 CAF cell line, a total of 30 TGF-β-treated cells and 29 untreated cells were imaged. Figure 16 displays representative images of three TGF-β-treated (+ TGF- β) (g-i) and three untreated (- TGF- β) CAFs (j-l), again showing localization of VinTS7 within the FAs. A statistically significant difference in mean FRET efficiency per FA per cell was observed in the stable cell line CAFs between the two conditions ($p = 0.0170$) (Fig. 17b). The TGF-β-treated CAFs exhibited a lower mean FRET efficiency of $16.32 \pm 2.63\%$, whereas the untreated CAFs displayed a higher mean FRET efficiency of $18.01 \pm 2.60\%$.

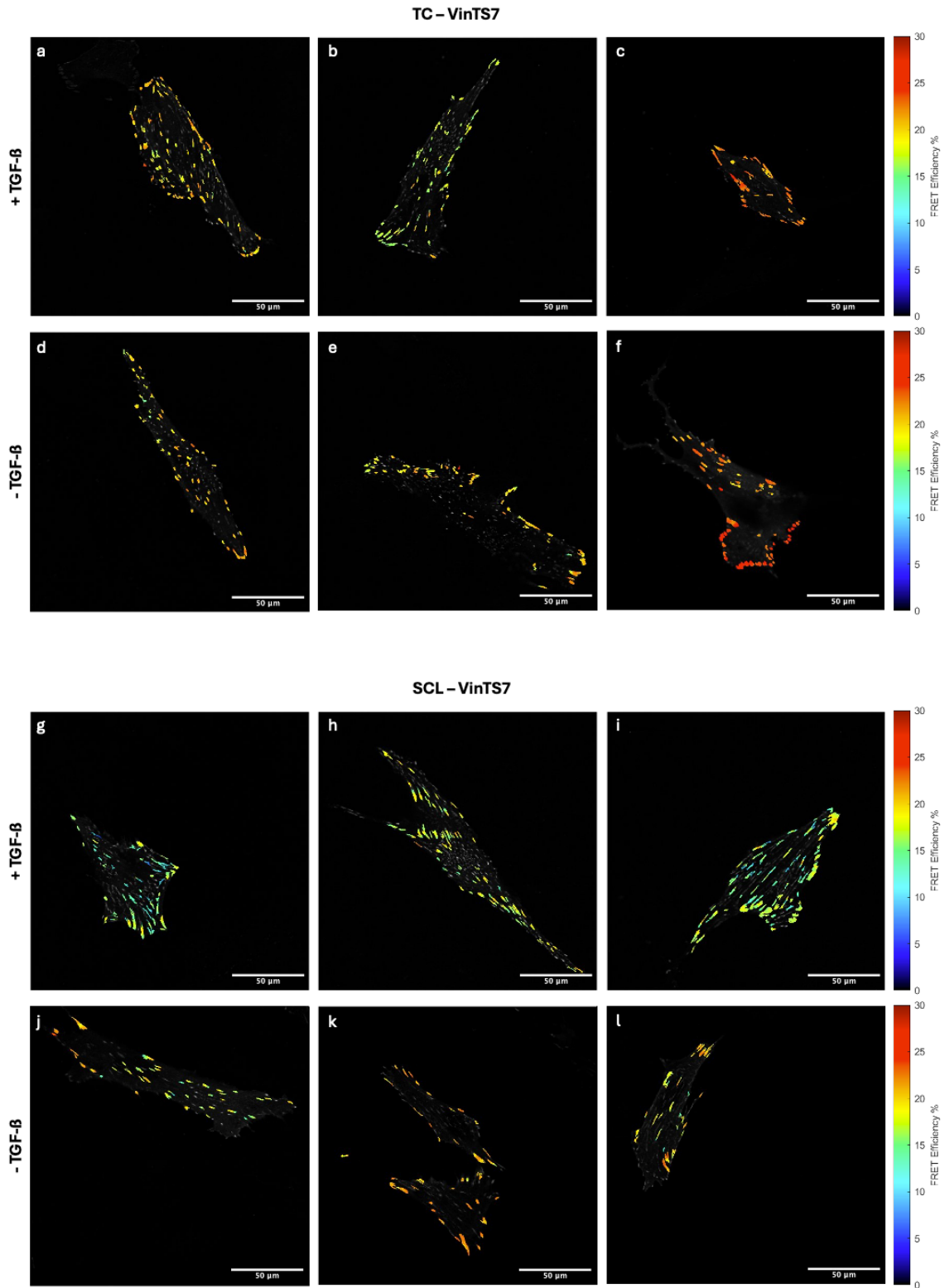


Figure 16. Representative microscopic images of CAFs expressing VinTS7, with FRET efficiency (%) visualized using a colour-coded scale. (a–c) Transiently transfected CAFs (TC–VinTS7) treated with TGF- β (+TGF- β). (d–f) Transiently transfected CAFs (TC–VinTS7) without TGF- β treatment (–TGF- β). (g–i) Stable VinTS7-expressing CAF cell line (SCL–VinTS7) treated with TGF- β (+TGF- β). (j–l) Stable VinTS7-expressing CAF cell line (SCL–VinTS7) without TGF- β treatment (–TGF- β).

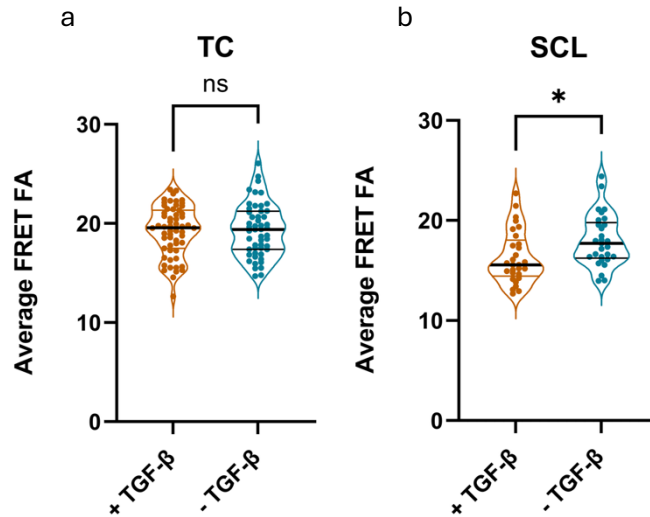


Figure 17. Mean FRET efficiency per focal adhesion per cell in VinTS7-expressing CAFs. (a) No statistically significant difference was found between TGF- β -treated (+ TGF- β) and untreated (- TGF- β) cells in TC CAFs ($p = 0.9099$; $19.25 \pm 2.51\%$ vs. $19.31 \pm 2.67\%$; $n=59$ vs 51 , respectively). (b) In the SCL-VinTS7, TGF- β treatment (+TGF- β) led to a significantly lower FRET efficiency compared to untreated (-TGF- β) cells ($p = 0.0170$; $16.32 \pm 2.63\%$ vs. $18.01 \pm 2.60\%$; $n=29$). Statistical analysis between two groups were performed using unpaired two-tailed parametric Student's t -tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

4.2.2 Amount FAs/Vinculin

Subsequently, the total number of FAs per cell was compared between TGF- β -treated and untreated CAFs. Only images showing clearly isolated individual cells were selected for this analysis. For the TC-VinTS7 CAFs, a total of 42 TGF- β -treated cells and 35 untreated cells were included. A statistically significant difference in the number of FAs per cell was observed between the two groups ($p = 0.0004$) (Fig. 18a). The TGF- β -treated cells exhibited an average of 39.86 ± 16.78 focal adhesions per cell, whereas the untreated cells showed an average of 27.80 ± 10.08 focal adhesions per cell. Similarly, in the SCL-VinTS7-expressing CAFs, a total of 22 TGF- β -treated cells and 14 untreated cells were analysed. Again, a statistically significant difference was found ($p = 0.0002$) (Fig. 18b). The TGF- β -treated cells had an average of 51.32 ± 17.16 FAs per cell, while the untreated cells had an average of 30.14 ± 9.70 .

In addition, we investigated whether there was a significant difference in the average amount of vinculin per FA between CAFs treated with TGF- β and untreated CAFs. Only cells from the SCL-VinTS7 were used for this analysis, because of their homogeneous expression levels and rather similar cell-to-cell fluorescence intensity. The analysis showed a statistically significant difference ($p = 0.0108$), with the untreated CAFs showing a higher mean vinculin intensity per FA (mean 358.01 ± 159.16) compared to the TGF- β -treated CAFs (mean 257.73 ± 132.22) (Fig. 18c).

