UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

Role of CFTR in Epithelial Differentiation by Functional Genomics

"Documento Definitivo"

Doutoramento em Biologia
Especialidade de Biologia de Sistemas

Luís Miguel dos Santos Sousa

Tese orientada por:
Professora Doutora Margarida Amaral e Professor Doutor Marc Chanson

Documento especialmente elaborado para a obtenção do grau de doutor

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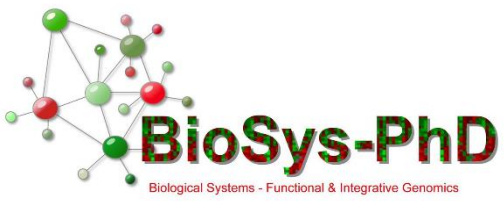
Luís Miguel dos Santos Sousa foi bolseiro de doutoramento no âmbito do programa doutoral BioSys - Sistemas Biológicos, Genómica Funcional & Integrativa (FCT /PD/00065/2012) da Faculdade de Ciências da Universidade de Lisboa, financiada pela Fundação para a Ciência e Tecnologia do Ministério da Educação e Ciência, Bolsa PD/BD/106087/2015.



De acordo com o disposto no artigo 24º do Regulamento de Estudos de Pós-Graduação da Universidade de Lisboa, Despacho nº 7024/2017, publicado no diário da República – 2a Série – nº 155 – 11 de Agosto de 2017, foram incluídos nesta dissertação três artigos científicos publicados:

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Sousa L*, Pankonien I*, Clarke LA, Silva I, Kunzelmann K, Amaral MD. KLF4 Acts as a wt-CFTR Suppressor through an AKT-Mediated Pathway. Cells. 2020 Jul 2;9(7):1607. doi: 10.3390/cells9071607. PMID: 32630830; PMCID: PMC7408019. - * igual contribuição

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Durante o período de realização deste trabalho, Luís Sousa foi co-autor dos seguintes trabalhos, não incluídos neste trabalho:

Silva JV, Cruz D, Gomes M, Correia BR, Freitas MJ, **Sousa L,** Silva V, Fardilha M. Study on the short-term effects of increased alcohol and cigarette consumption in healthy young men's seminal quality. Sci Rep. 2017;7:45457. Published 2017 Apr 3. doi:10.1038/srep45457 – publicado.

Felgueiras J*, **Sousa L*,** Teixeira AL, Regadas B, Korrodi-Gregório L, Luers G, Brauns A, Medeiros R. The oncogenic potential of TCTEX1D4 is modulated by the phosphoprotein phosphatase PPP1. Biochem Biophys Res Commun. – submetido. * - igual contribuição.

No cumprimento do disposto da referida deliberação, o autor esclarece serem da sua responsabilidade, exceto quando referido o contrário, a execução das experiências que permitiram a elaboração dos resultados apresentados, assim como da interpretação e discussão dos mesmos. Os resultados obtidos por outros autores foram incluídos com a autorização dos mesmos para facilitar a compreensão dos trabalhos e estão assinalados nas respetivas figuras e metodologias.

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Summary

Cystic Fibrosis (CF) is caused by mutations in the CFTR gene. The relationship between CFTR and differentiation has been firmly documented. However, the mechanisms that regulate this relationship are still unclear.

The aim of this work was to further characterize the differentiation defect in CF and to identify the underlying molecular mechanisms.

In the first chapter, our results show that CF cells display increased proliferation, acquire lower levels of TEER upon polarization, display slower wound healing, and have altered differentiation. Moreover, our data suggest that partial epithelial–mesenchymal transition occurs in CF cells.

Given that Krüppel-like factors (KLFs) play pivotal roles in the regulation of differentiation, in the second chapter we tested the levels of KLF2, 4 and 5 and found that only KLF4 is differentially expressed in CF vs non-CF cells.

Therefore, we then assessed whether KLF4 modulation had an impact in CFTR levels and function. Our data show that KLF4 acts as a negative regulator of wt-CFTR expression and function. Noteworthy, F508del cells are relatively insensitive to KLF4 modulation.

Next, we tested possible pathways by which KLF4 action can impact CFTR in the CF and non-CF contexts. Our data showed that AKT signalling acts as a modulator of CFTR in a KLF4-dependent way, whilst GSK3β was observed to be a negative regulator of CFTR independent of KLF4 in CF cells.

In chapter three, our data show that KLF4 knock-out has no major impact in proliferation but modulates TEER acquisition, wound healing, and differentiation markers differentially in CF vs non-CF cells. Moreover, we characterized the levels of KLFs, CFTR and proteins of interest during epithelial differentiation and showed that maximum KLF4 expression occurs prior to maximum CFTR expression.

Altogether these data contribute to mechanistic clarification of the relationship between CFTR and epithelial differentiation.

Key words: Cystic Fibrosis, KLF4, Epithelial differentiation, AKT signalling, EMT.

Resumo

A Fibrose Quística (FQ) é a doença autossómica recessiva letal mais comum entre a população caucasiana. A nível clínico, a FQ apresenta-se como uma doença multi-sistémica e progressiva sendo a doença pulmonar a principal causa de morte. A nível pulmonar, a desregulação no transporte iónico leva a um aumento da viscosidade e desidratação do muco, promovendo ciclos de inflamação e infeção com progressiva destruição da função pulmonar.

A FQ é provocada por mutações no gene CFTR (do inglês "*Cystic Fibrosis Transmembrane conductance Regulator*"). A proteína CFTR pertence à superfamília dos transportadores ABC (do inglês "*ATP-binding cassette*") e funciona como um canal de cloreto (Cl^-) e bicarbonato (HCO_3^-) na membrana apical de células epiteliais promovendo o movimento de água essencial para a manutenção da homeostase celular. Mais de 2000 mutações já foram descritas como possivelmente causadoras de doença, sendo a deleção do resíduo fenilalanina na posição 508 (F508del) a mutação mais comum, estando presente em pelo menos um dos alelos de cerca de 80% dos doentes a nível mundial.

A associação entre diferenciação epitelial e CFTR já se encontra amplamente descrita. Os níveis de expressão de CFTR são mais elevados em células diferenciadas e a função e tráfego de CFTR são dependentes da polarização e diferenciação. Variações nos níveis de expressão e localização intracelular ocorrem também durante o desenvolvimento embrionário. Mais ainda, a diferenciação epitelial parece não só afetar a proteína CFTR no estado normal (*wild-type* - wt) mas também a mutante (F508del-CFTR).

Outros estudos indicam que não apenas a diferenciação afeta os níveis de expressão e atividade de CFTR, mas que a relação inversa também se verifica. Mutações no CFTR estão associadas a maior risco de cancro e malformações fetais, bem como alterações na capacidade de regeneração.

Sendo a CFTR uma proteína de membrana, a sua função na diferenciação epitelial deverá ocorrer através da modulação de atividade de outros intervenientes ou pela modulação de vias de sinalização, sendo que a ligação entre a CFTR e diversas vias de sinalização como as do TNFα, NF-κB, AKT, ERK e WNT já se encontram descritas na literatura.

O objetivo deste trabalho era o de clarificar a nível mecanístico a interligação entre a proteína CFTR e a diferenciação no contexto da FQ.

No primeiro capítulo deste trabalho pretendemos caracterizar as alterações a nível de diferenciação e proliferação que existem nas células de FQ.

Os nossos resultados indicam claramente que as células de FQ apresentam uma maior proliferação, adquirem menores níveis de resistência transepitelial após polarização e regeneram mais lentamente após "*wounding*". Mostrámos ainda a existência de uma transição epitelial-mesenquimal (EMT, do inglês "*epithelial–mesenchymal transition*") parcial, nomeadamente pelo aumento dos níveis de expressão de marcadores mesenquimais sem, contudo, ocorrer a repressão vincada da expressão de marcadores epiteliais. Podemos então dizer que as células de FQ apresentam um fenótipo mais próximo das células cancerígenas. Verificámos ainda a existência de alterações na localização intracelular de certas proteínas como a citoqueratina 18 e N-caderina. Mostrámos que este fenómeno de EMT parcial é possivelmente mediado pelo fator de transcrição TWIST1.

Dado que os fatores de transcrição da família KLF (do inglês "*Krüppel-like factor*") regulam uma enorme variedade de vias de sinalização e desempenham papéis centrais na diferenciação e proliferação, no segundo capítulo deste trabalho decidimos avaliar os níveis de expressão de alguns membros desta família (KLF2, 4 e 5) em tecidos/células FQ vs não FQ.

Utilizando tecido pulmonar de indivíduos com FQ, identificámos a nível do mRNA que o KLF4 é mais expresso em pulmão de indivíduos com FQ vs controlos. No nosso modelo celular (CFBE, do inglês "*Cystic Fibrosis Bronchial Epithelial*"), também verificamos uma sobre-expressão de KLF4, ao nível do mRNA e a nível proteico relativamente a células controlo.

De seguida, avaliámos o possível impacto da modulação de KLF4 nos níveis de expressão e função da CFTR. Utilizando siRNA para KLF4, verificámos que o *knockdown* (KD) de KLF4 promove um aumento nos níveis de expressão da CFTR. Todavia, verificámos que tal apenas se verifica nas células que expressam wt-CFTR, ou seja, não mutada. No caso de células que expressam F508del-CFTR, o KD de KLF4 não apresenta um impacto nos respetivos níveis de expressão.

Usámos então de seguida uma abordagem de *knock-out* (KO) pela técnica CRISPR/Cas9 para abrogar a expressão de KLF4. Após validação do KO de KLF4 nas células CFBE expressando wt- ou F508del-CFTR, avaliámos o impacto deste KO nos níveis e função da CFTR. Em concordância com o observado aquando do KD, verificámos que o KO de KLF4 promove um marcado aumento nos níveis de expressão da wt-CFTR sem causar alterações significativas na expressão da F508del-CFTR.

De seguida, aplicamos uma abordagem complementar para avaliar a relação entre KLF4 e CFTR. Através da sobre-expressão exógena de KLF4 por transfeção do respetivo cDNA, verificámos que tal resultou numa diminuição dos níveis da wt-CFTR. Porém, mais uma vez a F508del-CFTR apresentou-se como refratária à modulação pelo KLF4, não ocorrendo alterações nos respetivos níveis de expressão mediante sobre-expressão de KLF4. Mais ainda, a expressão exógena de KLF4 traduziu-se numa diminuição da atividade de wt-CFTR medida por *patch-clamp*. Desta forma, concluímos que existe uma interação funcional entre KLF4 e CFTR, apresentando-se o KLF4 como regulador negativo de wt-CFTR, sendo que a F508del-CFTR é refratária à modulação pelo KLF4.

Tendo em conta que KLF4 e CFTR não interagem diretamente, postulámos que a interação funcional entre as duas proteínas deve ser mediada por vias de sinalização celular. Por análise bioinformática, identificámos como vias de sinalização potencialmente envolvidas nesta interação funcional as do EGFR-AKT e da β-Catenina-GSK3β. Assim, utilizámos de seguida inibidores destas duas vias de sinalização com o objetivo de determinar o seu impacto na proteína CFTR e, a verificar-se, se é dependente do KLF4. Os nossos resultados indicam que a o AKT é um regulador positivo da expressão da wt-CFTR, apresentando-se como um regulador negativo da expressão de F508del-CFTR. A utilização das células KO para KLF4, indica que este efeito é, pelo menos em parte, mediado pelo KLF4, devido à diminuição do impacto da inibição de AKT na expressão de wt-CFTR nas células KLF4 KO. No referente à GSK3β, esta apresenta-se como um regulador negativo tanto da wt-CFTR como da F508del-CFTR, sendo a sua ação independente da expressão de KLF4 em FQ.

Em conclusão neste segundo capítulo deste trabalho identificámos o KLF4 como um regulador negativo da wt-CFTR, mas não da F508del-CFTR, sendo o seu efeito potencialmente mediado pela via da AKT. Por outro lado, a via da GSK3β revelou ser uma via reguladora negativa da CFTR (normal e mutante) independente de KLF4 no contexto da FQ.

No terceiro capítulo da tese incluímos resultados relativos à caracterização do impacto do KO de KLF4 na proliferação celular e diferenciação epitelial bem como dos níveis de KLFs e marcadores de diferenciação durante a diferenciação epitelial.

O KO de KLF4 não parece afetar a proliferação, contudo tem impactos diferentes em processos celulares de polarização, regeneração e diferenciação consoante a célula estiver a expressar CFTR normal ou mutante. O KO de KLF4 diminui a aquisição de resistência transepitelial (e portanto, diminui o respetivo nível de polarização) e a regeneração nas células que expressam wt-CFTR, apresentado o efeito oposto nas células que expressam F508del-CFTR. Quanto aos marcadores de diferenciação epitelial, verificámos que de um modo geral, o KO de KLF4 produz uma diminuição dos marcadores de diferenciação epitelial e um aumento dos níveis de certos marcadores mesenquimais. Em paralelo, e paradoxalmente, o KO de KLF4 leva a uma diminuição dos níveis de TWIST (fator pro-EMT) mas apenas nas células F508del-CFTR.

Para compreender os efeitos do KLF4 durante a diferenciação epitelial, usámos o modelo celular BCi que é uma linha epitelial basal respiratória humana que se diferencia nos vários tipos de células do epitélio respiratório ao fim de 30 dias. Verificámos que os níveis de KLF4 aumentam ao longo da diferenciação epitelial, atingindo um máximo aos 15 dias de diferenciação. Tal padrão de expressão é semelhante ao que ocorre para KLF5, AKT e GSK3β, sendo que precede a expressão máxima da CFTR, a qual aumenta gradualmente até ao dia 30 de diferenciação.

Como conclusões finais podemos indicar que este trabalho permitiu uma melhor caracterização da existência de EMT parcial nas células FQ, bem com a identificação de KLF4 como um regulador negativo dos níveis de expressão e função da wt-CFTR. Adicionalmente, identificámos duas vias de sinalização com impacto no contexto da FQ, nomeadamente a da AKT e a da GSK3β, sendo que a primeira depende do KLF4 e a segunda não em células de FQ. Por último,

caracterizámos o impacto diferencial do KO de KLF4 na proliferação, polarização, regeneração e diferenciação epitelial e os níveis de expressão de proteínas de interesse durante a diferenciação.

Palavras-chave: Fibrose Quística, KLF4, Diferenciação epitelial, AKT, EMT.