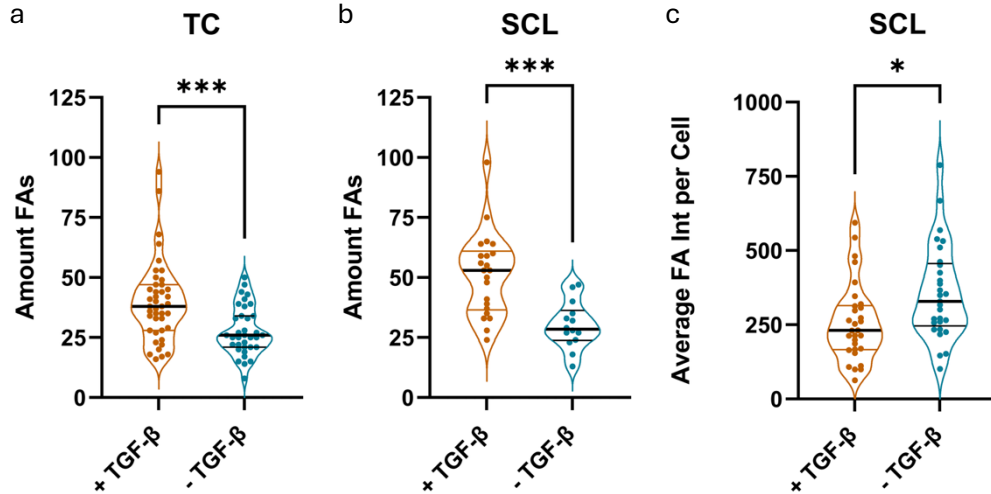


Figure 18. Quantification of focal adhesions (FAs) per cell in VinTS7-transfected and stable VinTS7-expressing CAFs. (a) In TC-VinTS7 CAFs, TGF- β treatment (+TGF- β) significantly increased the number of FAs per cell compared to untreated (-TGF- β) cells ($p = 0.0004$; 39.86 ± 16.78 vs. 27.80 ± 10.08 FAs per cell; $n = 42$ and 35 , respectively). (b) in the SCL-VinTS7 CAFs, TGF- β -treated (+TGF- β) cells displayed significantly more FAs than untreated (-TGF- β) cells ($p = 0.0002$; 51.32 ± 17.16 vs. 30.14 ± 9.70 FAs per cell; $n = 22$ and 14 , respectively). (c) In SCL-VinTS7 CAFs, TGF- β -treated (+TGF- β) cells exhibited a significantly lower average vinculin intensity per FA compared to untreated (-TGF- β) cells ($p = 0.0108$; 257.73 ± 132.22 vs. 358.01 ± 159.16 mean intensity units; $n = 30$ and 29 , respectively). Statistical analysis between two groups were performed using unpaired two-tailed parametric Student's t -tests: * $p < 0.05$, ** $p < 0.01$, $p^{***} < 0.001$ and **** $p < 0.0001$.

4.2.3 Controls

4.2.3.1 VinTL7

As a control construct for VinTS7, VinTL7 (Vinculin tailless) was employed. This control is essential to assess the accuracy of force measurements with VinTS7. VinTL7 lacks the tail domain of vinculin, resulting in the lack of mechanical coupling between the ECM and the cytoskeleton. Specifically, there is no force transmitted over vinculin, even though talin remains mechanically engaged with actin. Vinculin's role is primarily to stabilize focal adhesions under tension due to the lack of force transduction, the molecular spring of the sensor is expected to be in a relaxed state, reflected in increased FRET efficiency.

VinTL7 was introduced into the cells via transient transfection (TC-VinTL7). Subsequently, five TGF- β -treated cells five and untreated cells were analysed. The results from these groups were compared to the SCL-VinTS7 CAFs. Figure 19 presents representative fluorescence images of CAFs treated with TGF- β (a) compared to untreated control cells (d).

Within the stable cell line under TGF- β treatment, a statistically significant difference was detected between VinTS7 and VinTL7 (mean values: $16.32 \pm 2.63\%$ vs. $18.95 \pm 1.06\%$; $p = 0.0471$), suggesting altered tension in the VinTS7 construct under these conditions (Fig. 20a). Under the condition without TGF- β treatment, no significant differences were found between VinTS7 and VinTL7 (mean values: $18.01 \pm 2.60\%$ vs. $18.76 \pm 1.92\%$; $p = 0.9068$) (Fig. 20b).

4.2.3.2 VinTS-I997A

As a second control, a variant of VinTS7 containing a point mutation I997A in the tail domain of vinculin was used. In this mutation, the isoleucine (I) at position 997 is replaced by alanine (A). This mutation disrupts vinculin function and mimics the effect of the VinTL7, while still maintaining the full vinculin structure. As a result, the sensor is expected to remain in a relaxed state, resulting in increased FRET efficiency. Figure 19 shows representative fluorescence images of a TGF- β -treated CAF (b) and an untreated CAF (e).

As expected, VinTS-I997A exhibits a similar FRET profile to VinTL7. In TGF- β -treated cells, a significant difference was observed in the SCL-VinTS7 under TGF- β treatment (means $16.32 \pm 2.63\%$ and $18.97 \pm 1.72\%$, respectively; $p = 0.0053$) (Fig. 20a). In the condition without TGF- β , no significant difference was found in the SCL-VinTS7 (means $18.01 \pm 2.60\%$ and $18.61 \pm 2.94\%$, respectively; $p = 0.8904$) (Fig. 20b).

4.2.3.3 TSMoD7

As an additional no-tension control for the VinTS7 construct, a stable cell line expressing TSMoD7 (SCL-TSMoD7) was generated. TSMoD7 is a cytosolic FRET-based tension sensor that lacks vinculin or any anchoring domain, and therefore does not experience mechanical forces. TSMoD7 consists solely of the donor–acceptor fluorophore pair (Clover–mScarlet-I) separated by the elastic (GGSGGS)₇ linker and does not include any protein domains that tether the construct to cellular structures. As a result, TSMoD7 remains freely diffusive in the cytosol and is not subject to mechanical tension. Consequently, it serves as a reference for the maximum expected FRET efficiency in the absence of force transmission. In this experiment, CAFs treated with TGF- β , and untreated controls were analysed separately. A total of 10 CAFs expressing TSMoD7 were imaged for each condition. Representative microscopy images of TGF- β -stimulated CAFs (c) and untreated CAFs (f) are shown in figure 19.

Under TGF- β treatment, no statistically significant difference was observed between SCL-VinTS7 and TSMoD7 (mean values: $16.32 \pm 2.63\%$ vs. $14.65 \pm 1.23\%$; $p = 0.1175$) (Fig. 20a). In the absence of TGF- β , significant differences in FRET efficiency were observed between TSMoD7 and SCL-VinTS7 ($18.01 \pm 2.60\%$ vs. $12.31 \pm 2.54\%$; $p < 0.0001$) (Fig. 20b). These results contrast with the theoretical expectation of higher FRET efficiency in TSMoD7-expressing cells.

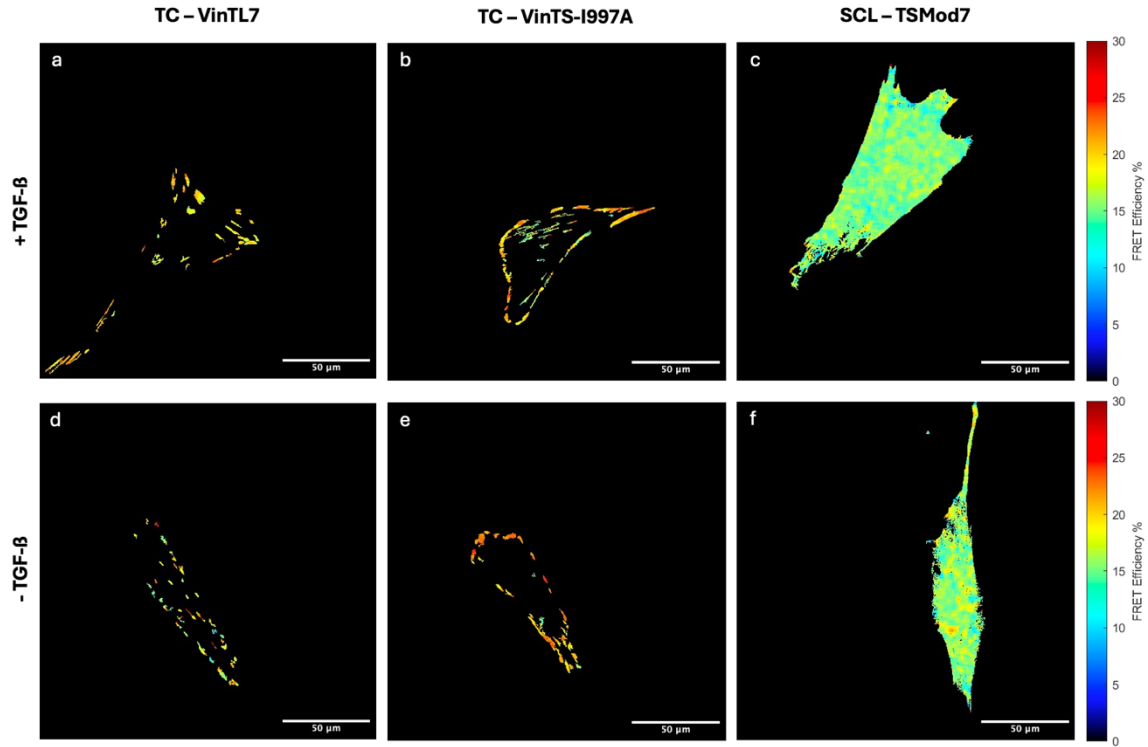


Figure 19. Representative microscopic images of CAFs expressing VinTL7, VinTS-I997A and TSMoD7, with FRET efficiency (%) visualized using a colour-coded scale. (a-c) Transiently transfected CAFs (TC-VinTL7 and TC-VinTS-I997A) and continuous transfected (SCL-TSMoD7) treated with TGF- β (+TGF- β). (d-f) Transiently transfected CAFs (TC-VinTL7 and TC-VinTS-I997A) and continuous transfected (SCL-TSMoD7) without TGF- β treatment (-TGF- β)