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Abbreviations

ABC – ATP-binding cassette

AJ – adherens junctions

ALI – air-liquid interface

ASL – air surface liquid

ATP - adenosine triphosphate

BCi-NS1.1 – basal cell immortalized-nonsmoker 1.1

BSA – bovine serum albumin

cAMP – cyclic adenosine 3',5' monophosphate

CBAVD – congenital bilateral absence of vas deferens

CF – cystic fibrosis

CFBE – cystic fibrosis bronchial epithelial

CFTR – cystic fibrosis transmembrane conductance regulator

CK - cytokeratin

CLDN1 – claudin 1

Clnx – calnexin

Co-IP – co-immunoprecipitation

Ctrl – control

Cx - connexin

DMEM – Dulbecco's modified Eagle's media

DMSO – dimethyl sulfoxide

DSPI/II – desmoplakin I/II

DTT - dithiotreitol

E-Cad – epithelial cadherin

EGFR – epithelial growth factor receptor

EMA – European Medicines Agency

EMEM – Eagle's minimum essential media with L-glutamine

EMT – epithelial to mesenchymal transition

EMTa-TF – EMT associated-transcription factor

ENaC – epithelial sodium channel

ER – endoplasmic reticulum

ERQC – ER quality control

ESC – embryonic stem cell

F508del – deletion of phenylalanine at position 508

FBS – foetal bovine serum

FDA – Food and Drugs Administration

FN – fibronectin

Fsk – forskolin

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GJ – gap junction

GSK3β - glycogen synthase kinase 3 beta

IF - immunofluorescence

KLF – Kruppel-like factors

KO – knock out

MCC – mucociliary clearance

MET – mesenchymal to epithelial transition

MRSA – methicillin-resistant *Staphylococcus aureus*

MSD – membrane spanning domains

MW – molecular weight

NBD – nucleotide binding domain

N-Cad – neural cadherin

NC – negative control

NFM – non-fat milk

NTC – non-treated control

PBS – phosphate buffer saline

PBST – PBS-Tween20®

PCR – polymerase chain reaction

PFA - paraformaldehyde

pHBE – primary human bronchial epithelial

PM – plasma membrane

RD – regulatory domain

RT- room temperature

qRT-PCR – quantitative reverse transcriptase PCR

SB – sample buffer

TBST – tris-buffered saline -Tween20®

TEER – transepithelial electrical resistance

TF – transcription factor

TGFβ – transforming growth factor β

TGFβR – TGFβ receptor

TJ – tight junction

TM – transmembrane

Vim - vimentin

WB – western blot

WCL – whole cell lysate

Wt – wild-type

ZO-1 – Zonula occludens-1

Section 1 – Introduction

1 Cystic Fibrosis

Cystic fibrosis (CF) was first described in 1938 when paediatrician and pathologist Dorothy Andersen noted the presence of mucus plugging the glandular ducts of the pancreas in infants dying of malnutrition (Andersen 1938). CF was later further characterized by Farber (Farber 1944) as causing fat and protein malabsorption, pulmonary disease and the presence of thick mucus causing obstruction of the ducts of mucus glands, designated “mucoviscidosis” (Farber 1944, Davis 2006, Strausbaugh and Davis 2007). In the following years, reports appeared describing pathological alterations in a number of other organs, such as intestine (Sinclair and Driver 1954), liver and lung (Pugsley and Spence 1949), with some authors describing it as a “generalized exocrinopathy” (Di Sant'Agnese 1956). By 1946, CF aetiology was clarified as presenting a Mendelian autosomal recessive pattern of inheritance by Andresen (Andersen and Hodges 1946). The basic electrolyte defect underlying CF was identified in 1953 (Di Sant'Agnese, Darling et al. 1953), but it took another 30 years until Quinton published a seminal paper describing the defective cAMP-mediated regulation of chloride (Cl^-) transport as the fundamental defect in CF (Quinton 1983), coupled with an increased sodium (Na^+) absorption (Boucher, Stutts et al. 1986). In 1989 the gene responsible *CFTR* (which encodes for the cystic fibrosis transmembrane conductance regulator) was successfully identified and cloned (Kerem, Rommens et al. 1989, Riordan, Rommens et al. 1989, Rommens, Iannuzzi et al. 1989).

CF is the most common lethal genetic disease among Caucasian populations (Collins 1992), with a prevalence of around 1:2500 in Europe (Mirtajani, Farnia et al. 2017). Table 1 presents the clear differences in CF incidence between regions and populations.

Table 1 - Cystic Fibrosis incidence rates in different countries/populations.

Continent	Country	Incidence	Reference
Europe	Ireland	1/1353	(Farrell 2008)
	United Kingdom	1/2381	
	Germany	1/3300	
	Spain	1/3750	
	Italy	1/4238	

	France	1/4700	
	Portugal	1/6000	
	Finland	1/25000	
Asia	Bahrain	1/5800	(Singh, Rebordosa et al. 2015)
	Jordan	1/2985	
	Japan	1/350000	
	United Arab Emirates	1/15876	(Silva Filho, Castaños et al. 2016)
	India	1/40000-100000	
Oceania	New Zealand (non-Maori)	1/3179	(Wesley and Stewart 1985)
	Australia	1/2500	(Silva Filho, Castaños et al. 2016)
Africa	South Africa (african ancestry)	1/7056	
South America	Chile	1/4000	
	Brazil (european)	1/1600	
	Brazil (afro)	1/14000	
North America	Mexico	1/8500	
	United States of America	1/3500	
	Canada	1/2714	(Dupuis, Hamilton et al. 2005)

CF is a multisystemic chronic and progressive disease caused by CFTR loss-of-function mutations. The multisystemic nature of CF arises from the fact that CFTR is expressed in many epithelial cells, including sweat ducts, airways, pancreatic ducts, intestine, biliary tree and vas deferens (Davis 2006). Therefore, CF clinical manifestations can include several systems and vary among individuals, with CFTR functional levels determining the severity of the symptoms (Strausbaugh and Davis 2007). Lung disease is the primary cause of morbidity and mortality in CF (Strausbaugh and Davis 2007). The lungs of children with CF are usually normal in appearance at birth but quickly become colonised by *Haemophilus influenza* or *Staphylococcus aureus*. These are eventually substituted by *Pseudomonas aeruginosa* which becomes almost always the primary coloniser of CF lungs, although co-infection with other pathogens like *Burkholderia cepacia,* methicillin-resistant *S. aureus* (MRSA) and S*tenotrophomonas maltophilia* is common. These chronic cycles of infection and inflammation promote bronchiectasis which ultimately cause irreversible lung damage leading to pulmonary insufficiency with marked hypoxaemia and hypercarbia. It is estimated that pulmonary insufficiency is responsible for at least

80% of CF-related deaths (O'Sullivan and Freedman 2009). Other relevant clinical manifestations include CF-related diabetes mellitus, osteoporosis, pancreatic insufficiency, meconium ileus, gastroesophageal reflux, liver diseases and male infertility (azoospermia) often caused by congenital bilateral absence of vas deferens (CBAVD) (Strausbaugh and Davis 2007, O'Sullivan and Freedman 2009).

CF diagnosis was initially based on clinical symptoms. After the development of the sweat test, the diagnosis of CF was made on the basis of a sweat Cl^- concentration of 60 mEq/L or greater plus a relative with CF, or clinically relevant lung disease, or pancreatic insufficiency (Davis and di Sant'Agnese 1984). Over the years with the identification of the CFTR gene and the development of complementary diagnostic techniques such as new-born genetic screening and nasal potential difference the guidelines for CF diagnosis evolved to a more comprehensive format (Farrell, White et al. 2017).

2 CFTR: from the gene to the protein

CFTR gene resides on chromosome 7, *locus* 7q31.3, and is about 190kb in length with 27 exons. It encodes a mRNA with 6,129 bp that is translated into a 1,480 amino acid polypeptidic chain (Ellsworth, Jamison et al. 2000, Davis 2006). CFTR protein (ABCC7), is a member of the ABC (ATP-binding cassette) transporter superfamily functioning as a cAMP-regulated Cl^- and bicarbonate (HCO_3^-) channel in the apical plasma membrane (PM) of epithelial cells, and is key to maintain ion and fluid homeostasis (Riordan 2008). Structurally, CFTR is composed by: a) two membrane spanning domains (MSD1 and MSD2), each one composed of six transmembrane segments (TM1-6 and TM7-12) forming the channel pore that conducts Cl^-/ HCO_3^-; b) two cytosolic nucleotide binding domains (NBD1 and NBD2) where channel gating is regulated via ATP binding/hydrolysis; c) a CFTR-exclusive regulatory domain (RD), containing multiple phosphorylation sites pivotal for channel activity modulation (Rommens, Iannuzzi et al. 1989). Fig.1 shows the schematic representation (A) and cryo-EM resolved (B) CFTR structure (Liu, Zhang et al. 2017).

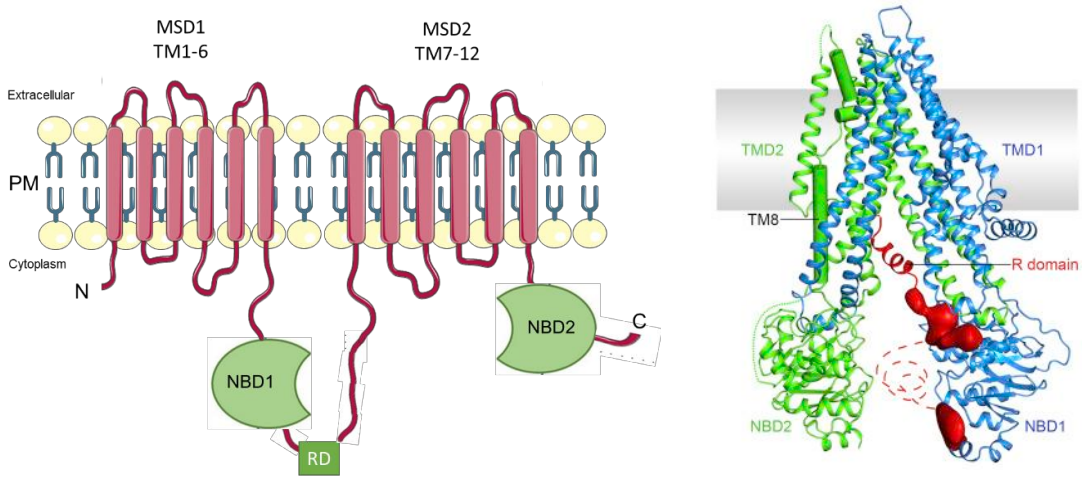


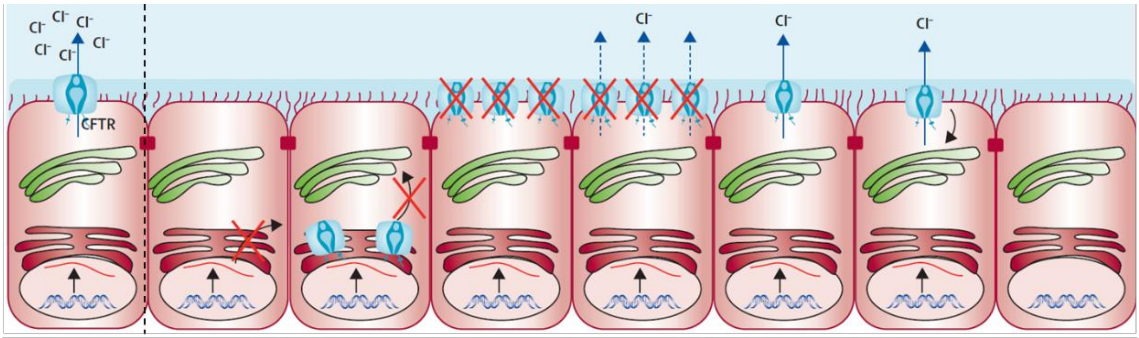
Figure 1 - Structure of CFTR protein.
CFTR is composed of two membrane spanning domains (MSD1 and MSD2) each one with 6 transmembrane segments (TM1-6 and TM7-12). These form the channel pore. CFTR additionally contains two nucleotide biding domains (NBD1 and NBD2) for gating regulation and one regulatory domain (RD) for activity modulation via phosphorylation.

CFTR-dependent movement of Cl^-/ HCO_3^- can be represented as a two-step process. Firstly, phosphorylation of CFTR by PKA (or PKC) is required to displace the RD. Secondly, NBD binding/hydrolysis of ATP is required for opening of the CFTR pore, i.e., channel-gating, with the two nucleotide binding domains acting cooperatively to regulate the binding (NBD1) and hydrolysis (NBD2) of ATP in CFTR (Mehta 2005). This sequence of phosphorylation and ATP hydrolysis does not happen in reverse, since PKA-dependent phosphorylation must precede ATP-dependent channel opening. The reason for this complexity during cell Cl^-/ HCO_3^- secretion is unclear (Mehta 2005).

At present 2,089 CFTR mutations have been reported, however only a subset of ~17% (352) has been confirmed as CF-causing mutations (2020-CFTR2Database). Several types of mutations are identified (missense, frameshift, splicing, nonsense, etc.) spanning all 27 exons, 26 introns, as well as the 3'UTR and the promoter (2020-CFTRDatabase). Therefore, to better understand the effects of these diverse mutations in the *CFTR* gene and their disease-causing mechanism, CFTR mutations have been grouped into seven functional classes.

Class I mutations lead to lack of protein production due to premature stop codons (PTCs); class II mutations are characterized by impaired traffic; class III mutations display impaired gating; class IV mutations show decreased conductance; class V mutations have less protein, often caused by alternative

splicing; class VI mutations have less stability; and class VII mutations determine no mRNA production. Due to the differences between the groups, different therapeutic strategies are required (De Boeck and Amaral 2016). In Fig. 2 we represent the different classes of mutations and the respective therapeutic approaches.



WT-CFTR	Class I	Class II	Class III	Class IV	Class V	Class VI	Class VII
CFTR defect	No protein	No traffic	Impaired gating	Decreased conductance	Less Protein	Less stable	No mRNA
Mutation examples	Gly542X	Phe508del	Gly551Asp	Arg117His	Ala455Glu	c.120del23	1717-1G→A
Therapeutic approach	Rescue synthesis	Rescue traffic	Restore activity	Restore activity	Correct splicing	Promote stability	Unrescuable
Drugs (approved)	Read-through compounds (no)	Correctors (yes)	Potentiators (yes)	Potentiators (no)	Antisense oligonucleotides (no)	Stabilizers (no)	Bypass therapies (no)

Figure 2 - Classes of CFTR mutation and respective therapeutic strategies.
CFTR mutations are grouped into seven classes, each one corresponding to the basic underlying defect causing CF. Mutations from each class are expected to have a common therapeutic strategy. [From: (De Boeck and Amaral 2016)].

The most common CF-causing mutation is by far F508del - the deletion of phenylalanine residue (Phe) at position 508 and located in NBD1. F508del occurs worldwide in approximately 80% of individuals with CF in at least one allele and leads to CFTR misfolding and Endoplasmic Reticulum (ER) retention. Consequently, this causes premature degradation of the mutant protein, preventing its delivery to the apical PM (Riordan 2008). F508del is therefore classified as a class II mutation (De Boeck and Amaral 2016).

CFTR processing and trafficking are highly regulated processes that aim to guarantee correct folding of this large multi-domain protein. CFTR undergoes several steps of processing, first co-translationally at the ER, then at the Golgi and lastly at the trans-Golgi network before reaching the PM (Farinha, Matos et al. 2013). Given its complex structure, it is already difficult for the wild-type (wt)-

CFTR protein to reach a native conformation (only 20-40% reaches it), and in the case of F508del-CFTR, almost all protein misfolds, being recognized by the ER quality control (ERQC) machinery and directed to degradation via the ubiquitin-proteasome pathway (UPP) (Amaral 2004, Amaral 2005). Therefore, almost all F508del-CFTR does not reach the PM (Amaral 2004). CFTR core-glycosylation occurs co-translationally at the ER leads to the formation of the immature form of 140 kDa, often referred to as band B (Farinha, Matos et al. 2013). Wild-type CFTR traffics through the Golgi complex where it is fully glycosylated, the mature form of 160-180 kDa, also known as Band C. This mature form of CFTR is absent in ER-retained variant of CFTR such as F508-del CFTR. This differential glycosylation allows for the distinction between wt- and F508del-CFTR by the pattern of bands displayed: wt-CFTR appears with 2 bands (native CFTR with 160-180 kDa and immature CFTR with 140 kDa) whilst F508-del CFTR appears as a single band at 140 kDa (Amaral, Farinha et al. 2016).