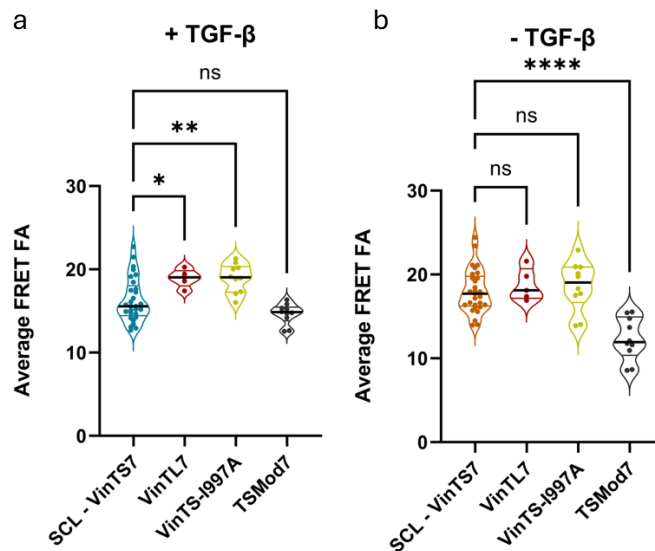


Figure 20. FRET efficiency (%) of VinTS7 and control constructs in CAFs under (a) +TGF- β and (b) -TGF- β conditions. (a) Under TGF- β treatment, SCL-VinTS7 showed significantly lower FRET efficiency compared with VinTL7 ($p = 0.0471$; $16.32 \pm 2.63\%$ vs. $18.95 \pm 1.06\%$; $n = 29$ and 5 , respectively)

and VinTS7-A99I ($p = 0.0053$; $16.32 \pm 2.63\%$ vs. $18.97 \pm 1.72\%$; $n=29$ and 10 , respectively). In addition, the FRET efficiency of SCL-VinTS7 was not significantly different from that of TSMoD7 ($p = 0.1175$; $16.32 \pm 2.63\%$ vs. $14.65 \pm 1.23\%$; $n=29$ and 10 , respectively). (b) Without TGF- β treatment (-TGF- β), there were no significant differences between VinTS7 and VinTL7 in SCL ($p = 0.9068$; $18.01 \pm 2.60\%$ vs. $18.76 \pm 1.92\%$; $n=29$ and 5 , respectively) or between VinTS7 and VinA99I ($p = 0.8904$; $18.01 \pm 2.60\%$ vs. $18.61 \pm 2.94\%$; $n=29$ and 10 , respectively). The FRET efficiency of SCL-VinTS7 was significantly higher than that of TSMoD7 ($p < 0.0001$; $18.01 \pm 2.60\%$ vs. $12.31 \pm 2.54\%$; $n=29$ and 10 , respectively). Statistical analysis between two groups were performed using ordinary one-way ANOVA: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$.

4.3 Objective III: Development of a fluorescent reporter for live-cell imaging of CAF differentiation

4.3.1 PCR fragments

The **pcDNA3.1 backbone (pBB)** was derived from the pcDNA3.1-Clover plasmid. To enable the stepwise assembly of the final construct, three distinct variants of the pcDNA3.1 backbone were generated, each containing a unique combination of restriction sites. **pBB1** includes the restriction sites NotI and BclI, **pBB2** contains NotI and EcoRI, and **pBB3** incorporates EcoRI and BclI. These variations enabled the generation of intermediate plasmids required for modular cloning. The first backbone was designed for insertion of **ACTA2 promoter (P1) for plasmid 1** (reporter plasmid without the ACTA2 enhancer), the second backbone was used for constructing an intermediate plasmid containing the **ACTA2 enhancer (Enh)**, and the third backbone allowed insertion of **ACTA2 promoter (P2) for plasmid 2** (reporter plasmid with ACTA2 enhancer). In all cases, the expected PCR product of approximately 4420 base pairs was successfully obtained (Suppl. Fig. 1a-b). The ACTA2 enhancer fragment was amplified from genomic DNA isolated from CAFs, resulting in the expected PCR product of 3795 base pairs (Suppl. Fig. 1c). In addition, the ACTA2 promoter regions were amplified from the same genomic DNA source. Two separate promoter constructs were designed: one for including the ACTA2 enhancer in the plasmid and one without. Both constructs yielded PCR products of identical length, with the expected fragment size of approximately 757 base pairs (Suppl. Fig. 1c). For the first fluorescent protein, **mTagBFP2 (mT)**, the mTagBFP2-Farnesyl-5 plasmid was used as a template. The PCR product of 1642 base pairs was successfully obtained (Suppl. Fig. 1d). For **mGarnet2 (mG)**, the LifeAct-mGarnet2 plasmid served as a template. The PCR product of 757 base pairs was successfully obtained, including sequences for nuclear localization (NLS) and a stop codon to ensure proper nuclear targeting and translation termination (Suppl. Fig. 1d).

4.3.2 Construction single-fragment intermediate plasmids

To facilitate the modular assembly of the final expression constructs, three intermediate single-fragment plasmids were generated, namely: i) **pBB1-P1**, consisting of pcDNA3.1 backbone 1 (pBB1) combined with ACTA2 promoter 1 (P1), ii) **pBB2-Enh**, consisting of pcDNA3.1

backbone 2 (pBB2) combined with the ACTA2 enhancer (Enh), and iii) **pBB3-P2**, consisting of pcDNA3.1 backbone 3 (pBB3) combined with ACTA2 promoter 2 (P2). All intermediate constructs were validated by Sanger sequencing.

4.3.3 Construction pcDNA3.1-ACTA2 Enhancer-ACTA2 Promotor

The next step involved generating a construct containing both the ACTA2 enhancer and ACTA2 promoter 2 within a single plasmid. Two strategies were employed. In the first approach, the ACTA2 enhancer was ligated into pBB3-P2. In the second approach, ACTA2 promoter 2 was introduced into pBB2-Enh. Both strategies aimed to generate a unified construct, designated **pBB-Enh-P2**, ACTA2 enhancer and ACTA2 promoter 2. The final plasmid was confirmed by Sanger sequencing.

4.3.4 Construction pcDNA3.1-mTagBFP2-ACTA2 Promotor-mGarnet_NLS

The two fluorescent proteins, mTagBFP2 and mGarnet2, were sequentially inserted into the intermediate construct pBB-P1, resulting in the final plasmid **pBB-mT-P1-mG**. This is the final product of the reporter plasmid without the ACTA2 enhancer, pcDNA3.1-mTagBFP2-ACTA2 Promotor-mGarnet_NLS. Successful integration of all genetic elements was confirmed by Sanger sequencing.

4.3.5 Construction pcDNA3.1-mTagBFP2 -ACTA2 Promotor-mGarnet_NLS

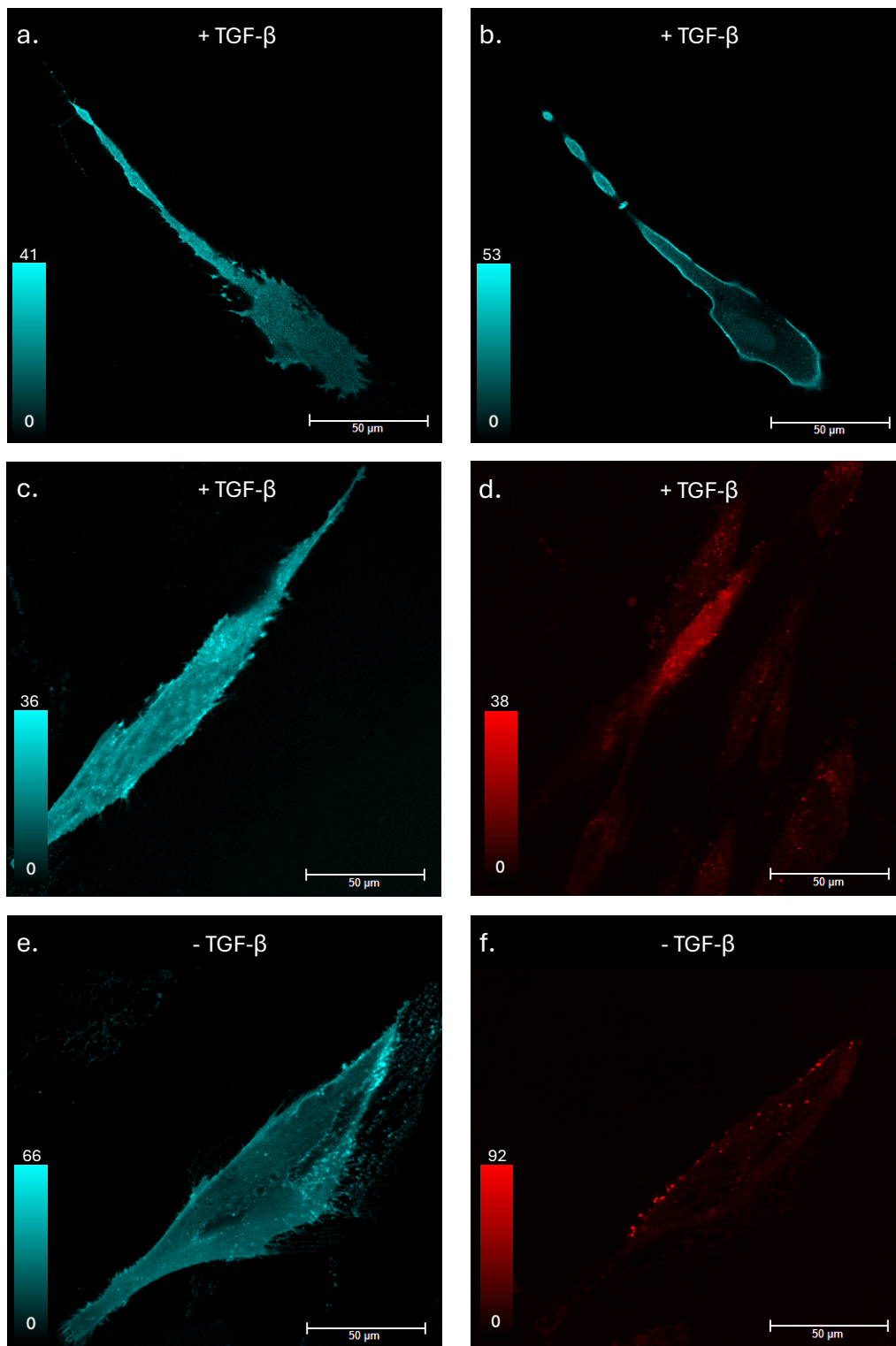
To generate the final product of the reporter plasmid with the enhancer, we aimed to integrate the **ACTA2 enhancer-ACTA2 promoter 2** fragment (as assembled in pBB-Enh-P2; §4.3.3) into the previously constructed plasmid **pBB-mT-P1-mG** (§4.3.4), thereby replacing ACTA2 promoter 1 with the ACTA2 enhancer-ACTA2 promoter 2 combination to create the final construct **pBB-mT-Enh-P2-mG**, pcDNA3.1-mTagBFP2 -ACTA2 Promotor-mGarnet_NLS. Despite several attempts, **this final cloning step was not successfully completed** within the timeframe of this master thesis. Further optimization and troubleshooting will be required to complete the assembly of this construct.