However, CFTR levels at the PM are not only determined by CFTR synthesis, maturation, and trafficking. Ultimately, they are dependent on three main factors: a) CFTR anterograde transport (from the ER to the PM, comprising synthesis, maturation and trafficking); b) CFTR endocytosis; c) CFTR recycling (Farinha and Canato 2017). These processes are under tight regulation to control CFTR levels/function at the apical PM under a variety of stimuli. Trafficking, turnover and retention of CFTR at the PM are regulated by several interacting proteins such as myosins, Rab family small GTPases and PDZ domain-containing proteins (Farinha and Canato 2017).

3 Cystic Fibrosis pathophysiology

Ultimately, all pathogenic CFTR mutations trigger a cascade of events that lead to CF lung disease (Fig.3): 1) lack of functional CFTR leads to deficient Cl^-/ HCO_3^- conductance, but also Na^+, due to the fact that the epithelial Na^+ channel (ENaC) is negatively regulated by CFTR; 2) the decreased Cl^-/ HCO_3^-permeability and increased Na^+ absorption leads to a decrease in the water content of the air-surface liquid (ASL) leading to a thick mucus contributing to a decrease of mucociliary clearance (MCC); 3) a cycle of destruction initiates with mucus obstruction of small airways causing disseminated bronchiectasis, recurrent

bacterial infections, chronic inflammation and lung tissue scarring/deterioration; 4) this cascade leads to progressive loss of lung function and ultimately end-stage pulmonary insufficiency (Regamey, Jeffery et al. 2011, Amaral 2015).

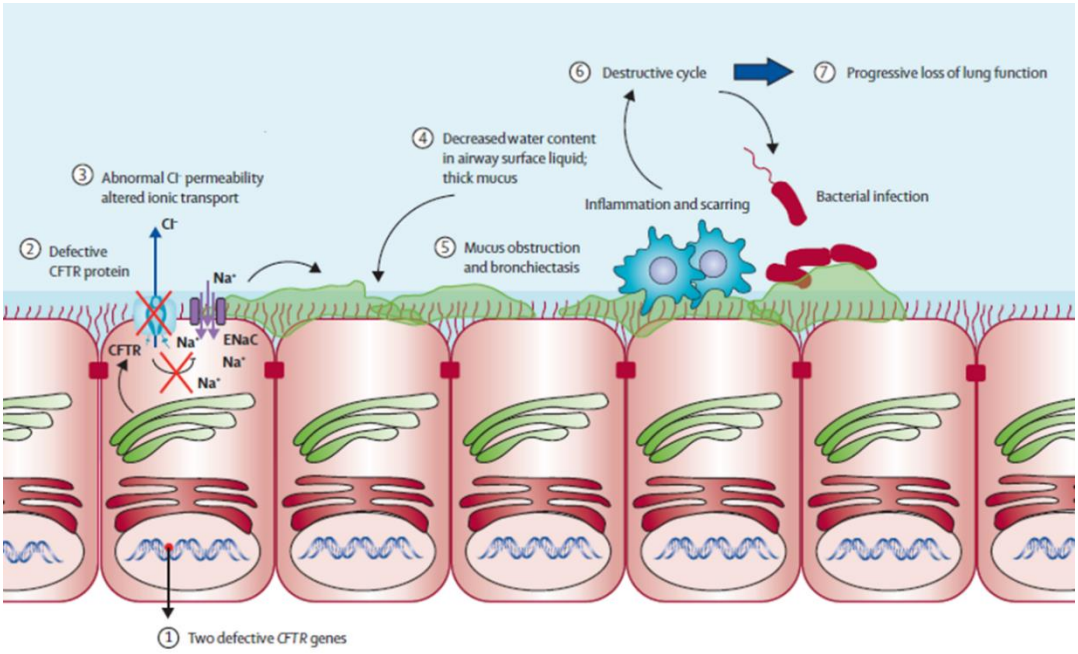


Figure 3 - Pathogenic cascade that causes CF lung disease.
CF is caused by mutations in the CFTR (1) gene that lead to defective CFTR protein (2) causing abnormal Cl-/bicarbonate function homeostasis and enhanced water and Na+ absorption (3). This leads to reduced ASL, dehydrated mucus (4) and impaired MCC (5). A continuous cycle of infection and inflammation (6) leads to progressive loss of lung function (7). [From: (De Boeck and Amaral 2016)].

Most CF treatments focus on the symptoms rather than focusing on the correction of the causing agent, acting on several aspects of CF pathophysiology. These include: bronchodilators, mucolytics and kinesitherapy to improve MCC, either by promoting airway hydration (like mannitol) or by decreasing sputum viscosity (dornase alfa); antibiotics to deal with bacterial infections (De Boeck and Amaral 2016); pancreatic enzymes, high food intake, vitamin/mineral supplements to overtake malabsorption; etc. Lung transplantation is the ultimate solution considered for individuals with CF and end-stage lung disease, having a 67 % survival rate at 5 years (Stephenson, Sykes et al. 2015). Noticeably, a wide variety of emerging symptomatic therapies are under study, targeting other aspects of CF pathophysiology, such as ENaC blockers, excessive inflammation regulators and biofilm disruptors (De Boeck and Amaral 2016).

More recently however, several agents that target mutant CFTR protein, i.e., the CF causative underlying defect, have been developed and reached the clinic. Two classes of CFTR modulators are currently available: correctors that increase F508del-CFTR processing and trafficking to the cell surface and potentiators that stimulate CFTR activity at the PM. Different combinations of CFTR modulators are available in the clinic and are recommended to individuals with CF according to the respective genotype. The most recently approved drug of this type (Trikafta™) is expected to be effective on individuals with CF carrying the F508del mutation in at least one allele, i.e., ~80-85% of individuals with CF worldwide (Hoy 2019). Indeed, the genotype of the patient determines the therapeutic strategy that is more suitable. Table 2 lists CFTR modulators currently approved by European Medicines Agency (EMA) and US Food and Drugs Administration (FDA) (CFF 2020).

Table 2 - FDA-approved CFTR modulators

Modulator	Commercial Name (US)	Function	Target	Examples	Approval
Ivacaftor	Kalydeco®	Potentiator	Gating mutations, residual function and lower conduction mutations	G551D, R74W, R117H	EMA FDA
Lumacaftor +Ivacaftor	Orkambi®	Corrector +Potentiator	Protein processing mutation	F508del homozygous	EMA FDA
Tezacaftor +Ivacaftor	Symkevi® - Eur Symdeko®- US	Corrector +Potentiator	Protein processing mutation, residual function mutations	F508del homozygous, R74W	EMA FDA
Elexacaftor +Tezacaftor +Ivacaftor	Trikafta™	Correctors +Potentiator	Protein processing mutation+Any	F508del + any other mutation	FDA

Over the years great improvements occurred in terms of life expectancy and quality of life of individuals with CF. Median age of survival is now around 40 years in Europe and US (Keogh and Stanojevic 2018), steadily raising from a median survival age from 10 years in 1962 (CFF 2020). The combination of conventional therapies with the emerging CFTR modulators, that correct the underlying molecular defect of CF, is expected to further improve the life

expectancy and quality of life of individuals with CF. Nevertheless, as the median age of individuals with CF increases, some new complications, like high cancer frequency, are emerging (Strausbaugh and Davis 2007).

4 Cystic Fibrosis and epithelial differentiation

The respiratory tract is composed of endoderm-derived epithelium surrounded by cells of mesodermal origin (Kimura and Deutsch 2007). Differentiation of the lung is mediated by key signalling pathways, including the fibroblast growth factor, hedgehog, retinoic acid, bone morphogenetic protein and Wnt signalling pathways (Kimura and Deutsch 2007).

The airways of mammals can be divided into two functional parts: the conducting airways and the respiratory airways. Conducting airways consists of the nose, trachea and bronchi. Their main functions are the transport of air to the respiratory airways, warming up the air and a key role in immune and microbial defence (Hollenhorst, Richter et al. 2011). Lungs display one of the largest surface areas in the human body and represent a direct contact surface with the exterior. Therefore, lung epithelium must act as a selective permeable barrier against inhaled pathogens and air surface liquid (ASL) secreted must allow for the continuous removal of the contaminants via the action of the ciliated cells (Wansleeben, Barkauskas et al. 2013). On the other hand, respiratory airways consists of the respiratory bronchi and alveoli, mediating gas exchange to provide an adequate intake of oxygen and removal of carbon dioxide (Hollenhorst, Richter et al. 2011).

The tracheobronchial epithelium consists of a continuous layer of columnar or cuboidal cells originating from the basement membrane, thus forming a pseudostratified epithelium. Various cell types can be found consisting mainly of basal cells, club cells, goblet cells and ciliated cells (Hollenhorst, Richter et al. 2011). Other cell types that can also be found are Tuft cells, Neuroendocrine cells and Ionocytes (Montoro, Haber et al. 2018). These cell types are expressed in different proportions with varying distribution between the type of airway epithelia (Hollenhorst, Richter et al. 2011). Key regulators of cell-type epithelial differentiation have been identified as well as specific molecular markers (Wansleeben, Barkauskas et al. 2013, Montoro, Haber et al. 2018).

The association between epithelial differentiation and CFTR has been described in several studies. CFTR has been shown to play a role in fundamental cellular processes related to differentiation such as foetal development (Larson and Cohen 2005, Cohen and Larson 2006, Meyerholz, Stoltz et al. 2010, Navis, Marjoram et al. 2013), epithelial differentiation/polarization (Puchelle, Gaillard et al. 1992, Brezillon, Dupuit et al. 1995, Dupuit, Kalin et al. 1995, Moyer, Denton et al. 1999, LeSimple, Liao et al. 2010), regeneration (Coraux, Hajj et al. 2005, Hajj, Lesimple et al. 2007, Schiller, Maniak et al. 2010) and epithelial-mesenchymal transition (EMT) (Zhang, Jiang et al. 2013, Rout-Pitt, Farrow et al. 2018, Narayanan, Schappell et al. 2020).

A noteworthy aspect is that CFTR levels in foetuses were found to increase from 10 weeks to mid-gestation, with its levels and location becoming more restricted afterwards, as differentiation takes place, as only a subset of cells maintains CFTR expression. Noticeably, the major difference between CF and non-CF foetuses was a 3-week delay in the appearance of detectable CFTR in CF (Marcorelles, Montier et al. 2007). Since CFTR levels on adult lung epithelia are much lower than the ones observed in foetuses and neonates, it indicates that the CFTR protein may exert functions at an early developmental stage with an impact much later in adulthood (Larson and Cohen 2005). Supporting this hypothesis is the fact that CFTR loss of function has been associated with several developmental deficiencies. Indeed, a possible role of CFTR in differentiation is further supported by the observation of tracheal development abnormalities in the cartilaginous rings in mice (Bonvin, Le Rouzic et al. 2008), piglets and young children lacking functional CFTR (Meyerholz, Stoltz et al. 2010). Also, male infertility in CF is almost always associated with the developmental condition CBAVD (Chen, Ruan et al. 2012). Noteworthy, CF mouse models display severe intestinal developmental issues which are corrected via CFTR rescue *in vitro* without the need for a continuous expression of CFTR (Cohen, Morrow et al. 1998).

CFTR has also been associated with epithelial differentiation (Buchwald, Sood et al. 1991, Montrose-Rafizadeh, Guggino et al. 1991, Montrose-Rafizadeh, Blackmon et al. 1992, Sood, Bear et al. 1992). Differentiation seems to affect not only CFTR levels but also its localization both in normal or mutant forms (Puchelle, Gaillard et al. 1992, Brezillon, Dupuit et al. 1995, Dupuit, Kalin et al.

1995, Brézillon, Hamm et al. 1997, Kalin, Claass et al. 1999, Penque, Mendes et al. 2000).

Another aspect related to differentiation is epithelial regeneration which has also been reported as dependent on functional CFTR (Tran-Paterson, Davin et al. 1992, Castillon, Hinnrasky et al. 2002, Coraux, Hajj et al. 2005, Hajj, Lesimple et al. 2007, Schiller, Maniak et al. 2010, Adam, Bilodeau et al. 2018). Regeneration is also dependent on the inflammation (Adam, Roux-Delrieu et al. 2015).

It has also been established that there is an excessive inflammatory response in CF and that it contributes to the CF lung disease (Heeckeren, Walenga et al. 1997) and that this pro-inflammatory state may even start in utero (Verhaeghe, Delbecque et al. 2007). In fact, inhibition of CFTR mimics the CF inflammatory profile (Perez, Issler et al. 2007). This may occur via several different pathways, such as the NF-kB overproduction (Chen, Jiang et al. 2012, Gao and Su 2015). CFTR also has a marked impacted in the levels of several inflammatory cytokines (Moss, Bocian et al. 1996, Becker, Sauer et al. 2004, Veit, Bossard et al. 2012, Kim, Beyer et al. 2013) and some cytokines may affect CFTR levels and function (Cafferata, Gonzalez-Guerrico et al. 2000, Skowron-zwarg, Boland et al. 2007, Tamanini, Borgatti et al. 2011, Wu, Ahrens et al. 2011).

A more comprehensive study shows that CF transcriptome leads to major alterations in calcium and membrane pathways, inflammation, wound healing, proliferation, oestrogen signalling and defence response (Clarke, Sousa et al. 2013). Other transcriptomic studies also corroborate these underlying differences (Zoso and Sofoluwe 2019). Several transcription factors, including Krüppel-like factors have been found to be regulating CFTR (Mutolo, Leir et al. 2018). Other relevant signalling pathways in the CF context that may have impact in differentiation and wound healing have been described like TNF-α, Src and JNK (Huang, Dudez et al. 2003, Huang, Jornot et al. 2003, Dudez, Borot et al. 2008, Losa, Kohler et al. 2014).

Objectives

The present doctoral work aimed at understanding the relationship between CF and epithelial differentiation and the possible roles of KLF4 in the regulation of CFTR.

The general goals of this work can be subdivided into the following, each one corresponding to one of the chapters of this work:

1 - Understand the relationship between epithelial differentiation and cystic fibrosis.

2 - Unravel the role of KLF4 in CFTR expression/activity in the context of cystic fibrosis.

3 - Characterize the impact of KLF4 knock out in epithelial differentiation in the context of cystic fibrosis.

Section 2 - Materials and methods

Materials and Methods

1 Cell culture techniques

1.1 Native human lung tissue

Explanted CF lungs and control tissues were collected in the Paediatrics Department of Motol University Hospital (Prague, Czech Republic) under approval of applied regulations and the hospital's Ethics Committee and shipped over 24h to Lisboa. After cleaning, primary, secondary and tertiary bronchi were used for primary HBE (pHBE) cell isolation, RNA extraction and immunohistochemistry. For RNA extraction, pieces of secondary bronchi were collected after lung cleaning in extraction buffer (see qRT-PCR), snap frozen in liquid nitrogen and kept at -80ºC. For immunohistochemistry, tissue was fixed overnight with electron microscopy grade PFA (0.2% or 4% v/v, Electron Microscopy Sciences, 15710) and then dehydrated and slowly frozen as previously described (Bajanca, Luz et al. 2004). Briefly, after lung cleaning and fixation, the pieces of secondary/tertiary bronchi were kept for 12h at a time in phosphate buffers with increasing sucrose (Fluka, 84100) content (4% to 15%) and then incubated in a final solution with 15% sucrose and 7.5% gelatine (Sigma-Aldrich, G9391) for 1h at 37°C. Dry ice-chilled isopentane (VWR, 24872) was then used to slowly freeze the tissues, which were kept at -80ºC until sectioning. Tissue sections were cryocut using a Leica CM1850 UV cryostat. Cryosections 5 to 10 µm thick were generated on silane-prep slides (glass slides coated with aminoalkylsilane, Sigma-Aldrich, S4651), left to dry overnight at 37ºC and used the following day for immunohistochemistry.

1.2 Cell lines and growth conditions

CF-relevant immortalized bronchial epithelial cell lines, Cystic Fibrosis Bronchial Epithelial cells (CFBE41o-) stably overexpressing wt- and F508del-CFTR (Bebok, Collawn et al. 2005) were used in this work. CFBE cells were grown in Eagle's Minimum Essential Medium (EMEM) with Earl salts and L-glutamine (Corning, 10-010-CVR) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Gibco, 10270), 1% Pen/Strep and puromycin (Sigma-Aldrich, P8833) at 2.5µg/mL for selection. To achieve polarization, cells were seeded on

collagen IV pre-coated transwell permeable supports at a density of 1.25, 2.5 or 10 x105 cells, depending on the diameter of the filter (6.5mm, 12 mm or 24mm insert, Corning 3470, 3460 and 3450, respectively). On the following day, media was changed from 10% to 2% (v/v) FBS to promote differentiation/polarization. The transepithelial electrical resistance (TEER) was measured regularly using a Millicell© ERS meter. Polarized CFBE cells were grown with media in both apical and basolateral compartments since a similar bronchial cell line (16HBE14o-) was found to only express occludin, claudin1, connexin (Cx) 43 and E-cadherin (E-Cad) when grown as submerged cultures (Berube, Prytherch et al. 2010). The KLF4 knockout CFBE cell lines generated by the CRISPR-Cas9 technique were grown under the same conditions as the other CFBE cells.

BCi-NS1.1 cells were cultured with Pneumacult-Ex Medium supplemented with Pneumacult-Ex 50X supplement (#05008; STEMCELL Technologies), 96 µg/ml hydrocortisone (H0888; Sigma-Aldrich), and 1% penicillin–streptomycin (10,000 U/ml) (15140-148; Gibco) in a 37°C, 5% CO2 humidified incubator. Following expansion, the cells were seeded onto either 6.5- or 12-mm diameter size transwell inserts with 0.4 µm pore polyester membrane (#3470, #3460; Corning Incorporated), coated with human type IV collagen (C7521; Sigma-Aldrich) at a density of 1.5×10^5 or 3.0×10^5, respectively. Cells were cultured with 1:1 DMEM/F-12 (15-090-CM; Corning Incorporated) supplemented with 5% FBS (Gibco), 1% penicillin–streptomycin, 0.5% amphotericin B (15290-026; Gibco), and 0.1% gentamicin (G1272; Gibco). On the following day, the medium in both chambers was replaced with DMEM/F12 supplemented with 2% Ultroser G (15950-017; Pall Life Sciences), 1% penicillin–streptomycin, 0.5% amphotericin B, and 0.1% gentamicin. Air-liquid interface (ALI) was established once cells reached full confluency by aspirating the medium from the apical chamber. The medium was replaced every 2–3 d for 30 d and polarization was monitored by measurements of the TEER using a chopstick electrode (Millicell-ERS, Millipore). Every % is v/v, except when otherwise stated.

Primary human bronchial epithelial (pHBE) cells were isolated as previously described (Li 2013). Briefly, cells were dissociated from the bronchial tissue by protease/DNase treatment and were then collected by centrifugation at 500g for 5min at 4ºC. pHBE cells were cultured in BEpiCM (ScienceCell, 3211) in 6-well culture plates previously coated with PureCol (type I collagen) (30

µg/mL, Advanced Biomatrix, 5005). To generate a differentiated epithelium $2x10^5$ cells were seeded onto collagen IV (Sigma-Aldrich, C7521) pre-coated transwell 6.5 mm permeable supports (Corning, 3470) and, when confluent, culture medium was removed from the apical surface. Cells were kept on ALI for 21 days in order to fully differentiate before experiments (e.g. Western blot, wound healing) were performed. Growth curves were performed on non-polarized HBE cells plated in PureCol coated 24-well culture plates.

All cell lines were grown at 37ºC in 5% CO_2.

1.3 TEER measurements

TEER measurements were carried out on polarizing CFBE and differentiating BCi-NS1.1 and pHBE cells using a volt-ohmmeter (Millicell-ERS, Millipore, MER5000001), as a first indicator that the cells were differentiated and ready for further experiments. The volt-ohmmeter was allowed to incubate with the medium for 30 minutes at RT before measuring the resistance. The measurement was performed always before changing the media of the cells.

1.4 Treatment with compounds

CFBE cells were seeded (100 000 cells) in a P-24 plate and allowed to grow until fully confluent (24h). AKT inhibitor MK-2206 (Selleckchem, S1078) and GSK3β inhibitor TWS119 (Selleckchem, S1590) were added for 48h at a working concentration of 1 µM. Inhibitors were diluted in DMSO at a stock concentration of 40 mM and DMSO was therefore used as a negative control. Other inhibitors tested are described in Table S5.

When applicable, cells were incubated with the corrector VX-809 (Lumacaftor) or vehicle control (DMSO) – 3 µM for 48h in the appropriate media with 0.1% (v/v) FBS.

When applicable, cells were treated with CFTR-Inhibitor-172 - 30 µM for 48h and compared with vehicle control (DMSO), using media with 0.1% (v/v) FBS.

When applicable, IL-4 was used to treat BCi-NS1.1 cells to promote mucous production. The standard ALI method was used and 5 ng/ml of IL-4 (BMS337; eBioscience) was added to the basolateral side for up to 20 days.

All tested compounds and conditions are shown in more detail in Table S5.

1.5 siRNA transfection

For siRNA transfection, 75 000 cells were transfected in suspension in 24-well plates 24h after being split. Transfection mixture using Lipofectamine 2000 (1 mg/ml, Invitrogen #11668019) was prepared containing 50 nM of siRNA (Table S4) and 3 ng Lipofectamine to a final volume of 400 µL OptiMEM:EMEM (1:3), according to the manufacturer´s instructions. After transfection, cells were grown in FBS-free media. After 24h the media was changed to EMEM supplemented with 5% FBS (v/v). 72h after transfection, cells were harvested. The respective negative controls were used (Ambion or Dharmacon).

1.6 cDNA transfection

Suspension transfection of CFBE wt- and F508del-CFTR expressing cells with plasmids containing GFP tagged KLF4 (or GFP for negative control) was performed in 24 well-plates 24h after being split. Two transfection mixtures were prepared, one with 3 ng Lipofectamine® 2000 (1 mg/ml, Invitrogen #11668019) in 50 µl of OptiMEM and one with 1500 ng cDNA in 50 µL of OptiMEM, according to the manufacturer instructions. These two were allowed to incubate for 20 min and then added dropwise to the well containing 300 µl of EMEM 2% FBS and 75 000 cells in suspension. 24h later, media was changed to EMEM supplemented with 10%(v/v) FBS. 48h post-transfection the protein extraction was performed.

KLF4-GFP used was from Origene (RG206691) and the GFP control used was pEGFP-C2 from Clontech.

1.7 KLF4 KO cell generation

The Cas9 plasmid was obtained from Addgene (pCas9_GFP, #44719). pCas9_GFP was used with two guide RNAs (1. 5’-GGGGCGGCCGGGAAGCACTG-3’), 2. 5’- GAAACCTTACCACTGTGACT-3’) targeting the genomic region of KLF4, constructed using Invitrogen's GeneArt® Gene synthesis system. The knockout of KLF4 was carried out using the

CRISPR/Cas9 system as previously described (Mali, Yang et al. 2013) using Lipofectamine 2000 for cell transfection. For clone selection a plasmid containing the hygromycin resistance gene was co-transfected. Cell clones were isolated using clonal discs. Once expanded, genomic DNA from each clone was isolated and amplified by PCR with primers recognizing sequences covering the gRNA targeted region. PCR products were sequenced to identify KLF4 KO clones, which were also confirmed by WB using KLF4 antibody for detection.

2 Cellular and molecular biology assays

2.1 Production of competent bacteria

The bacterial strain XL1-B (*Escherichia coli* – E-coli) was inoculated overnight into 2 ml of LB medium at 37ºC with shaking (220 rpm). The pre-inoculum was used to inoculate 200 ml LB medium and incubated 37ºC at 220rpm until reaching a final concentration of 5 x 10^7 bacteria/ml which correspond to an absorbance of 0.3 at 600 nm. Then, bacteria were transferred to ice and centrifuged at 1000g for 15 min at 4ºC. The bacterial pellet was resuspended in ice-cold RF1 buffer (100 mM RbCl, 50 mM MnOH, 30 mM KCH_3CO_2, 10 mM CaOH, pH 7.5, 15% (w/v) glycerol, pH 5.8) and incubated in ice for 15 min and centrifuged at 1.000 g for 15 min at 4ºC. The pellet was resuspended in ice-cold RF2 buffer (10 mM RbCl, 75 mM CaOH, 10 mM MOPS, 15% (w/v) glycerol, pH 6.5) and incubated at 4ºC. Finally, 200 µl aliquots were prepared, frozen immediately in liquid nitrogen and stored at -80ºC.

2.2 Transformation of competent bacteria

E. coli XL1-Blue bacterial cells were transformed by incubating a 200 µl aliquot of competent bacteria with approximately 100 ng of DNA for 30 min at 4ºC. Then, bacteria were exposed to a heat-shock at 42ºC for 90 seconds and after the mixture was incubated for 2 min at 4ºC. After that, LB medium without antibiotic was added and incubation in an orbital incubation for 45 min at 37ºC and 220 rpm followed. Bacteria were pelleted at 5000 g for 2 min and the pellet was resuspended in the remaining supernatant which was plated into LB-agar supplemented with the specific antibiotic namely 100 µg/ml ampicillin (Sigma-

Aldrich #A9518-56) or kanamycin A (Fisher Bioreagents, # BP906-5). Transformed bacteria grown overnight at 37ºC.

2.3 DNA plasmid extraction

The NZYMiniprep kit (NZYtech, #MBO1001) was used to perform small scale plasmid DNA purification according to the manufacturer´s instructions. In summary, this protocol is based on an alkaline lysis of the bacterial cells followed by centrifugation steps to remove cellular residues, genomic DNA and denatured proteins. The plasmid DNA is absorbed onto a silica matrix in the presence of high saline concentrations. Finally, DNA is washed and eluted in RNAse and DNAse free water. The DNA concentration was determined using a Nanodrop spectrophotometer DN1000 (Alpha-gene) measuring the absorbance at 260 nm and its purity was assessed by the ratios A260/A280 and A260/A230.

2.4 Confluency assay

Cells were seeded at different densities in a 12-well plate, namely 100 000, 250 000 and 500 000 cells and allowed to grow for 24h, respectively originating low confluency, medium confluency and full confluency cell layers. Protein extraction was then performed after washing 1x with PBS.

2.5 Proliferation assays (Ki-67+)

Proliferation was assessed by the evaluation of Ki-67+ staining. 100 000 cells were seeded in P24 well plates and grown until fully confluent (circa 24h). A wound was performed by scratching with a pipette tip and cells were grown for 24h. Then, cells were fixed with PFA for 20 minutes and left over night with PBS at 4ºC. Cells were then permeabilized with 0.3% Triton X-100 solution for 15 min and treated with ammonium chloride 0.5 M for 15 minutes to reduce background. Cells were then blocked with PBS 2% BSA for 30 min, followed by 90 minutes of incubation with primary antibody against Ki-67 (1/100 – M7240 Dako™). Cells were then washed with PBS and incubated with DAPI (1/20 – Amersham PA53021) and secondary antibody for 1 hour (1/1500 – Alexa568® anti mouse).

After washing with PBS, images were taken at a fluorescence microscope and Ki-67+ ratio assessed.

2.6 Growth curves

Control and CF pHBE cells were seeded in 24-well plates previously coated with PureCol at a density of 50000 cells/well (day 0). Cells were kept in BEpiCM and were harvested and counted every 2-4d to assess the growth rate. Media was changed regularly. At least three different wells were considered for all time points in all controls and CF pHBE cells.

For CFBE cells, 50 000 CFBE cells were seeded in a P24 well plate. After 24 hours, pictures were then taken at given timepoints using the brightfield microscope at given timepoints and cell number was counted in the central area using ImageJ for image analysis.

2.7 Wound healing assays

Fully polarized CFBE cells or fully differentiated pHBE cells were mechanically injured by scraping a sterile P10 pipette tip across the cell monolayer. For cell wounding PBS was added to the apical side of the filters. After wounding the apical surface was washed twice with PBS to remove cell debris. Fresh media was added to the basolateral (on both cell types) and apical side (only on CFBE cells).

Wound closure was monitored by live cell imaging (48h, 37ºC, 5% CO2) with an automated Leica DMI6000 widefield microscope coupled to a Hamamatsu Flash4 sCMOS camera, using a HCX 4x W 4x/0.1 objective. Images were taken every 2-3h. Software used for acquisition was Leica's LAS x, and image processing was performed on ImageJ FIJI (Schindelin, Arganda-Carreras et al. 2012). FIJI was used to segment, and measure wound area. Wound closure was then calculated as Wound size (%)=(A_t/A_0)×100, where A_t is the area for a given time point and A_0 is the initial wound area. Wound size was plotted as a function of time (h) and was used to calculate the rate (slope) of wound closure (%/h).

For comparison of CFBE wt- and F508del-CFTR cells and their KLF4 KO counterparts, a different approach was used. Cells split the previous day were

counted and 16 000 cells were seeded on a 96-well plate using a cell seeder. After 48h, 5µM of CellTracker (C7025, Invitrogen) was added for 1 hour. Using a 96-pin scratch tool, developed by our group in collaboration with the mechanical workshop of EMBL, Heidelberg, Germany, 96 wounds were simultaneously created on the confluent cell monolayer. This tool allows for more reproducible wounds and decreases inter-wound variability. Cells were washed twice with Opti-MEM before adding imaging media (EMEM without phenol red). Live cell imaging took place 12 hours with imaging every hour using a Leica SP8 confocal microscope. After imaging, cells were washed and fixed for 20 min at RT with 4% PFA, permeabilized with 0.1% Triton X-100 for 5 min at RT and incubated with SiR-actin (1:5000 SpiroChrome SC001) and Hoechst (1:1000 Sigma #94403) for 30 min at RT. Fluorescence images were taken at the Leica 6000b widefield fluorescence microscope.

2.8 Neurobiotin and Lucifer Yellow dye coupling

For analysis of cell-to-cell coupling, one CFBE cell within a cluster was impaled with a thin tip microelectrode filled with 1% Lucifer yellow and 2% neurobiotin solution prepared in 150 mM KCl and buffered to pH 7.2 with 10 mM N-2-hydroxyethylpiperazine-N#-2-ethanesulfonic acid–KOH. The tracers were allowed to fill the cells by simple diffusion for 3 min. Lucifer yellow was added to the microelectrode solution to visualize and mark the injected cell. At the end of the injection, the electrode was removed, and cells were fixed with 4% paraformaldehyde. After permeabilization with 0.3% Triton X-100, neurobiotin diffusion was revealed with streptavidine–rhodamine (1/3000) for 60 min at room temperature (RT). Fluorescent cells were observed with an inverted TMD-300 microscope (Nikon, Egg, Switzerland) equipped with a x40 phase 3 dark medium objective with a numerical aperture of 0.7 (Carl Zeiss). Images were captured with a Visicam digital camera (Visitron Systems, Puchheim, Germany) connected to a personal computer running Metafluor 4.01 software (Universal Imaging, Sunnyvale, CA). Numbers of stained cells were counted. (Morel, Burnier et al. 2010).