4.3.6 Testing pcDNA3.1-mTagBFP2-ACTA2 Promotor-mGarnet_NLS

To functionally validate the plasmid **pcDNA3.1-mTagBFP2-ACTA2 promoter-mGarnet2 (pBB-mT-P1-mG)**, CAFs were transiently transfected with the construct. Transfection efficiency was assessed by selecting cells that expressed mTagBFP2, visualized through fluorescence at the cell membrane. This membrane localization is mediated by a C-terminal amino acid sequence included in the mTagBFP2 construct (Fig. 21a: basal cell surface in contact with the glass coverslip; b: apical membrane at the upper cell surface).

To evaluate the activity of mGarnet2, which is under the control of the ACTA2 promoter, the TC-CAFs were analysed under two experimental conditions. In the first condition, cells were treated with TGF- β , which induces α SMA expression and is expected to activate the ACTA2 promoter. This should lead to nuclear expression of mGarnet2, due to the presence of two NLS sequences in the construct. In the second condition, cells were not treated with TGF- β , and therefore no activation of the ACTA2 promoter or expression of mGarnet2 was anticipated. As shown in Figure 21(d), little to no expression of mGarnet2 was observed in the TGF- β -treated condition and in the untreated group (Fig. 21(f)), resulting in minimal differences between the two groups. To confirm that the CAFs transfected with plasmid 2 (pcDNA3.1-mTagBFP2-ACTA2 Enhancer-ACTA2 Promotor-mGarnet_NLS) were indeed expressing α SMA following TGF- β stimulation, immunostaining for α SMA was performed in parallel. Interestingly, most of the transfected cells appeared to lack detectable α SMA expression (Fig. 22), whereas surrounding, non-transfected cells did show clear α SMA expression. This observation suggests that the α SMA pathway was effectively activated by TGF- β treatment in the overall cell population. The absence of mGarnet2 expression in the transiently transfected cells may therefore be attributed to insufficient promoter activity or another limitation within the plasmid construct, rather than a failure of pathway activation.

To assess whether the NLSs located at the 3' end of the mGarnet2 sequence influence its subcellular localization, **two new constructs were designed**. In both constructs, the mGarnet2 coding sequence was placed under the control of the CMV promoter within the pcDNA3.1 backbone. In the first construct, the original configuration of mGarnet2 was maintained, in which two consecutive NLS sequences were positioned at the 3' terminus of the fluorophore (referred to as **mGarnet2_NLS**). To insert this construct into pcDNA3.1, custom-designed primers 14 and 15 were used (Suppl. Table 3). In the second construct, the two consecutive NLS sequences were relocated to the 5' end of the mGarnet2 using primers 18 and 19 (Suppl. Table 3) (referred to as **NLS_mGarnet2**). Integration into the pcDNA3.1 vector was achieved using primers 16 and 17. Due to time constraints, the cloning of the first construct (mGarnet2_NLS) could not be completed within the scope of this thesis. However, the second construct (NLS_mGarnet2) was successfully generated and subsequently transfected into CAFs. As shown in Figure 21(g), the expressed NLS_mGarnet2 localizes predominantly to the nucleus, confirming both successful expression and nuclear targeting of the construct.



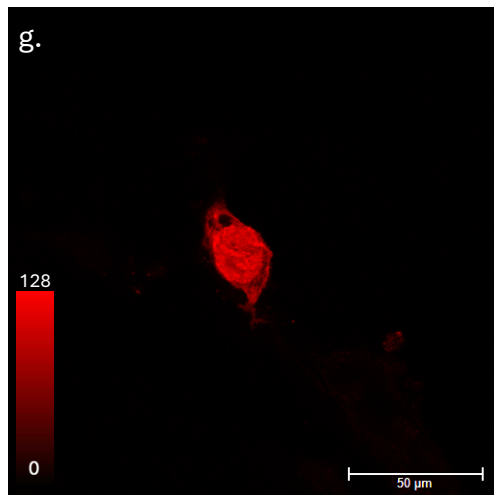


Figure 21. Microscopic images showing mTagBFP2 and mGarnet2 expression in CAFs with and without TGF- β treatment. (a–b) mTagBFP2 localized at the cell membrane of CAFs; (a) Surface view, (b) Same cell imaged at a higher Z-plane. (c–d) CAF treated with TGF- β : (c) Membrane-localized mTagBFP2, (d) Nuclear mGarnet2 expression in the same cell. (e–f) CAF without TGF- β treatment: (e) Membrane mTagBFP2, (f) Nuclear mGarnet2 in the same cell. (g) Nuclear localization of mGarnet2 under control of the CMV promoter in CAFs.

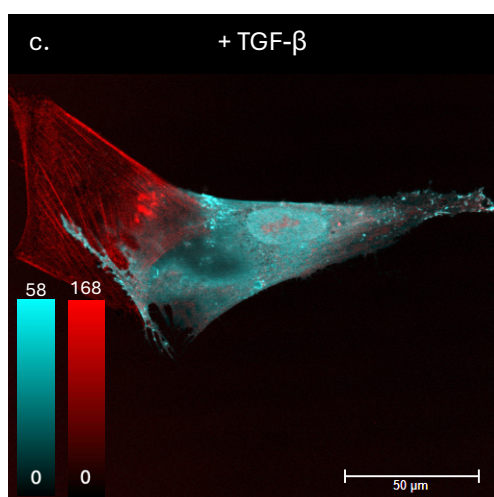
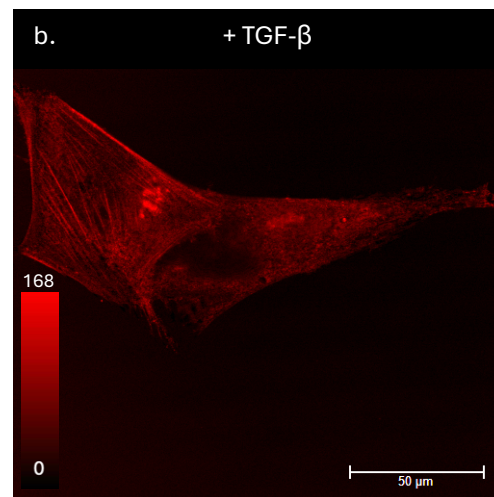
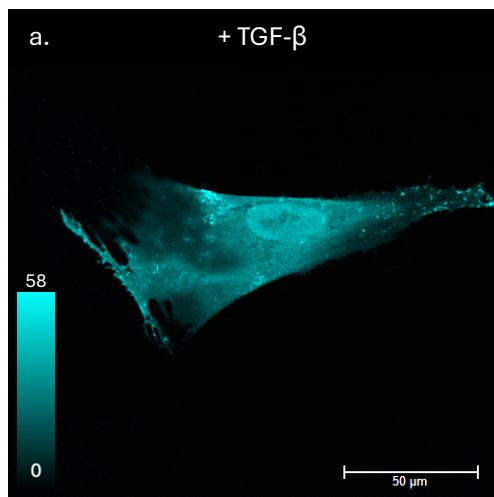


Figure 22. Representative fluorescence microscopy images showing mTagBFP2 and α SMA expression in CAFs following TGF- β treatment. (a) mTagBFP2 fluorescence, illustrating membrane localization in a single CAF. (b) The same field of view after α SMA immunostaining, showing α SMA expression in the surrounding CAFs. (c) Merged image of (a) and (b).

5 DISCUSSION

5.1 Objective I: Activation assay

5.1.1 Protocol

The first part of this thesis introduces an optimised protocol for the activation for 2D *in vitro* differentiation of CAFs. By systematically optimising medium composition and the concentration of TGF- β , it was possible to reduce the time and compounds required to perform the activation assay. This optimisation contributes to improved reproducibility and facilitates the comparability of future studies.

Our results show that an initial cell growth time of one day is sufficient, although this may vary depending on the fibroblast type used. At a seed density of 125 cells/mm², differentiation of CAFs can be initiated by the addition of 1 ng/mL TGF- β . The induction of differentiation requires an incubation time of about 24 hours, after which almost complete efficiency of differentiation is observed. The activated CAFs can then be verified for expression of the α SMA marker. In this study, immunostaining was applied for detection, but alternative techniques such as western blotting can also be used. A notable finding is that a concentration as low as 1 ng/mL is already sufficient to induce a high efficiency of the differentiation response. This is illustrated in figure 14, where similar expression levels of α SMA are observed. Consequently, a higher concentration, such as 3 ng/mL or 5 ng/mL, does not appear necessary. This avoids unnecessary and excessive exposure to TGF- β , as well as the associated cellular stress. In addition, we investigated whether preconditioning the cells in starvation medium would promote the differentiation process by first exposing them to a stressful environment. The results showed that there was no real difference in α SMA expression between cells grown in starvation medium and those in normal medium. This implies that an additional preconditioning step is not necessary, increasing the efficiency of the protocol.

Quantitative analysis revealed a **differentiation efficiency of 87.72% following 24-hour treatment with 1 ng/mL TGF- β** . In contrast, non-treated CAFs displayed a baseline α SMA expression of only 0.085%. While low, this residual expression indicates that a small subset of untreated CAFs still express α SMA. This may be attributed to the inherent cellular memory of these CAFs, which may have previously expressed α SMA in their original tumour microenvironment before reverting to a more quiescent state. As such, these cells may retain a heightened sensitivity to cues promoting α SMA expression. Furthermore, mechanical stress exerted through the underlying 2D substrate, such as the glass surface, may activate signalling pathways involved in α SMA expression. Given the relatively high cell density at the time of fixation, it is plausible that intercellular mechanical interactions contributed to α SMA expression in some untreated cells.

The literature shows that protocols for the *in vitro* 2D differentiation of fibroblasts with TGF- β vary considerably. This variability includes the TGF- β concentrations used, growth conditions and preconditioning strategies. For example, some studies use a 48-hour incubation time, a preconditioning starvation condition or a higher TGF- β concentration up to 10 ng/mL^{84–86}. However, our results show that a 24-hour incubation time and a TGF- β concentration as low as 1 ng/mL are already sufficient to induce efficient differentiation. One of the main objectives of this optimisation is to potentially standardise CAF activation. This would facilitate the comparability of research results between different research groups.

5.1.2 Limitations

Some limitations should be considered. For example, this protocol was tested only on the specific CAF cell line CT5.3hTERT. Other CAF subtypes or fibroblast cells could potentially respond differently to TGF- β , which requires additional validation to assess the broader applicability of the protocol. In addition, immunostaining was used for the visualisation of α SMA. Although this method is suitable for qualitative evaluation, or in other words, shows whether a CAF cell is ‘activated’ or not, quantitative analysis techniques, such as western blotting, would be better suited for more accurate quantification of α SMA expression and hence the degree of CAF activation.

5.2 Objective II: Investigation Mechanobiology in CAFs

5.2.1 FRET efficiency

The second objective of this thesis was to investigate the mechanical tension within FAs of CAFs under two distinct conditions: one in which the CAFs were treated with TGF- β and one in which they remained untreated. To quantitatively assess the intracellular tension within FAs, we employed the vinculin-based tension sensor VinTS7. This biosensor allows precise measurement of mechanical forces exerted at vinculin-containing adhesion sites. It was hypothesised that TGF- β -treated CAFs would exhibit higher mechanical tension at their FAs compared to untreated cells. This expectation is based on the premise that TGF- β -treated CAFs are in an active state of spreading and remodelling, processes that involve increased cytoskeletal contractility and traction force generation⁸⁷. In this context, FAs serve as critical anchoring points that transmit these intracellular forces to the ECM⁸⁸.

To measure the tension across the VinTS7 sensor, FLIM-FRET approach was employed. This technique enables quantification of FRET efficiency through the measurement of donor fluorescence lifetime, which reflects the distance between the two fluorophores linked by the (GGSGGS)₇ elastic spring. Higher tension results in increased separation of the fluorophores, thereby reducing FRET efficiency, which is detected through the increase of Clover fluorescence lifetime. Initially, VinTS7 was introduced into the CAFs via transient transfection. However, this approach led to considerable heterogeneity in fluorescence intensity and FRET

efficiency among cells that successfully incorporated the plasmid. Since transient transfection does not integrate the plasmid into the host genome, the number of plasmid copies taken up varies from cell-to-cell^{93,94}. As a result, some cells expressed higher levels of VinTS7 within their focal adhesions than others, leading to substantial variability in sensor intensity across the cell population. This variability complicated the selection of cells with comparable vinculin levels for analysis. Attempts to select cells based on uniform fluorescence intensity were inherently prone to bias and lacked reproducibility. Furthermore, the transient transfection process itself introduces cellular stress, which may alter the physiological state of the cells and confound the interpretation of tension measurements. These confounding factors likely influenced the mechanical tension detected at vinculin sites within FAs and may explain why no significant difference in FLIM-derived tension was observed between TGF- β -treated and untreated CAFs in the transiently transfected cells.

Due to the limitations associated with transient transfection, we opted to generate a stable cell line in which the VinTS7 construct was stably integrated into the genomic DNA of the CAFs. This approach offers several advantages. First, it enabled the analysis of a larger number of cells per sample, as a greater proportion of CAFs consistently expressed the VinTS7 sensor. Second, stable integration significantly reduces the likelihood of multiple copies of the construct being incorporated into the genome of individual cells, resulting in more homogeneous expression levels of VinTS7 across the cell population⁹³. Consequently, this uniformity led to more consistent fluorescence intensity within FAs, minimizing experimental variability and reducing selection bias. In addition, the cellular stress typically induced by the transient transfection procedure is avoided in the stable cell line, thus better preserving the native physiological state of the cells. When using this SCL-VinTS7, a significant difference in mechanical tension was observed between the two experimental conditions. Specifically, **CAFs treated with TGF- β exhibited significantly higher tension over vinculin** compared to untreated CAFs. These results confirm the initial hypothesis and support the notion that TGF- β -treated CAFs, being in a more contractile and activated state, generate higher intracellular forces that are transmitted through FAs⁸⁷. This observation aligns with previous studies demonstrating that differentiated CAFs exhibit enhanced contractile behaviour and actively modulate the mechanical properties of the extracellular matrix⁹¹. Therefore, the establishment of a stable VinTS7-expressing CAF-line was essential for reliably detecting differences in mechanical tension and for overcoming the technical and biological limitations associated with transient expression.

5.2.2 Amount FAs

Another parameter of interest in this study was the number of FAs per cell. We hypothesised that CAFs treated with TGF- β would display a higher number of FAs per cell compared to untreated CAFs. This expectation is consistent with the hypothesis discussed in §5.2.1. As these differentiated CAFs spread more extensively, they are expected to form more adhesion points with the ECM, manifested as an increased number of focal adhesions. Thus, an

increased number of FAs in TGF- β -treated cells likely reflects their enhanced adhesive and contractile behaviour, which is strongly associated with elevated FA formation and maturation⁹¹.

In both experimental conditions, TC-VinTS7 CAFs and the SCL-VinTS7 CAFs, a significant difference in the number of FAs per cell was observed. Specifically, **CAFs treated with TGF- β consistently exhibited a higher number of FAs** compared to untreated CAFs. These findings support our hypothesis that TGF- β -treated CAFs adopt a more activated and contractile phenotype (myofibroblast), which is reflected in their increased formation of FA sites. This elevated number of FAs likely facilitates enhanced interaction with the ECM, enabling the cells to generate traction and migrate more effectively. The results confirm that myofibroblast-like CAFs, induced by TGF- β , utilise a greater number of FAs to establish stronger and more numerous anchorage points, thereby supporting their motile and invasive behaviour within the cellular microenvironment.

5.2.3 Amount Vinculin FA per cell

When assessing the quantitative distribution of vinculin within FAs per cell, measured as the average fluorescence intensity per FA, a significant difference was observed between TGF- β -treated and untreated CAFs in the SCL-VinTS7 CAFs. **Untreated CAFs exhibited slightly but significantly higher average vinculin intensity per FA**, indicating increased vinculin density at individual adhesion sites. A plausible explanation for this finding is that untreated CAFs may compensate for their lower number of FAs by recruiting more vinculin per FA site. This enhanced recruitment could reinforce the integrin–actin linkage, thereby sustaining cytoskeletal tension through a limited number of mechanically robust adhesions. This hypothesis aligns with previous studies demonstrating the role of vinculin as a force-sensitive adaptor that stabilizes FAs under mechanical load by strengthening integrin-cytoskeleton coupling^{92,93}. Elevated vinculin expression and recruitment per FA may thus enhance the mechanical stability of each FA, ensuring adequate contractile force transmission even when fewer FAs are present. Taken together with the results discussed in §5.2.2., an additional interpretation emerges: TGF- β -treated CAFs appear to form a greater number of FAs and experience increased tension across vinculin molecules but exhibit relatively reduced vinculin content per individual FA. In contrast, untreated CAFs rely on fewer but more vinculin-enriched FAs, potentially as a compensatory mechanism to preserve mechanical integrity and adhesive functionality in the absence of TGF- β stimulation.