3 Biochemical assays

3.1 Protein extraction

For protein extraction cells were washed three times with 1x PBS and lysed in 1x sample buffer (SB) (2x SB –Tris-HCl (Sigma, 30721) 62.5 mM, pH 6.8, SDS 3% (Gibco, 15553), glycerol 20% (Sigma, 92025), Bromophenol Blue 0.02%(w/v), DTT (Sigma, D0632) 100 mM) supplemented with protease inhibitor cocktail (Roche, 11697498001), 25U Benzonase (Sigma-Aldrich, #E1014-25G) and 3.125 mM of $MgCl_2$ (Merck, 105833). Lysates were prepared by repeated pipetting and then collected.

3.2 Protein quantification assay

The Bio-rad protein assay (Bio-rad, 5000006) was used to quantify protein extracts. This method is based on the Bradford method. Basically, reagent is diluted in water (20:80) a standard curve is made using different concentrations of the BSA (Bio-rad, 5000002) applying 0-20 µl of the standard into 1000-980 µl of the reagent. 10 µl of the samples are applied in 990 µl of the reagent. All of these are incubated for 5 min at RT and assessed in a spectrophotometer by measuring the absorbance at 595 nm. Standards are used to create a linear standard curve and the concentration of the samples calculated by plotting the results against the standard curve.

3.3 Western blot

25-40 µg of protein were loaded onto polyacrylamide gels (4% for stacking and 7% or 10% for resolving gels) in order to perform SDS/PAGE. Transfer onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, IPVH00010) was preformed using a wet-transfer system. The membranes were blocked for 1h with 5% (w/v) non-fat milk (NFM) in PBS supplemented with Tween 20 (Fisher BioReagents, BP337-100). This was followed by incubation with the primary antibody overnight at 4ºC, with gentle shaking. Horseradish peroxidase (HRP)-conjugated secondary antibodies were applied for 1h at RT. All the antibodies were diluted in the blocking solution. Membrane luminescence was detected on a Chemidoc XRS+ system (BioRad, 170-8265). Quantification of band intensity

was performed using the Image Lab software (BioRad, 170-9690), which integrates peak area. All measurements were normalized against loading controls (calnexin - Clnx, vinculin, tubulin or GAPDH). A list of primary and secondary antibodies can be found in Table S1 and S2.

3.4 Co-Immunoprecipitation

CFBE wt-CFTR and F508del-CFTR cells were lysed at 4°C with PD buffer [50 mM Tris-HCl, 0.1 M NaCl, 1% (v/v) NP40, 10% (v/v) glycerol, pH 7.5] supplemented with protease inhibitor cocktail (Roche). Lysates were centrifuged. Pellets were discarded and the supernatants were pre-cleared through incubation with Protein-G agarose beads (Invitrogen). The supernatants were then incubated overnight with the appropriate antibody, either anti-CFTR or anti-KLF4, at 4°C. For control reactions no antibody was added. Beads were washed three times with wash buffer (Tris-HCl 0.1M, NaCl 0.3M, Triton X-100 1% (v/v), pH 7.5), followed by elution with 1x sample buffer and further separation on SDS-PAGE and western blot analysis.

3.5 gDNA extraction and sequencing

Genomic DNA was extracted using the Genomic DNA purification kit (Promega #A1120) and samples were sent to be sequenced by STABvida and sequences analysed using the SnapGene viewer software.

3.6 RNA extraction, reverse transcription and RT-qPCR

Total RNA was extracted from human CF (F508del/F508del) and non CF lung samples using the Direct-zol RNA Miniprep Plus kit (Zymo Research), and reverse transcription of cDNA was then performed using 100 ng of each RNA sample, M-MuLV Reverse Transcriptase (NZYtech) and random primers. Similar protocol was used for CFBE and BCi cells, using for RNA extraction the NZY total RNA isolation kit (NZYtech MB13402) according to the protocol provided.

Then, a mix containing forward and reverse primers, cDNA (5ng) and 1x Evagreen SsoFast PCR reagent (Bio-Rad, 172-5204) was used along with a Bio-Rad CFX96 system. Bio-Rad CFX Manager 2.0 software (Bio-Rad, 1845000) was

used for analysis. A standard cycle protocol was used for PCR amplification (1 min at 95°C followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C).

Technical duplicates were used in amplification, melt curves were examined to confirm the amplification of specific products, and negative controls were confirmed to be free of amplification after 40 PCR cycles. Mean relative levels of expression were calculated for the target genes using the ΔΔCT method, where Fold Change = $2(-^{\Delta\Delta CT})$, using mean levels of expression in non CF samples as the baseline (or WT-CFTR cells).

Information regarding the primer sequences used is presented in Table S3. ACTB and GAPDH were used as housekeeping genes for the experiments on lung and cells (BCi and CFBE), respectively.

3.7 Immunofluorescence assay

CFBE cells were grown on glass coverslips and then fixed with PFA (Merck Millipore, 104003) 4% (v/v), permeabilized with Triton X-100 (Amersham Biosciences, 17-1315-01) 0.5% (v/v) and blocked with Bovine Serum Albumin (BSA) 1% (w/v). Cells were then incubated 2 hours with the primary antibody at room temperature, after which a mix of the secondary antibody and nuclear dye Hoechst 33258 (1µg/mL, Sigma-Aldrich, 94403) was applied for 1h at RT. Coverslips were then mounted in a mix of N-propylgallate (Sigma-Aldrich, P3130) and glycerol for microscopy (Merck, 104095).

In polarized CFBEs, cells were fixed with PFA 4% (v/v), permeabilized with Triton X-100 0.5% (v/v) and blocked with BSA 1% (w/v) before being removed from their supports using a scalpel.

Lung tissue staining were performed similar but permeabilization was achieved with a 0.2% (v/v) Triton X-100 solution and a quenching step with $NaBH_4$ (1mg/mL, Sigma-Aldrich, 213462) was additionally performed before blocking. Hoechst 33258 was used to stain the nuclei. The tissues stained were secondary/tertiary bronchi and were as similar as possible for comparison. Areas of extensive shedding/remodelling in patients were avoided in the analysis, and areas of intact epithelia preferred.

Imaging was performed using a Leica TCS SP8 confocal microscope coupled to a Hamamatsu Flash4 sCMOS camera, using HC Plan Apo 20x/0.75

and HC Plan Apo 63x/1.4 objectives. Software used for acquisition was Leica's LAS x, and image processing was performed on ImageJ FIJI.

A list of primary and secondary antibodies can be found in Tables S1 and S2.

4 Functional assays

4.1 Ussing chamber experiments

Polarized CFBE cells grown for 7 days on permeable supports (Costar) were mounted into a micro-Ussing chamber and analysed under open-circuit conditions at 37°C. Apical and basolateral sides were continuously perfused with ringer solutions containing 30 and 145 mM Cl^- concentrations (pH 7.4), respectively. After an equilibrium period, 30 µM amiloride (A7410; Sigma-Aldrich) was added apically to block ENaC. Subsequently, the cyclic adenosine 3'-5' monophosphate (cAMP) agonist, Forskolin (Fsk – 2 µM), the CFTR potentiator Gen (50 µM), and the CFTR channel blocker CFTR-Inh_{172} (30 µM) were added sequentially. Values for transepithelial voltages (Vte) were referenced to the basal surface of the epithelium. Transepithelial resistance (Rte) was determined by applying short current pulses (1 s) of 0.5 µA (5-s period). The equivalent short circuit (Ieq-sc) was calculated according to Ohm's law (Ieq-sc = Vte/Rte). Resistance was evaluated before performing the Ussing chamber experiments and a minimum of 500 $Ohm.cm^2$ was required to perform the experiment.

4.2 Patch Clamp assays

For patch clamping cells were grown on cover slips and transfected with KLF4-GFP and GFP only as control. The GFP signal allowed the detection of transfected cells. After 48-72 h the cells were used for measurements. Mounted in a perfused bath on the stage of an inverted microscope the cells were perfused continuously and held at 37°C via a water tubing system. Patch clamp recordings were performed in whole-cell configuration. A glass microelectrode was filled with an intracellular cell-like solution and attached to the cell surface which leads to a high resistance seal formed between the pipette and the cell when applying a slight negative pressure. To achieve the whole-cell configuration the patch was disrupted by a hard suction and whole-cell currents were measured using a

computer-controlled amplifier, which is connected to the microelectrode. Membrane voltages (V_m) from -100 to 100 mV were clamped in 20 mV steps.

5 Databases and bioinformatics

During this work some databases/websites were used.

For the description of CFTR mutations and their clinical significance both CFTR1 and CFTR2 databases were used, in which one can retrieve the total number of described mutation and those that are CF-causing, respectively. These databases are located respectively in the following websites http://www.genet.sickkids.on.ca (CFTR1) and https://www.cftr2.org (CFTR2).

For information regarding the FDA-approved therapies for CF the Cystic Fibrosis Foundation website (https://www.cff.org/) was consulted and the relevant information retrieved.

For the characterization of transcription factor binding sites and gene targets the Genecards website https://www.genecards.org was consulted and the relevant information was extracted from the Genomics section.

For protein characterization and information regarding protein tissue specificity the Human Protein Atlas was consulted and the relevant information obtained https://www.proteinatlas.org/.

For protein-protein interaction networks generation, the database STRING (https://string-db.org/) was used and the networks of interest generated.

To retrieve information and acquire an overview of the relevant signalling pathways the KEGG website was used (https://www.kegg.jp/kegg/kegg2.html).

6 Statistical analysis

Data are always presented as mean ± SEM. Student's t-test for unpaired samples was used for statistical analyses. Prism 6 software (GraphPad, Inc., San Diego, CA) was used for graph design and statistical analyses. Significant differences were defined for $p \leq 0.05$ and marked with an asterisk. Other trends or tests may be stated in the legend. N=3 unless stated otherwise in the figure or in its legend. Compared WB images are always from the same blot.

Supplementary information

Table S1 - Primary antibodies list and conditions

Target	Application	Dilution	Host	MW (kDa)	Company	Reference
CFTR	IF (Lung)	1/200	Mouse	120-180	RD Biotech	MATG1104
	IF (CFBE)	1/500			CFF	570
	WB (CFBE)	1/3000				596
	WB (HBE and BCi)	1/500 (1% NFM-TBST)				
Calnexin	WB	1/3000	Mouse	90	BD Biosciences	610523
Claudin1	WB	1/100	Mouse	20	Santa Cruz	sc-166338
Collagen I	IF (CFBE)	1/50	Rabbit	250	Abcam	ab34710
	IF (lung)	1/100				
	WB	1/1000				
Connexin 26	WB	1/200	Rabbit	30	Santa Cruz	sc-7261-R
Connexin 30	WB	1/200	Mouse	30	Santa Cruz	sc-514847
Connexin 31	WB	1/200	Mouse	35	Santa Cruz	sc-81803
Connexin 43	WB (CFBE)	1/200	Mouse	40	Santa Cruz	sc-13558
	IF (CFBE)	1/100			Invitrogen	71-0700
	WB (HBE)	1/100				
Cytokeratin 5	WB	1/1000	Rabbit	60	Invitrogen	PA1-37974
Cytokeratin 13	WB	1/500	Goat	50	Santa Cruz	sc-31703
Cytokeratin 14	WB	1/500	Mouse	50	Santa Cruz	sc-53253
Cytokeratin 18	IF & WB	1/100	Mouse	45	Santa Cruz	sc-323229
DSPI/II	WB	1/500	Rabbit	250	Santa Cruz	sc-33555
E-Cadherin	IF	1/200	Mouse	135	BD Biosciences	610181
	WB	1/5000				
EGFR	WB	1/500	Mouse	180	Santa Cruz	sc-373746
Fibronectin 1	WB	1/1000	Mouse	250	Santa Cruz	sc-8422
	IF	1/100				
Foxj1	WB	1/500	Mouse	60	eBioscience	14-9965
GAPDH	WB	1/10000	Mouse	37	Abcam	ab8245
GSK3beta	WB	1/1000	Rabbit	50	Cell signaling	12456s
Ki67	IF	1/100	Rabbit	250	Abcam	ab16667
	WB	1/2500				
	IF (Ki67+)	1/100	Mouse		Dako	M7240
KLF2	WB	1/200	Mouse	37	Abcam	ab194486
KLF4	WB	1/1000	Rabbit	60	Santa Cruz	sc-20691
	IF	1/200				
KLF5	WB	1/500	Rabbit	50	Abcam	ab24331
N-Cadherin	IF (CFBE)	1/100	Mouse	135	BD Biosciences	610920
	IF (lung)	1/200				
	WB (HBE)	1/500				
	WB (CFBE)	1/1000				
Occludin	WB	1/100	Mouse	90	Santa Cruz	sc-133256
pAKT	WB	1/1000	Rabbit	60	Cell signaling	13038s
pGSK3beta	WB	1/1000	Rabbit	50	Cell signaling	9336s
pSmad2	WB	1/200	Rabbit	60	Cell signaling	3101s
Smad7	WB	1/1000	Rabbit	50	Aviva	ARP32008
SNAIL+SLUG	IF & WB	1/1000	Rabbit	25	Abcam	ab180714
TEAD4	WB	1/200	Mouse	50	Abcam	ab58310
TGFbetaRI	WB	1/200	Rabbit	50	Cell signaling	3712s
TGFbetaRII	WB	1/200	Mouse	80	Santa Cruz	sc-17719
TWIST	WB (CFBE)	1/50	Mouse	25	Abcam	ab50887
	WB (HBE)	1/25				
Vimentin	IF (Lung)	1/250	Rabbit	60	Abcam	ab92547
	IF (CFBE)	1/100				
	WB (CFBE)	1/1000				
	WB (HBE)	1/100 (NFM-TBST)				
Vinculin	WB	1/1000	Mouse	130	Santa Cruz	sc-73614
ZO1	IF	1/100	Mouse	250	Invitrogen	33-9100
	WB (HBE)	1/2500				
	WB (CFBE)	1/5000				
α-SMA	WB	1/500	Rabbit	40	Abcam	ab5694
alphaTubulin	WB	1/10000	Mouse	50	Sigma	T5168
beta-catenin	IF (CFBE)	1/100	Rabbit	90	Abcam	ab32572
	IF (Lung)	1/200				
	WB	1/5000				

Information regarding the used primary antibodies, their application, the respective dilution, the host, the molecular weight, the company and reference are clarified.

Table S2 - Secondary antibodies list and conditions.

Antibody	Target	Use	Dilution	Host	Company	Reference
Alexa Fluor 488	Mouse IgG	IF	1/500	Donkey	Life Technologies	A21202
Alexa Fluor 488	Rabbit IgG		1/500			A21206
Alexa Fluor 568	Rabbit IgG		1/500			A10042
Alexa Fluor 568	Mouse IgG		1/500			A10037
(H+L)-HRP Conjugate	Mouse IgG	WB	1/3000	Goat	Bio-Rad	170-6515
(H+L)-HRP Conjugate	Rabbit IgG	WB	1/3000			170-6516
(H+L)-HRP Conjugate	Goat IgG	WB	1/5000	Donkey	Invitrogen	A15999

Information regarding the secondary antibodies, their application, the respective dilution, the host, the company and reference are clarified.

Table S3 - Primers information.