5.2.4 VinTS Controls

To validate the functionality and sensitivity of the VinTS7 tension sensor, three control sensors were employed: VinTL7, VinTS-I997A, and TSMoD7. All three are expected to exhibit higher FRET efficiencies compared to VinTS7, as they are designed to reflect conditions with minimal or no mechanical tension across the elastic spring element. A significant difference in FRET

efficiency was observed between VinTS7 and both VinTL7 and VinTS-I997A under the condition where CAFs were treated with TGF- β . In this more 'activated' state, **VinTL7 and VinTS-I997A exhibited significantly higher FRET efficiencies than VinTS7**, consistent with the expectation that VinTS7 reports increased mechanical strain in FAs, while the control constructs remain mechanically relaxed. These results are consistent with previous studies, which have similarly reported that control constructs lacking mechanical load-bearing capacity exhibit higher FRET efficiencies due to the absence of tension across the sensor module^{74,94}. However, it should be noted that the FRET efficiencies reported for VinTL7 and VinTS-I997A in the present study were somewhat lower than those observed in other studies, potentially due to differences in experimental setup, sensor design, or cellular context.

However, the results obtained from the TSMo7 control deviated from expectations. No significant difference was observed between SCL-TSMo7 and SCL-VinTS7 in TGF- β -treated cells. In fact, **the average FRET efficiency of TSMo7 was slightly lower than that of VinTS7**. These findings contradict the anticipated outcome, as TSMo7, being a cytosolic, untargeted sensor, should display minimal mechanical load and therefore a higher FRET efficiency. These findings are in contrast with previously published data, where TSMo7-based constructs consistently demonstrated higher FRET efficiencies than force-sensitive biosensors such as VinTS, due to the absence of mechanical strain across the spring module^{74,95}. Several factors may account for this unexpected behaviour of TSMo7. First, because TSMo7 is freely distributed in the cytoplasm, high expression levels could promote intermolecular FRET between donor and acceptor fluorophores from different sensor molecules, resulting in artifactual energy transfer and a net decrease in measurable FRET efficiency. Second, the elastic linker (GGSGGS)₇ used in TSMo7 may adopt a more extended conformation in the cytoplasmic environment, increasing the spatial separation between donor and acceptor and thereby reducing maximal FRET efficiency. Additionally, differences in local physicochemical conditions (such as pH, temperature, or ionic strength) between the cytoplasm and FAs may influence fluorophore maturation or sensor conformation, ultimately impacting FRET performance. It can also be to do with FLIM, where the lifetime of fluorescent proteins depend on their microenvironment, hence the lifetime of cytosolic control can be different from the lifetime of same fluorescent protein located in FAs.

Under the condition in which CAFs were not treated with TGF- β , no significant differences were found between VinTS7 and either VinTL7 or VinTS-I997A. This suggests that in the absence of TGF- β stimulation, FAs are in a more relaxed state, exerting little to no mechanical tension on vinculin. As a result, VinTS7 behaves similarly to the control sensors, in line with their relaxed conformation.

5.2.5 Limitations

Several limitations of the experimental setup must be acknowledged when interpreting the results of this study. First, all force measurements were conducted using a single tension

sensor, VinTS7. While VinTS7 provides valuable insights into the mechanical forces at FAs, the literature increasingly highlights a diverse array of tension sensors, each with distinct force sensitivity ranges (in the piconewton scale). A meaningful extension of this work would be to incorporate a broader panel of force sensors. This would allow for the determination of the most appropriate sensor that matches the force range exerted at CAF FAs, thereby enabling more accurate and physiologically relevant quantification.

Second, the control constructs VinTL7 and VinTS-I997A were introduced into the CAFs via transient transfection. This approach introduces variability in the number of plasmids taken up by individual cells, resulting in inconsistent expression levels and, consequently, heterogeneous fluorescence intensities across the sample population. In addition, transient transfection itself is a known source of cellular stress, which can alter cellular behaviour and potentially confound experimental outcomes. The variability in expression made it challenging to select CAFs with comparable fluorescence intensities across conditions, leading to potential bias in cell selection. The morphology and physiological behaviour of TC-VinTL7 CAFs were also noticeably abnormal. This further complicated the selection of representative cells for analysis, and as a result, only five cells per condition could be reliably selected for VinTL7 measurements. This limited sample size reduces the robustness of conclusions drawn from this control condition. In contrast, TSMoD7 was introduced via stable integration alongside VinTS7, which eliminated the confounding effects associated with transient transfection. However, the unexpected results obtained with TSMoD7 are likely attributable to the properties of its elastic linker. The (GGSGGS)₇ spring used in this construct may not be optimal for cytosolic applications. Its high elasticity could lead to a naturally extended conformation that increases the donor–acceptor distance and reduces the baseline FRET efficiency, even in the absence of mechanical tension. To confirm this hypothesis, it would be necessary to compare TSMoD7 with other cytosolic tension sensors that incorporate alternative spring sequences with varying stiffness and contour lengths. Such an approach would help validate the suitability of cytosolic controls and provide a more accurate benchmark for interpreting the FRET efficiency of VinTS7.

5.3 Objective III: Development of a fluorescent reporter for live-cell imaging of CAF differentiation

The aim of developing the two reporter plasmids was to enable live-cell imaging to detect α SMA expression in live CAFs in real-time. In this way, the differentiation of CAFs to a myofibroblast-like phenotype can be validated without the need for cell fixation required by traditional detection methods such as immunostaining. Avoiding fixation offers significant advantages, including the ability to track dynamic processes in the same cell population over time. The ultimate goal of this approach was to use the reporter plasmids simultaneously with FRET-based tension sensors, allowing simultaneous evaluation of the mechanical stresses in FAs and the myofibroblast differentiation status of individual live cells.

5.3.1 Reporter Plasmid 1: pcDNA3.1-mTagBFP2-ACTA2 Promotor-mGarnet_NLS

Within the timeframe of this thesis, **reporter plasmid 1 (pcDNA3.1-mTagBFP2-ACTA2 Promotor-mGarnet_NLS) was successfully constructed**. To evaluate its functionality, the plasmid was transiently transfected into CAFs under two experimental conditions: one in which the CAFs were treated with TGF- β , and one untreated control condition. The membrane-localized mTagBFP2 reporter was robustly expressed in transiently transfected cells, enabling the identification and selection of successfully transiently transfected populations. However, expression levels of mTagBFP2 varied between cells, suggesting heterogeneous plasmid uptake and potentially varying copy numbers among individual CAFs. This variability in fluorescence intensity is commonly observed in transient transfection experiments, where inconsistent transgene expression levels across the cell population are a well-known limitation⁹⁶. Subsequently, mTagBFP2-positive cells were assessed for mGarnet2 expression. In both treatment conditions, the expression of mGarnet2 was minimal. Under TGF- β -treated conditions, where activation of the ACTA2 promoter was expected to drive mGarnet2 expression and nuclear localization, only weak or negligible expression was observed. In the untreated condition, no mGarnet2 signal was detected, which is consistent with expectations, given the inactivity of the ACTA2 promoter in the absence of TGF- β stimulation. These results suggest either suboptimal activation of the ACTA2 promoter under the tested conditions or potential limitations in the design or expression dynamics of the reporter construct.

To investigate whether the presence of two consecutive NLS sequences at the 3'-end of mGarnet2 possibly interfered with the correct folding or functional localisation of the protein, this construct was placed under the control of the constitutively active CMV promoter in the pcDNA3.1 backbone. In parallel, an alternative construct was generated in which the two NLS sequences were added to the 5' end of the mGarnet2 sequence (NLS_mGarnet2). Within the timeframe of this thesis, the construction with mGarnet2_NLS under the CMV promoter could not be successfully completed, so validation of this also remained delayed. In contrast, the alternative construct (NLS_mGarnet2) was completed in time and functionally validated. Transient transfection in CAFs showed that this construct was correctly expressed and efficiently localised to the nucleus, indicating that placement of the NLS sequences at the 5' end does not adversely affect the folding or nuclear targeting of mGarnet2. The likely cause for the low or absent mGarnet2 expression under the control of the ACTA2 promoter is probably not due to the construct design, but to the limited strength of the promoter itself. The addition of the ACTA2-enhancer should be considered in future experiments, as it plays a crucial role in the recruitment of RNA-polymerase II and can significantly increase the overall transcriptional efficiency of ACTA2-driven expression systems⁹⁷.

A notable observation during this experiment was that most of the CAFs that had successfully incorporated the reporter plasmid showed no detectable expression of α SMA. This observation was confirmed via immunostaining directed against α SMA. This finding suggests that uptake of the plasmid may induce an additional cellular stress response, which in turn

suppresses α SMA expression. One possible explanation is that the presence of the plasmid, or the transient transfection process itself, interferes with molecular signalling pathways essential for ACTA2 transcription and consequently α SMA expression. This could imply that certain transcriptional regulators or epigenetic mechanisms are disrupted, leading to impaired differentiation of CAFs to a myofibroblast phenotype. Further experimental validation is needed to determine whether this suppression is caused by the plasmid construct itself, the transient transfection mechanism, or by activation of specific stress-related pathways.

5.3.2 Reporter plasmid 2: pcDNA3.1-mTagBFP2-ACTA2 Enhancer- ACTA2 Promotor-mGarnet_NLS

For the construction of **plasmid 2 (pcDNA3.1-mTagBFP2-ACTA2 Enhancer- ACTA2 Promotor-mGarnet_NLS)**, all required DNA fragments were generated separately and **selected successfully**. However, within the time frame of this thesis, it was not possible to assemble the complete construct. In the final cloning step, where both the ACTA2 promoter and ACTA2 enhancer had to be simultaneously integrated into the pre-existing plasmid 1 (pcDNA3.1-mTagBFP2-ACTA2 Promotor-mGarnet_NLS), the desired ligation could not be successfully performed, despite several attempts. A possible explanation for the failure of this cloning is the considerable size of the combined ACTA2 regulatory elements (4332 bp), which together with the pcDNA3.1 backbone including mTagBFP2 and mGarnet2 (6819 bp) form a construct of more than 11 kb. This increases the technical complexity of ligation and lowers the efficiency of recombination. Due to the failure of successful cloning, the functional validation of plasmid 1 (pcDNA3.1-mTagBFP2-ACTA2 Promotor-mGarnet_NLS), whose only structural difference from plasmid 2 (pcDNA3.1-mTagBFP2-ACTA2 Enhancer-ACTA2 Promotor-mGarnet_NLS) is the presence of the ACTA2-enhancer, could not be performed within this thesis.

5.3.3 Limitations

Due to the time constraints of this thesis, the planned comparison between plasmid 1 (pcDNA3.1-mTagBFP2-ACTA2 Promotor-mGarnet_NLS) and plasmid 2 (pcDNA3.1-mTagBFP2-ACTA2 Enhancer-ACTA2 Promotor-mGarnet_NLS) could not be performed. As a result, the influence of the ACTA2-enhancer on the promoter's transcriptional activity remained untested. The preliminary results with plasmid 1 (pcDNA3.1-mTagBFP2-ACTA2 Promotor-mGarnet_NLS) suggest that the ACTA2 promoter without enhancer is insufficiently active to induce detectable expression of mGarnet2 in TGF- β -treated CAFs. This finding potentially points to a need for additional regulatory elements, such as the ACTA2 enhancer, to ensure adequate transcription. However, it is important to highlight that this interpretation is speculative. A similar requirement for enhancer activity has been demonstrated in a zebrafish study, where a transgenic reporter construct combining the ACTA2 promoter with its enhancer was necessary to drive robust expression of α SMA in vascular mural and visceral smooth muscle cells⁹⁸. Alternative explanations for the low or absent expression of mGarnet2 should

be considered. For instance, the position or presence of the two NLS sequences could affect the proper folding, stability or nuclear localisation of the mGarnet2 protein. An additional control experiment would therefore consist of replacing mGarnet2 with another fluorescent protein within the same construct to evaluate whether the problem is bound specifically to mGarnet2. Thus, before the reporter constructs can be reliably employed for live-cell imaging of α SMA expression, several steps of validation are required. Only through these additional experiments can it be determined with certainty where the current bottleneck is located, and the effectiveness and applicability of the plasmid constructs be evaluated.

6 CONCLUSIONS AND FUTUTRE PROSPECTS

The overall aim of this study was to investigate the mechanobiology of CAFs under two different phenotypic states: the untreated CAF phenotype and the TGF- β -induced myofibroblast phenotype. As a precondition for this study, the first objective was to establish an efficient and reproducible protocol for inducing CAF differentiation using TGF- β within the shortest possible time frame. The final result shows that successful differentiation to the myofibroblast phenotype was achieved within four days, from cell seeding to fixation, when using a minimum TGF- β concentration of only 1 ng/mL. This result indicates that an effective and time-efficient differentiation protocol was developed. However, it is important to note that the protocol was only validated in a single CAF cell line (CT5.3hTERT). To determine its wider applicability and robustness, further validation in other CAF cell lines is needed. Such crossline assays will provide optimisation and possibly lead to the establishment of a standardised protocol for TGF- β -induced CAF differentiation.

Following the successful establishment of a TGF- β -induced differentiation protocol, the core objective of this thesis focused on investigating the mechanobiology of CAFs by quantifying molecular tension within FAs using the VinTS7 FRET-based tension sensor. Specifically, FRET efficiency was measured in both untreated and TGF- β -treated CAFs to assess differences in force transmission across the protein vinculin. The results supported the initial hypothesis: differentiated CAFs, representing a more activated myofibroblast phenotype, exhibited significantly lower FRET efficiency values, indicative of higher mechanical tension across vinculin within their FAs. This finding is consistent with the increased contractile behaviour typically associated with myofibroblasts, which are known to exert greater traction forces on their microenvironment. In addition, differentiated CAFs were found to possess a higher number of FAs per cell compared to untreated CAFs. This increase in adhesion sites further reflects their heightened mechanical activity and the need for additional anchor points to transmit forces to the extracellular matrix. Interestingly, the average vinculin fluorescence intensity per FA was higher in untreated CAFs, suggesting a greater concentration of vinculin at individual adhesion sites. A possible explanation for this observation is that undifferentiated CAFs, despite forming fewer FAs, compensate by recruiting more vinculin per FA to maintain cytoskeletal tension and adhesion stability. Future studies should aim to refine these findings by incorporating additional FRET-based tension sensors, such as the ones utilizing spring-like F40 or semi-digital FL and HP35 peptide domains as the flexible linker between the donor and acceptor. This would help to more precisely match the sensor's dynamic range to the specific force profile of CAFs, ultimately improving measurement accuracy and enabling more detailed mechanobiological analyses.

Finally, this thesis also aimed to develop a reporter plasmid for the direct identification of differentiated CAFs, thereby eliminating the need for fixation during sample preparation and allowing live-cell imaging. The goal was to construct two similar reporter plasmids: one

incorporating both the ACTA2 enhancer and ACTA2 promoter, and another containing only the ACTA2 promoter. Due to time constraints, only reporter plasmid 1 (pcDNA3.1-mTagBFP2-ACTA2 Enhancer-ACTA2 Promotor-mGarnet_NLS)) was successfully developed during the course of this project. The reporter plasmid was designed to express two fluorescent proteins: mTagBFP2, targeted to the cell membrane, as a transient transfection marker, and mGarnet2, under the control of the ACTA2 promoter and localized to the nucleus, to indicate differentiation into a myofibroblast phenotype. While mTagBFP2 expression and membrane localization functioned as intended, serving as a reliable indicator of successful plasmid uptake, mGarnet2 expression was minimal to absent. These findings suggest that the ACTA2 promoter alone lacks sufficient transcriptional activity to drive robust expression of the reporter in this context. This outcome supports the hypothesis that the ACTA2 enhancer is essential for effective promoter activity. To validate this assumption, future work should prioritize the construction and evaluation of plasmid 2 (pcDNA3.1-mTagBFP2-ACTA2 Enhancer-ACTA2 Promotor-mGarnet_NLS).

Alternatively, replacing mGarnet2 with a brighter or more efficiently folding fluorescent protein may enhance nuclear signal detection. Additionally, the positioning of the NLS should be reassessed, as its current configuration may interfere with proper folding or nuclear import of mGarnet2. In summary, this thesis presents a solid foundation in the form of reporter plasmid 1 (pcDNA3.1-mTagBFP2-ACTA2 Promotor-mGarnet_NLS), which, though not yet fully optimized, holds promise for future refinement. With further development, it may serve as a valuable tool for both live-cell imaging and fixed-sample analysis of CAF differentiation status.

7 CONTRIBUTION STATEMENT

All experiments were carried out by the candidate under the supervision of mentor Samet Aytekin. Primer design was supported by Guillermo Solis Fernandez and Samet Aytekin. The stable cell lines were generated in collaboration with Sarah Vorselmans. The thesis was revised multiple times throughout the year by my mentor, Samet Aytekin. Within this master's thesis, AI tools were employed exclusively for language-related support, including grammar correction, translation, and rewording.

The student declares that the submitted thesis text (including graphs) is original work, written using the wording of the student and reflecting the student's English language proficiency.

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9 ADDENDUM

9.1 Risk analysis

Given that the experiments were conducted within biochemical, chemical, and microscopy laboratory environments, strict safety precautions were necessary to ensure a safe working environment. Prior to gaining access to the building and laboratory facilities, all researchers were required to complete a mandatory Health, Safety, and Environment (HSE) training. This assessment provided essential knowledge on safe laboratory conduct, proper disposal of hazardous materials, emergency response protocols, and identified key contact persons responsible for laboratory safety oversight. General laboratory safety rules applied across all workspaces, including the mandatory use of gloves, safety goggles, and lab coats. Upon exiting the laboratory, handwashing was compulsory. For every experimental procedure, a risk assessment was either consulted or created to ensure prior awareness of any potential hazards related to equipment setups or chemical use.

Work involving living cells was performed in a biosafety level 2 (L2) laboratory. Access to this lab required the use of dedicated lab coats, and gloves had to be changed immediately before entering. These gloves were disposed of directly after leaving the lab, followed by thorough handwashing and disinfection with 70% ethanol to prevent any cross-contamination.

Most cell culture experiments were conducted under a sterile biosafety cabinet. All materials introduced into the cabinet were disinfected with 70% ethanol. Additionally, the cabinet was sterilized with ethanol before and after each session, and ultraviolet (UV) radiation was applied to further maintain aseptic conditions. Samples were consistently stored in a designated incubator to prevent contamination. All biological waste was immediately disposed of in containers containing bleach to ensure initial decontamination, after which it was transferred to certified biohazard waste bins for proper disposal. During the thawing of cryopreserved cell lines from liquid nitrogen, additional safety measures were taken by wearing heat-resistant gloves and a protective face shield to minimize the risk of injury from extreme temperatures.