Target	Fw/Rv primer	Sequence (5'-3')
ACTA2	Forward	GTGTTGCCCCTGAAGAGCAT
	Reverse	GCTGGGACATTGAAAGTCTCA
ACTB	Forward	CTCTTCCAGCCTTCCTTCCT
	Reverse	AGCACTGTGTTGGCGTACAG
CDH1	Forward	TGCCCAGAAAATGAAAAAGG
	Reverse	GTC ATGTGGCAATGCGTTC
CDH2	Forward	GGACAGTTCCTGAGGGATCA
	Reverse	GGATTGCCTTCCATGTCTGT
CLDN1	Forward	CCTCCTGGGAGTGATAGCAAT
	Reverse	GGCAACTAAAATAGCCAGACCT
COL1A1	Forward	GAGGGCCAAGACGAAGACATC
	Reverse	CAGATCACGTCATCGCACAAC
CTNNB1	Forward	AAAGCGGCTGTTAGTCACTGG
	Reverse	CGAGTCATTGCATACTGTCCAT
DSP	Forward	GCAGGATGTACTATTCTCGGC
	Reverse	CCTGGATGGTGTTCTGGTTCT
FN	Forward	CGGTGGCTGTCAGTCAAAG
	Reverse	AAACCTCGGCTTCCTCCATAA
GAPDH	Forward	ATGGGGAAGGTGAAGGTCG
	Reverse	GGGGTCATTGATGGCAACAATA
GJA1	Forward	CAATCACTTGGCGTGACTTC
	Reverse	AACGAAAGGCAGACTGCTCA
GJB2	Forward	TCGCATTATGATCCTCGTTGTG
	Reverse	GGGGAAGTAGTGATCGTAGCAC
GJB3	Forward	GGACTGCTACATTGCCCGAC
	Reverse	ATGGTGAGTACGATGCAGACG
CK18	Forward	AAGGTGAAGCTTGAGGCAGA
	Reverse	CTGCACAGTTTGCATGGAGT

KLF2	Forward	CTACACCAAGAGTTCGCATCTG
	Reverse	CCGTGTGCTTTCGGTAGTG
KLF4	Forward	CCCACATGAAGCGACTTCCC
	Reverse	CAGGTCCAGGAGATCGTTGAA
KLF5	Forward	GAACTGGTCTACGACTGAGGC
	Reverse	CCTGGTCCAGACAAGATGTGA
OCLN	Forward	ACAAGCGGTTTTATCCAGAGTC
	Reverse	GTCATCCACAGGCGAAGTTAAT
SNAI1	Forward	TCGGAAGCCTAACTACAGCGA
	Reverse	AGATGAGCATTGGCAGCGAG
SNAI2	Forward	CGAACTGGACACACATACAGTG
	Reverse	CTGAGGATCTCTGGTTGTGGT
TJP1	Forward	CGGTCCTCTGAGCCTGTAAG
	Reverse	GGATCTACATGCGACGACAA
TWIST1	Forward	GTCCGCAGTCTTACGAGGAG
	Reverse	GCTTGAGGGTCTGAATCTTGCT
VIM	Forward	AGTCCACTGAGTACCGGAGAC
	Reverse	CATTTCACGCATCTGGCGTTC
ZEB1	Forward	TTACACCTTTGCATACAGAACCC
	Reverse	TTTACGATTACACCCAGACTGC
ZEB2	Forward	CAAGAGGCGCAAACAAGCC
	Reverse	GGTTGGCAATACCGTCATCC

Description of the primers used in RT-qPCR. Sequences for the primers were found at Harvard Primerbank.

Table S4 - siRNAs information.

siRNA	Company	Line	Stock	Reference
KLF2	Ambion	Silencer Select	30 μM	s20270
KLF4	Dharmacon	siGENOME pool	20 μM	M-005089-03
KLF5	Ambion	Silencer Select	30 μM	s2116
CFTR	Ambion	Silencer Select	30 μM	s2947
NC1	Ambion	Silencer Select	30 μM	4390843
NC3	Dharmacon	siGENOME	20 μM	D-001210

Information regarding the siRNAs used and their references.

Table S5 -Compounds information.

Compound	Target	working Conc.	time	Company	Reference
mk-2206	AKT inhibitor	1 μM	48h	Selleckchem	S107807
TWS119	gsk3b inhibitor	1 μM	48h	Selleckchem	S159001
erlotinib	EGFR inhibitor	10 μM	72h	Selleckchem	S1023
XAV-939	WNT inhibitor	1 μM	12h	Selleckchem	S118004
CHIR-99021	GSK3 inhibitor	20 μM	24h	Selleckchem	S2924
VX-809	F508del-CFTR rescue	3 μM	48h	Selleckchem	S1565
Inhibitor-172	wt-CFTR inhibitor	30 μM	48h	Selleckchem	S7139
IL-4	mucous production	333nM	5 to 15d	eBioscience	BMS337

Information regarding the compounds used/tested, their targets, conditions and references. DMSO was used as a control at a 1 μM concentration.

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Section 3 – Results and discussion

Chapter I – Impaired Differentiation in CF: Mutant CFTR Impacts on Proliferation and Differentiation

Some of the data present in this chapter was included in the following work: Quaresma MC, Pankonien I, Clarke L, **Sousa LS**, Silva IAL, Railean V, Doušová T, Fuxe J, Amaral MD (2020) Mutant CFTR Drives TWIST1 Mediated Epithelial-Mesenchymal Transition. Cell Death Dis. – submitted, under revision.

Abstract

Cystic fibrosis (CF) is a monogenic disease resulting from mutations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene encoding a chloride/bicarbonate channel. Recent evidence indicates that CFTR plays a role in other cellular processes, namely in development, cellular differentiation, and wound healing. Moreover, CFTR has been proposed to function as a tumour suppressor in a wide range of cancers. Along these lines, CF was recently suggested to be associated with epithelial-mesenchymal transition (EMT), a latent developmental process, which can be re-activated in fibrosis and cancer. However, it is unknown whether EMT is indeed active in CF and if EMT is triggered by dysfunctional CFTR or a consequence of secondary complications of CF.

In this study, we aimed to investigate the occurrence of EMT in the context of CF. To this end, we first observed that CF cells expressing mutant CFTR were in a hyper-proliferative state, evidenced impaired wound-healing and displayed altered expression of epithelial and mesenchymal markers, consistent with a partial EMT. Importantly, we found that EMT-associated transcription factor TWIST1 was consistently upregulated in CF cells.

Altogether, these results identify for the first time that EMT is intrinsically triggered by absence of functional CFTR and indicate that CFTR plays a direct role in EMT protection.

Introduction

CFTR through its chloride (Cl^-)/ bicarbonate (HCO_3^-) channel function is a key regulator of epithelial ion and fluid homeostasis. However, the correction of the Cl^-/ HCO_3^- homeostasis may not be sufficient to prevent the progress of lung disease (Roomans 2003). Besides the aforementioned role on Cl^-/ HCO_3^- transport, CFTR also exerts its action on local pH and regulates a variety of other ion channels, transporters and other proteins, like mucins or digestive enzymes (Borowitz 2015).

Moreover, it has been postulated that CFTR plays other key cellular functions that are not related to ion transport. Among these, it has been proposed

that CFTR may provide the platform for docking sites of several apical proteins, including other ion channels. CFTR dysfunction has also been associated with inflammation, destruction of the protease/antiprotease balance and alterations in the oxidant/antioxidant balance (Bergeron and Cantin 2019).

Furthermore, the relationship between epithelial differentiation and CFTR has been described in several studies. Firstly, several studies have shown that higher levels of CFTR mRNA are present in well-differentiated epithelial cells vs. undifferentiated cells (Buchwald, Sood et al. 1991, Sood, Bear et al. 1992) (Montrose-Rafizadeh, Guggino et al. 1991, Montrose-Rafizadeh, Blackmon et al. 1992). Secondly, it was observed that wt-CFTR expression at the apical plasma membrane (PM) is only observed in well differentiated cells, being epithelial differentiation and polarization required for CFTR PM traffic, maturation, and CFTR-mediated Cl^-/ HCO_3^- secretion (Morris, Cunningham et al. 1992, Puchelle, Gaillard et al. 1992, Morris, Cunningham et al. 1994). Indeed, marked differences in CFTR expression levels were observed in different tissues during development (Tizzano, Chitayat et al. 1993, Broackes-Carter, Mouchel et al. 2002). Abnormal expression and diffuse intracellular distribution of wt-CFTR was observed in remodelled and dedifferentiated tissue, indicating that incomplete differentiation may cause retention of wt-CFTR in the endoplasmic reticulum (ER) (Dupuit, Kalin et al. 1995). Moreover, stimulation of epithelial differentiation was shown to increase the delivery of F508del-CFTR to post-ER compartments, namely to the Golgi (Bebok, Venglarik et al. 1998). Finally, in turn tissue-specific variation of F508del-CFTR PM expression patterns varies from null to normal, also indicating that differentiation may affect mutant CFTR localization pattern (Kalin, Claass et al. 1999, Penque, Mendes et al. 2000). In fact, differentiated cells seem to be more permissive to F508del-CFTR PM expression.

One relevant observation was that CK8/CK18 heterodimers, a phenotype of epithelial differentiation, may be an essential component for the proper targeting of CFTR to the apical PM (Stanke, Hedtfeld et al. 2011). Similar observations were reported for components of the actin cytoskeleton (Matos and Pinto 2019). On the other hand, it has been established that CFTR expression is heavily dependent on a variety of transcription factors and miRNAs (Viart, Bergougnoux et al. 2015). Therefore, altogether this evidence suggest that differentiation may modulate the levels of CFTR and its targeting to the PM.

However, not only differentiation seems to affect CFTR PM traffic, but also several studies indicate that apically localized, functional CFTR in turn seems to be required for efficient epithelial differentiation. CFTR has been described as modulating proliferation of lung secretory cells, epithelial differentiation, tight junction (TJ) assembly and secretory activity (Larson, Delcarpio et al. 2000, Bertrand and Frizzell 2003, Ruan, Wang et al. 2014).

Moreover, downregulation of CFTR appears to promote junctional abnormalities, EMT and increased cancer risk and poor tumour prognosis (Maisonneuve, Marshall et al. 2007, Maisonneuve, Marshall et al. 2013, Zhang, Jiang et al. 2013, Molina, Stauffer et al. 2015, Maisonneuve, Lowenfels et al. 2016, Hadjiliadis, Khoruts et al. 2018, Maisonneuve, Lowenfels et al. 2018).

In parallel, cells from individuals with CF have been described as having higher proliferation rates (Leigh, Kylander et al. 1995), but this difference may not occur on certain less differentiated cell types such as fibroblasts (Bolton and Barranco 1975). Comparing the regeneration in CF vs non-CF xenografts, a higher proliferation rate (rather than decreased apoptosis (Gallagher and Gottlieb 2001)) is indeed observed in the regenerating CF epithelium, but a delay in the differentiation was also reported (Hajj, Lesimple et al. 2007). Ultimately, recent wound healing studies indicate that this process is slower in CF monolayers (Trinh, Prive et al. 2008). This concept was further confirmed as CFTR inhibition caused a reduction in wound healing through a reduced migration rate (Schiller, Maniak et al. 2010). Consistently, migration rate in wound healing has also been reported to be higher in cells expressing functional CFTR vs those with dysfunctional channel (Marino and Kotsias 2014). Furthermore, rescue of mutant CFTR reverts the situation and increases wound healing rate (Trinh, Bardou et al. 2012). Interestingly, a dynamic pattern of CFTR expression during cutaneous wound healing was observed: while wt-CFTR expression levels decrease during proliferation and increase during differentiation, F508del-CFTR lacks this dynamically regulated expression (Dong, Jiang et al. 2015). A transcriptome profiling meta-analysis study also revealed an EMT signature in the airways of individuals with CF (Clarke, Sousa et al. 2013).

It is therefore clear that a complex relationship between CFTR and epithelial differentiation occurs, and we aim to further clarify this relationship with this work.

Thus, our aim here is to investigate how dysfunctional CFTR impacts on epithelial differentiation/regeneration and whether EMT plays a role in CF lung disease progression. Our findings clearly show that both differentiation and regeneration are impaired in CF cells and that EMT is present in native CF bronchial tissues and cells.

Materials and methods

Cells and tissues

Native Human lung tissues, parental human bronchial epithelial cells and CFBE cells were used in this part of the work as described in the materials and methods section of the thesis.

Chemicals, antibodies and primers

Lists of primary and secondary antibodies used in both IF and WB are in Tables S1 and S2, respectively. Sequences for the primers used in qRT-PCR are in Table S3.

Transepithelial electrical resistance measurements

Transepithelial electrical resistance (TEER) measurements were carried out as described in the materials and methods section of the thesis.

Immunofluorescence staining

The IF protocol used been previously described (Simões, Quaresma et al. 2019). For further details, check the materials and methods section of the thesis.

qRT-PCR

qRT-PCR was performed as previously (Simões, Quaresma et al. 2019) and as described in the materials and methods section of the thesis.

Western Blotting

Western Blots (WB) were performed as previously described (Amaral, Farinha et al. 2016). For further details, check the materials and methods section of the thesis.

Growth curves

Growth curves were performed as previously described in the materials and methods section of the thesis.

Proliferation assays

Proliferation assays were based on marker of proliferation Ki-67 staining and were performed as described in the materials and methods section of the thesis.

Wound healing

Wound healing assays were performed as previously described in the materials and methods section of the thesis.

Neurobiotin and Lucifer Yellow assays

Neurobiotin and lucifer yellow assays were performed as previously described in the materials and methods section of the thesis.

Statistical analysis

Data are always presented as mean ± SEM. Student's t-test for unpaired samples was used for statistical analyses. Prism 6 software (GraphPad, Inc., San Diego, CA) was used for graph design and statistical analyses. Significant differences were defined for $p \leq 0.05$ and marked with an asterisk. Other trends or tests may be stated in the legend. N=3 unless stated otherwise in the figure or in its legend. Only WB from the same blot are analysed and compared together.

Results

1 CF cells display increased proliferation

We undertook a series of experiments to evaluate cellular proliferation in the context of CF. Firstly, we evaluated growth rate of CFBE cells expressing either wt- or F508del-CFTR. Our data show that F508del-CFTR CFBE cells have a higher growth rate in comparison to wt-CFTR cells (Fig.1.1A, Table 1.1).

We then evaluated the levels of proliferation by assessing the expression of proliferation marker Ki-67, by IF. Our data indicate that non-polarized F508del-

CFBE cells display higher levels of proliferating cells as shown by increased number of cells staining positive for Ki-67 (Fig.1.1B, 1.1C).

Data on polarized CFBE cells also show a trend (albeit not significant) towards higher Ki-67 expression levels in F508del-CFTR vs. wt-CFTR cells as assessed by Western blot (WB) (Fig.S1.1A).

These observations were further studied by our group. IF was performed in native human bronchial tissue and show increased Ki-67 expression in CF tissue vs non-CF (Fig.S1.1B). The most striking results were, however, observed for pHBE cells growth rates (Fig.S1.1C), in which expression of any of the three mutant CFTR genotypes resulted in 3-fold higher cell proliferation rates in comparison to control cells.

Data by colleagues in the lab also assessed the role of CFTR in the context of wound healing. The results showed that functional CFTR-expressing cells closed the wounds 1.5-2 times faster than CF cells in both polarized CFBE (Fig.S1.1D) and fully differentiated pHBE cells (Fig.S1.1E). Thus, despite the fact that cells expressing mutant CFTR proliferated faster, they were significantly less capable of closing the wound and regenerating an intact epithelial cell layer. This was also confirmed by the low TEER values measured in CFBE cells after wound healing (Fig.S1.2B).