Bacterial experiments were conducted using a non-pathogenic strain of *E. coli* in a designated biosafety level 1 (L1) laboratory, where the use of separate lab coats was mandatory to prevent cross-contamination. Gel electrophoresis was carried out in a dedicated L1 gel room, while other procedures involving non-living biological material took place in standard L1 laboratory facilities.

9.2 PCR settings and primers

COMPONENT	Volume x1 sample (μL)
Milli-Q H ₂ O	33.5
GC-buffer	10
DMSO	1.5
10 mM DNTP	1
FW primer	1.25
REV primer	1.25
DNA (100ng)	1
Phusion DNA Polymerase	0.5

Supplementary Table 1. PCR-Mix. Reaction mix composition for a 50 μL PCR reaction using Phusion® High-Fidelity DNA Polymerase.

COMPONENT	Volume x1 sample (μL)
Milli-Q H ₂ O	35
DreamTaq-buffer	10
10 mM DNTP	1
FW primer	1.25
REV primer	1.25
DNA (100ng)	1
DreamTaq DNA Polymerase	0.5

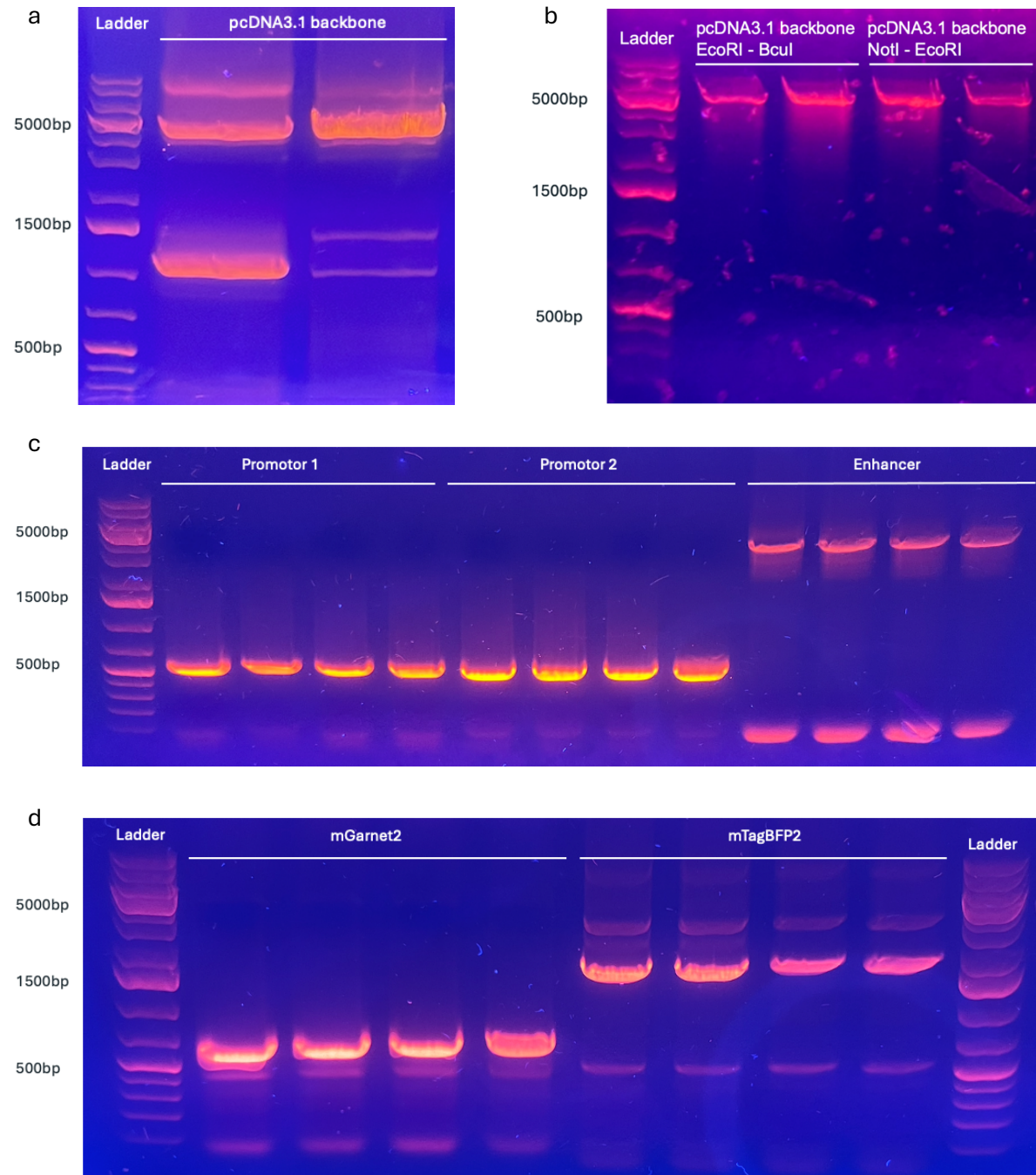
Supplementary Table 2. PCR-Mix. Reaction mix composition for a 50 μL PCR reaction using DreamTaq™ DNA Polymerase, used for colony PCR.

1	pcDNA3.1 Forward primer	CTGCGG ACTAGT CTCAGG GGTACC AGCTCGCTGATCAGCCTCGA
2	pcDNA3.1 Forward primer (EcoRI)	CTGCGG GAATTC ACTAGT GGTACC AGCTCGCTGATCAGCCTCGA
3	pcDNA3.1 Reverse primer	ATGAGAG GCGGCCGC ACCGGT AACGGT ATATCTGGCCCGT
4	pcDNA3.1 Reverse primer (EcoRI)	ATGAGAG GAATTC GCGGCCGC AACGGT ATATCTGGCCCGT
5	ACTA2 enhancer Forward primer	ATGAGAG GCGGCCGC GTAAGTGGCGCC AGGCCA
6	ACTA2 enhancer Reverse primer	ATGAGAG GAATTC TGGAGCTGCTT CACAGGATT
7	ACTA2 promotor Forward primer I	ATGAGAG GCGGCCGC ATTCTATT CCACTCACCTCC
8	ACTA2 promotor Forward primer II	ATGAGAG GAATTC ATTCTATT CCACTCACCTCC
9	ACTA2 promotor Reverse primer	ATGAGAA CTAGT CCACTTGCTT CCCAAACA
10	mTagBFP2 Forward primer	ATGAGAA ACGGT TAGCCCATATAT GGAGTTCCG
11	mTagBFP2 Reverse primer	ATGAGAG GCGGCCGC TAAGATACATTGATGAGTTTGG
12	mGarnet2 Forward primer	ATGAGAA ACTAGT ATGAACAGCCTGATCAAAGAAAACA
13	mGarnet2 Reverse primer	ATGAGAG GGTACCCTA TACCTTTCTCTTCTTTTTTGGT GATACCTTTC TCTTCTTTTTTG TGGCGCGCC CCCTCCGCC CAGGCC
14	mGarnet2 Forward primer insertion pcDNA3.1 (NLS at 3'-end)	CTGCGG AAGCTT ATGAACAGCCTGATCAAAGAAAACATG
15	mGarnet2 Reverse primer insertion pcDNA3.1 (NLS at 3'-end)	CTGCGG GAATTC CTATACCTTTCTCTTCTTTTTTGGT G
16	mGarnet2 Forward primer insertion pcDNA3.1 (NLS at 5'-end)	CTGCGG AAGCTT ATGGTGGAT CCAAAAAAGAAGAGAAAGGTAGA TCCAAAGAAGAAAAGGAAGGTTGGCAGCGGCTCCATGAACAGCC TGATCAAAGA

17	mGarnet2 Revers primer insertion pcDNA3.1 (NLS at 5'-end)	CTGCGGGAATTCTACCCCTCCGCCCAGGCCGGCGAACTTG
18	mGarnet2 Forward primer insertion pBB-T-P2-G (NLS at 5'-end)	CTGCGGACTAGTATGGTGGATCCAAAAAGAAGAGAAAGGTAGA TCCAAAGAAGAAAAGGAAGGTTGGCAGCGGCTCCATGAACAGCC TGATCAAAGA
19	mGarnet2 Reverse primer insertion pBB-T-P2-G (NLS at 5'-end)	CTGCGGGGTACCTACCCCTCCGCCCAGGCCGGCGAACTTG

Supplementary Table 3. Overview of primer sequences used for amplification of individual reporter plasmid components. This table lists the specific forward and reverse primers designed for the amplification and cloning of each functional element of the reporter plasmid. These include the pcDNA3.1 backbone, the ACTA2 promoter, the ACTA2 enhancer, the nuclear-localised fluorescent protein mGarnet2, and the membrane-localised fluorescent protein mTagBFP2. The primers were designed to include restriction sites and overhangs compatible with subsequent cloning steps. Colour coding: red = *BclI*, green = *KpnI*, dark blue = *NotI*, yellow = *MluI*, orange = *EcoRI*, light blue = *HindIII*, grey = NLS, black = STOP codon.

9.3 Identification PCR fragments



Supplementary Figure 1. Agarose gel analysis of PCR-amplified fragments for the construction of ACTA2 reporter gene constructs. (a) pcDNA3.1 backbone (~4420 bp) as a base vector. (b) Backbone fragments with restriction sites EcoRI-BclI and NotI-EcoRI (~4420 bp). (c) ACTA2 promoter 1, promoter 2 (~757 bp), and enhancer fragment (~3795 bp) from CAF genomic DNA. (d) Fluorescent protein fragments mTagBFP2 (~1642 bp) and mGarnet2 (~757 bp) with respective membrane and nuclear localisation signals.

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