Table 1.1 - Average number of cells during cell growth.

Time (h)	wt-CFTR	F508del-CFTR
0	42.3	76.3
3	57.0	80.0
6	52.0	76.0
8	52.0	78.7
22	47.0	101.0
24	50.3	96.3
30	67.3	100.7

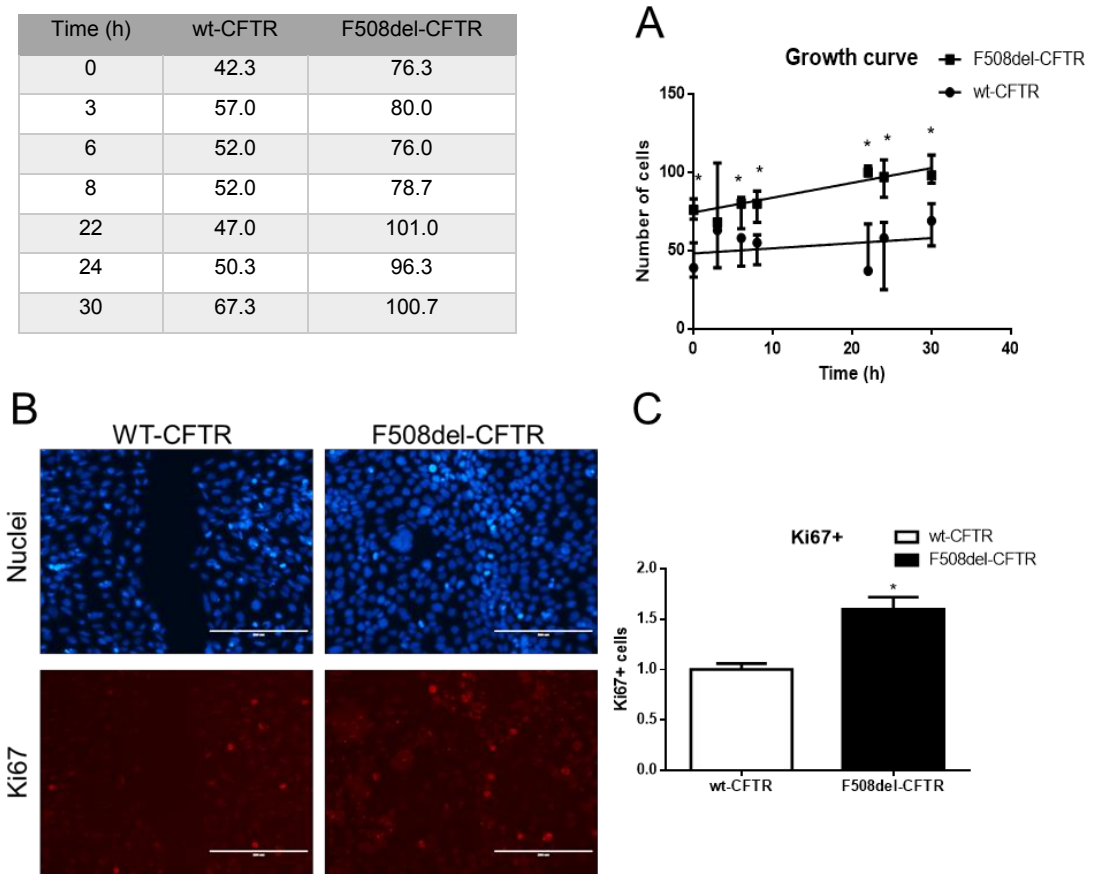

Figure 1.1 – CF cells display increased proliferation.
(A) *wt- and F508del-CFTR CFBE growth rate curves. (n=3, unpaired t-test, p<0.05).* ***(B)*** *Ki-67+ staining in proliferating cells after wounding. Results for wt- and F508del-CFTR CFBE are quantified in* ***(C)*** *(n=7, unpaired t-test, p<0.05).*

2 CF cells display reduced levels of TEER

Our first initial assessment of TEER in our cell models showed a striking difference between wt- and F508del-CFTR CFBE cells. Our results (Figure 1.2A Table 1.2) clearly indicate that F508del-CFTR cells reach much lower levels of TEER within the same time period.

These data were further confirmed by colleagues who evaluated the TEER levels on polarized primary pHBE cells and in CFBEs in the context of wound healing. Those data (Fig.S1.2A) indicate that CF pHBE cells also display lower levels of TEER after 21d of polarization when compared to non-CF cells. In the context of wound healing (Fig.S1.2B), it was also observed that wt-CFTR CFBE cells recovered TEER values much faster after wounding, being their resistance almost back to normal 48h after wounding. In contrast, F508del-CFTR cells still

had very low resistance 48h after wounding and did not fully recover until 96h after wounding.

Another technique that we applied was the injection of neurobiotin dye to assess gap junction (GJ) connectivity through dye diffusion. Results in Fig.1.2B show that wt-CFTR CFBE cells have significantly higher gap junction connectivity as measured by neurobiotin. No differences were found using Lucifer Yellow (data not shown).

Table 1.2 - Average TEER (ohm.cm^2) over time.

Time (d)	wt-CFTR	F508del-CFTR
3	1876.3	527.0
4	2001.3	583.0
5	2088.0	528.0
6	2423.3	542.7
7	2700.7	796.0

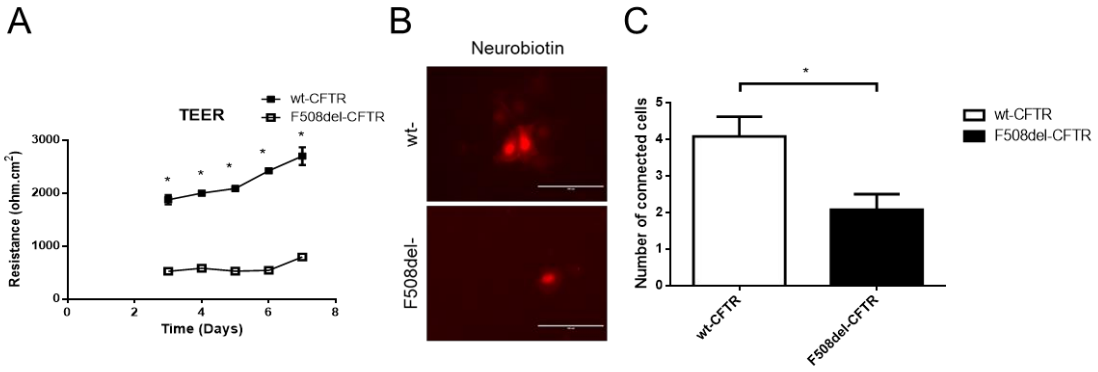

***Figure 1.2 - CF cells have lower TEER and gap junction connectivity vs non-CF cells**. **(A)** TEER measurements of wt- and F505del-CFTR expressing CFBE cells over time. Data are represented as resistance (expressed in ohm.cm^2) over 7 days, (n=3). **(B)** Neurobiotin microinjection assays in wt- vs F508del-CFTR CFBE cells. **(C)** Quantification of data from B (n=6, unpaired t-test, p<0.05) bar = 100 µm.*

3 CF cells display a partial EMT signature

Next, we evaluated the levels of several differentiation markers using IF and WB techniques.

Initially we used non-polarized cells. We started by assessing the levels of E-Cadherin (E-Cad) and N-Cadherin (N-Cad) in both wt- vs F508del-CFTR using WB, as their ratio is usually used as a measure of EMT. No major differences were found, although a trend towards higher E-Cad/N-Cad ratio was observed in F508del- vs wt-CFTR expressing cells (Fig.1.3A), consistent with partial EMT in

mutant cells. Our WB data also indicate that F508del-CFTR cells have higher levels of cytokeratin (CK) 18 (differentiated) and of CK5 (basal) epithelial cell markers. In parallel, Zonula occludens-1 (ZO-1) and Claudin 1 (CLDN1), both TJ markers, seem to be up and downregulated in CF cells, respectively (Fig.1.3B). Some other markers that were tested and showed no major alterations are shown in Fig.S1.3.

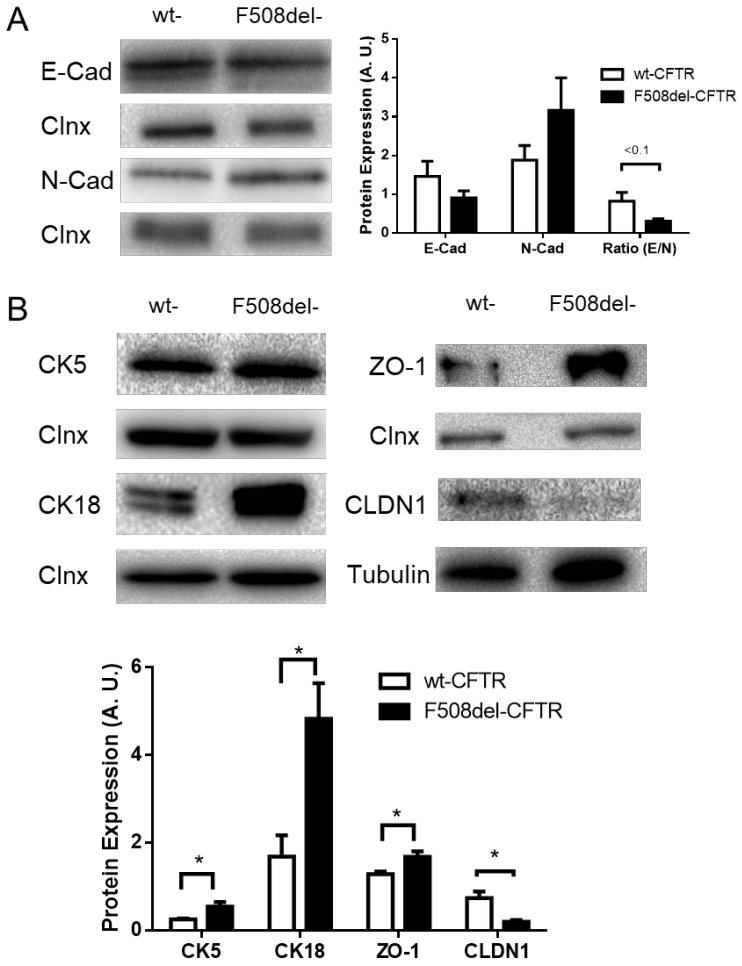

Figure 1.3 - Expression levels of differentiation markers in non-polarized CFBE cells.
(A) *Representative WB showing E-cad and N-Cad protein levels in non-polarized wt- and F508del-CFTR CFBE cells. Calnexin was used as loading control. Quantification by densitometry of the protein expression detected by WB. Data are normalized to loading control and shown as arbitrary units (A.U.) (n=3, unpaired t-test, p<0.10).* ***(B)*** *Representative WB showing CK5, ZO-1, CK18 and CLDN1 levels in non-polarized wt- and F508del-CFTR CFBE cells. Calnexin or ß-Tubulin were used as loading controls. Quantification by densitometry of the protein expression detected by WB. Data are normalized to loading controls and shown as arbitrary units (A.U.) (n=3, unpaired t-test, p-value < 0.5).*

By IF, N-Cad localization is observed to be altered in F508del-CFTR as the cells lose its nuclear expression as it becomes restricted to the PM (Fig.1.4A). Noticeably, CK18 pattern also appears to be overexpressed and disrupted in CF cells as it is expressed throughout the cytoplasm (Fig.1.4B). ZO-1 staining appears more intense in F508del-CFTR cells (Fig.1.4C). No major differences in

the expression patterns of E-Cad, β-Catenin or Vimentin were observed by IF (Fig.1.4D-F).

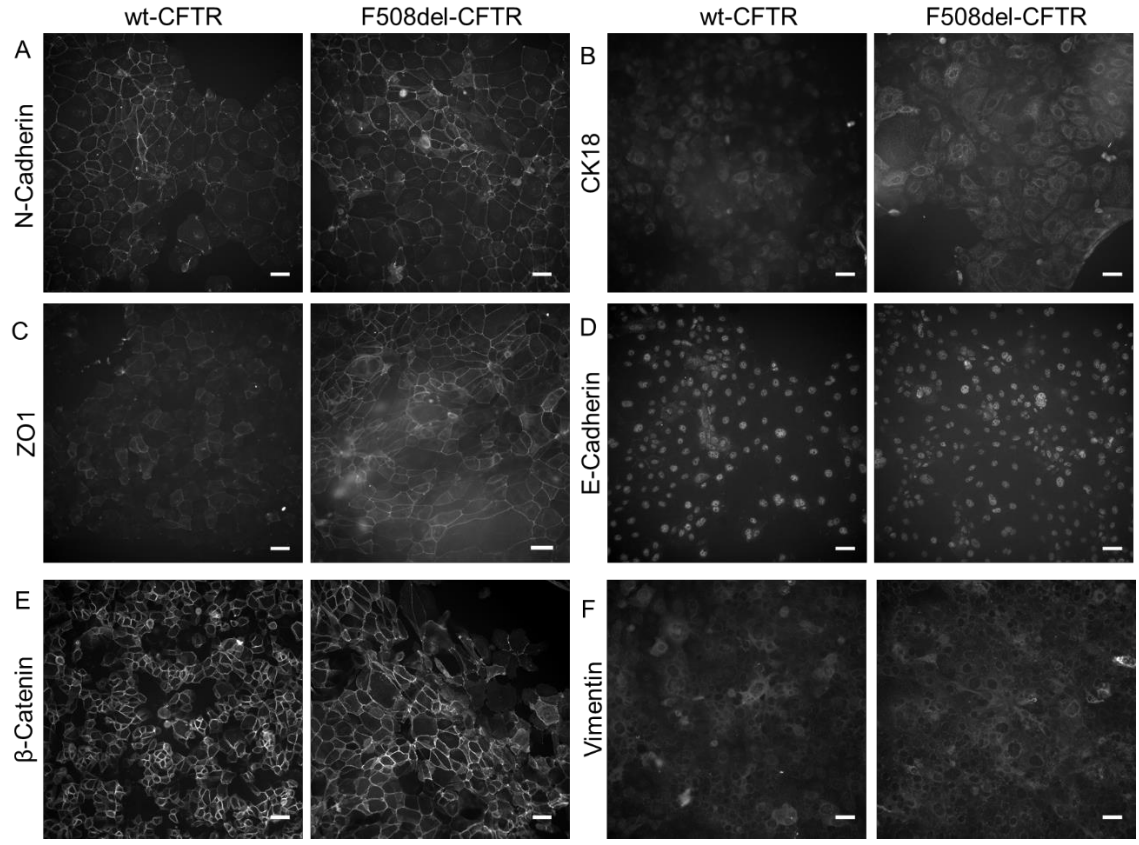

Figure 1.4 – Altered expression of epithelial/mesenchymal markers on non-polarized CFBE cells. *IF was performed on wt- and F508del-CFTR CFBE cells for N-Cad **(A)**, CK18 **(B)** and ZO-1 **(C)**. No major alterations were found in the expression levels/patterns of E-Cad **(D)**, β-catenin **(E)** and Vimentin **(F)**. Bar=50μm. n=2-4.*

In order to further investigate whether EMT is occurring on CF cells, we decided to do a more thorough characterization of differentiation markers in these cells and to this end, we used polarized cells that are more suitable to study differentiation processes.

Regarding protein levels as assessed by WB (Fig.1.5), major differences occur for Desmoplakin I/II (DSPI/II) and CLDN1, which are increased by 3- and 15-fold respectively in wt-CFTR cells. On the other hand, ZO-1 and Connexin 31 (Cx31) seem to be upregulated on F508del-CFTR, which is not expected in the context of a classic (or 'full') EMT. Mesenchymal markers, namely N-cad, Vimentin and collagen I are significantly increased in F508del-CFTR cells. Other mesenchymal and epithelial markers were assessed but showed no statistically significant differences between wt- and F508del-CFTR cells (Fig.S1.4).

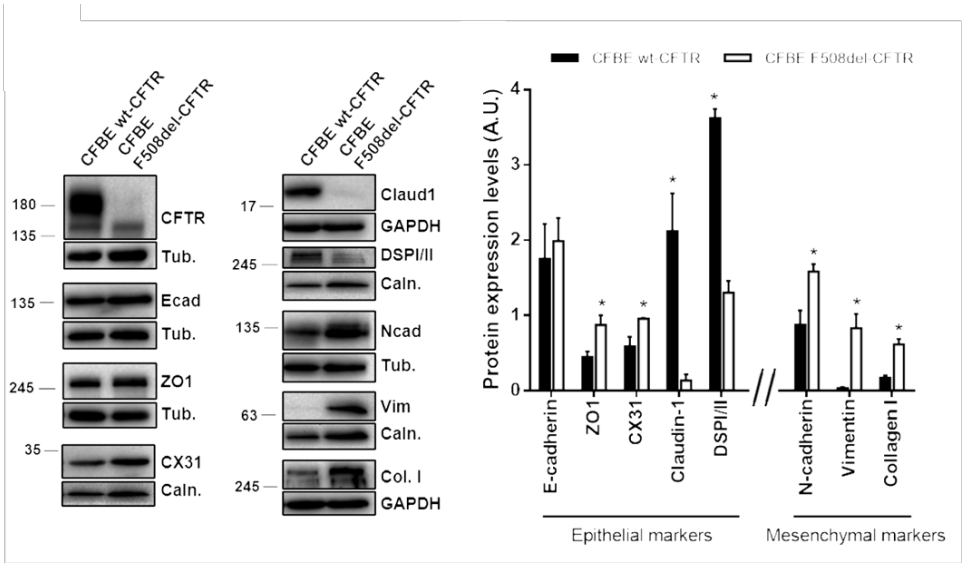

Figure 1.5 - Expression of differentiation markers in polarized CFBE cells.
Left: Representative WBs showing E-Cad, ZO-1, Cx31, CLDN1, DSPI/II, N-Cad, Vimentin (Vim) and Collagen I levels in polarized wt- and F508del-CFTR CFBE cells. Calnexin, GAPDH or ß-Tubulin were used as loading controls. Right: Quantification by densitometry of the WBs in left. Data are normalized to loading controls and shown as arbitrary units (A.U.) (n=3, unpaired t-test, p-value < 0.5). [WBs for CFTR, E-Cad, ZO-1, CLDN1, N-Cad, Vimentin and Collagen I in this image were performed by Margarida Quaresma and are included here with permission].

By confocal microscopy, colleagues in the lab also show that, as previously reported (Carvalho-Oliveira, Efthymiadou et al. 2004), CFTR levels are much lower in F508del-CFTR and CFTR localization is also aberrant (intracellular) compared to wt-CFTR (apical PM). Moreover, we also found differences in the general architecture of the cells, with wt-CFTR CFBE cells forming a single cell monolayer, unlike F508del-CFTR cells which tend to grow in a more disorganized pattern (Fig.S1.5A)

IF data (Fig.S1.5A) also indicate that there is a general disorganization of some epithelial markers in F508del-CFTR cells. Namely, E-cad localization seems to be less confined to the adherens junctions (AJs), collagen I is present in the ECM at higher amounts and N-Cad is expressed at higher levels and with a more diffuse distribution throughout the cell.

Further IF studies (Fig.S1.5B) performed by colleagues in the lab revealed differences in the localization of EMT markers. Indeed, ZO-1, E-cad and β-Catenin are increased in cytoplasm of F508del-CFTR compared to wt-CFTR, where they localize correctly at the PM. On the other hand, CK18, fibronectin (FN), CLDN1 and Cx43, also reveal some degree of disorganization, whereas in

wt-CFTR cells they retain their normal localization at the cytoskeleton, TJs and GJs, respectively.

These data were further validated by stainings of human lung tissue and primary human bronchial epithelial (pHBE) cells by colleagues on the lab. Briefly, on lung tissue mRNA levels show overexpression of the mesenchymal markers vimentin and collagen 1 coupled with overexpression of CK18, ZO-1, Occludin, Cx43 and Cx26 in F508del homozygous lung tissue (Fig.S1.6A). IF data on lung also showed altered epithelial morphology in CF, with marked disruption of the pseudostratified epithelium with differences found in the expression patterns of β-catenin, ZO-1, E-Cad, N-cad, FN, Vimentin and collagen 1 (Fig.S1.6B). our group also found increased levels of N-Cad and Vimentin in CF pHBE cells (Fig.S1.7).

To further assess the process of EMT in CF and to elucidate which pathways may be involved, we have assessed the expression of the main EMT associated transcription factors (EMTa-TF) SNAIL1, SNAIL2, TWIST1, ZEB1 and ZEB2. The data generated indicated that a significant upregulation of TWIST1 and ZEB1 mRNAs in native CF lung tissue vs non-CF lung tissue (Fig.S1.8). TWIST1 was also found to be significantly increased in non-polarized F508del-CFTR CFBE cells, unlike TEAD4 and SNAIL+SLUG (Fig.1.6), and in polarized CFBE cells (Fig.S1.9). Moreover, such a trend was also found in pHBE cells (Fig.S1.9).

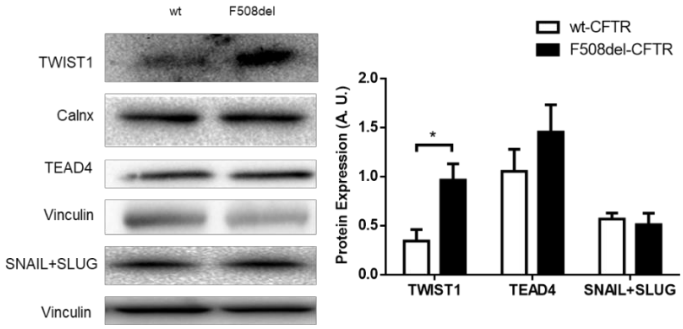

Figure 1.6 - TWIST1 is overexpressed in non-polarized F508del-CFTR CFBE cells.
Representative WB showing TWIST1, TEAD4 and SNAIL+SLUG levels in non-polarized wt- and F508del-CFTR CFBE cells. Calnexin and Vinculin were used as loading control. Quantification by densitometry of the protein expression detected by WB. Data are normalized to loading control and shown as arbitrary units (A.U.) (n=3, unpaired t-test, p-value < 0.5).

Discussion

The main goal of this study was to determine whether defective CFTR is linked to impaired epithelial cell differentiation and whether EMT program is active in the CF airways. By combining studies in native human lung tissue, primary human bronchial epithelial (pHBE) cells and immortalized bronchial epithelial cell lines, we found evidence of abnormal epithelial-mesenchymal plasticity in CF tissues and cells expressing diverse mutant forms of CFTR. These included mislocalization of cell junction proteins, disruption of the epithelial architecture, and aberrant expression of both mesenchymal markers and EMT-associated TFs.

Our data (Fig.1.1 and Fig.S1.1) show that CF cells display increased proliferation and reach lower levels of epithelial differentiation resulting in the presence of a leaky epithelia (Figs.1.2, S1.2) as shown by lower TEER values. In fact, a higher proliferation rate which is often associated with lower levels of differentiation (Ruijtenberg and van den Heuvel 2016). These data are in accordance with previous reports that associate CF cells with higher proliferation rates (Leigh, Kylander et al. 1995) and impaired differentiation (Hajj, Lesimple et al. 2007).

Moreover, it is worth noticing that the increased proliferation is not coupled to higher wound healing ability. In contrast, despite their intrinsic higher proliferation, CF cells take longer to recover upon wounding (Figs.S 1.1, S1.2), which we attribute to impaired migration, as previously reported (Hajj, Lesimple et al. 2007, Trinh, Prive et al. 2008, Schiller, Maniak et al. 2010, Trinh, Bardou et al. 2012).

We then characterized the levels of several differentiation markers in our cell models (Figs.1.3, S1.3). In a first approach we used non-polarized cells and we found that F508del-CFTR CFBE cells display increased levels of CK5 and decreased levels of CLDN1. As a marker of basal cells (Gusterson, Ross et al. 2005) higher expression of CK5 is therefore indicative that CF cells may have a more mesenchymal phenotype. In contrast, as an epithelial differentiation marker that is a constituent of TJs (Gudjonsson, Villadsen et al. 2002), downregulation of CLDN1 in CF cells indicates a more mesenchymal phenotype. On the other hand, we also observed the upregulation of some epithelial markers like CK18

and ZO-1. CK18 has been previously reported to be overexpressed in CF cells and it has been functionally associated with the delivery of mutant CFTR (Davezac, Tondelier et al. 2004, Duan, Sun et al. 2012). Although unexpected we also observed higher levels of ZO-1 expression in CF cells. ZO-1 has been previously shown to interact with CFTR in order to exert functions that promote TJ assembly and epithelial differentiation via the ZONAB pathway (Ruan, Wang et al. 2014). Since the CFBE cells that we used here rely on the overexpression of F508del-CFTR, we speculate that this may have a direct impact in the levels of ZO-1 or on its activity. We also report that CF cells display a modest increase in N-Cad and a slight decrease in the E-Cad expression. This trend is better illustrated by a slight decrease in the E-Cad/N-Cad ratio in CF cells (Loh, Chai et al. 2019), thus showing that CF cells display a slightly more mesenchymal-like phenotype, i.e., partial EMT (Li, Zhang et al. 2015). No alterations were observed in the levels of β-catenin, DSPI/II, Vimentin, Occludin, CK13, CK14 or FN using this methodological approach.

We then evaluated if some differences in terms of protein localization could be detected in CF cells. Our data (Fig.1.4) show some alterations in the expression patterns of N-Cad (loss of nuclear expression), CK18 (higher and more diffuse expression throughout the cell) and ZO-1 (higher expression) in CF cells. No major alterations in the expression patterns of E-Cad, β-catenin or Vimentin were observed. Once again, our data show some degree of alteration in the expression patterns of differentiation markers in CF, namely C18, N-Cad and ZO-1.

To further expand our work in this area, we then used polarized CFBE cells as they are more physiologically relevant to study differentiation and intracellular organization (Varga, Jurkuvenaite et al. 2004, Mendes, Wakefield et al. 2005, Berube, Prytherch et al. 2010, Monterisi, Favia et al. 2012).

Our WB data in polarized cells (Figs.1.5, S1.4) showed upregulation of several mesenchymal markers (N-Cad, Vimentin, Collagen I) coupled with the downregulation of some epithelial markers (CLDN1, DSPI/II) in F508del-CFTR cells. Taken together, again these data point towards a more mesenchymal phenotype. Nevertheless, we have observed upregulation of epithelial markers like Cx31 and ZO-1, compounds of GPs and TJs, respectively. However, since Cx31 has been associated to the TJs of basal cells, we speculate that this may

be the reason why we observe Cx31 upregulation in CF cells (Foglia, Scerri et al. 2009). Other epithelial and mesenchymal markers tested (E-cad, Occludin, β-catenin, Cx26, Cx43, CK18, α-SMA and FN) showed no marked alteration in CF cells. Combining these data with IF data obtained by colleagues in the lab for polarized CFBE cells (Fig.S1.5), qPCR and IF in lung tissue (Fig.S1.6) and WB data in pHBE (Fig.S1.7), we can put together an overall pattern of differences across the different cell models. While mesenchymal markers, including vimentin and N-cad, were consistently upregulated in CF tissues and cells, E-cad and most of the epithelial genes encoding components of cell junctions were not repressed, with the exceptions of CLDN1 (a TJ component) and DSPI/II (a desmosome component of the AJ). In fact, some of the epithelial markers, including ZO-1 and CK18, were upregulated in CF cells/tissues, albeit evidencing abnormal subcellular localization or organization. This is consistent with multiple reports showing that under pathological conditions, as in cancer, fibrosis and other chronic lung diseases, cells undergo partial rather than complete EMT (Grande, Sánchez-Laorden et al. 2015, Jolly, Ward et al. 2018). Indeed, increasing evidence indicates that pathological EMT is not a binary process but rather a spectrum of states, during which cells express both epithelial and mesenchymal phenotypes simultaneously (Saitoh 2018). Complete transition from an epithelial to a mesenchymal phenotype may only be applicable to developmental EMT (Dongre and Weinberg 2019). A possible explanation for the lack of downregulation of epithelial markers is the possibility that aberrant positive feedback loops may be activated trying to compensate for the partial EMT, leading to more protein even if dysfunctional or mislocalized, a feature evident in our IF experiments (Figs.1.4, S1.5, S1.6). For instance, there are studies reporting ZO-1 mislocalization and/or loss of function in the absence of CFTR with no impact in the ZO-1 expression levels (Castellani, Guerra et al. 2012). Therefore, we conclude that CF cells display an overall more cancer-like phenotype.

Our data centred in the EMTa-TFs (Figs.9, S8, S9) also give some insight in the way by which this partial EMT occurs. TWIST1 is the most consistently increased EMTa-TF in CF tissues/cells, among those we tested. On the other hand, other TFs like SNAI1 and ZEB1 are less consistently overexpressed. It is important to note that TWIST1 is a more potent mesenchymal inducer than

epithelial repressor, whereas SNAI1 and ZEB1 are strong epithelial repressors and weaker mesenchymal promoters (Nieto, Huang et al. 2016), which is line with our observation of mesenchymal markers upregulation without substantial repression of epithelial markers.

Unpublished data from our lab have shown that CF cells are more sensitive to TGFβ1-induced EMT. This indicates that CFTR may play a role in preserving cell junction integrity and protection from TGFβ1-induced EMT. Those data also showed that partial rescue of F508del-CFTR also promotes partial correction of the EMT. These data may provide the functional link between CFTR and the observed partial EMT in CF.

Altogether, our data suggest that mutant CFTR triggers EMT possibly through TWIST1 upregulation. In parallel to EMT occurrence, absence of functional PM CFTR could also lead to activation of signalling pathways which would normally be active in undifferentiated cells, like cell proliferation. Hyperproliferation and impaired wound healing were here confirmed as part of CF pathology, but we show for the first time that they occur in conjunction with EMT.

Therefore, we envisage a “two-hit” effect hypothesis. Firstly, CFTR mutation promotes partial EMT via upregulation of TWIST1. Secondly, the partial EMT leads to less resistance of the tissues to EMT-inducing factors such as TGFβ1.

With the extension of life expectancy in CF (Dodge, Lewis et al. 2007), previously underlying aspects of the disease, such as fibrosis and high cancer prevalence, are likely to emerge. Targeting EMT might be a novel therapeutic approach to preserve epithelial integrity and protect against developing cancer and epithelial dysfunction. On the other hand, this work also sheds some light on the role of CFTR as a protector of EMT and tumor suppressor, which can lead to novel avenues in the treatment/prevention of carcinogenesis.

Supplementary Data

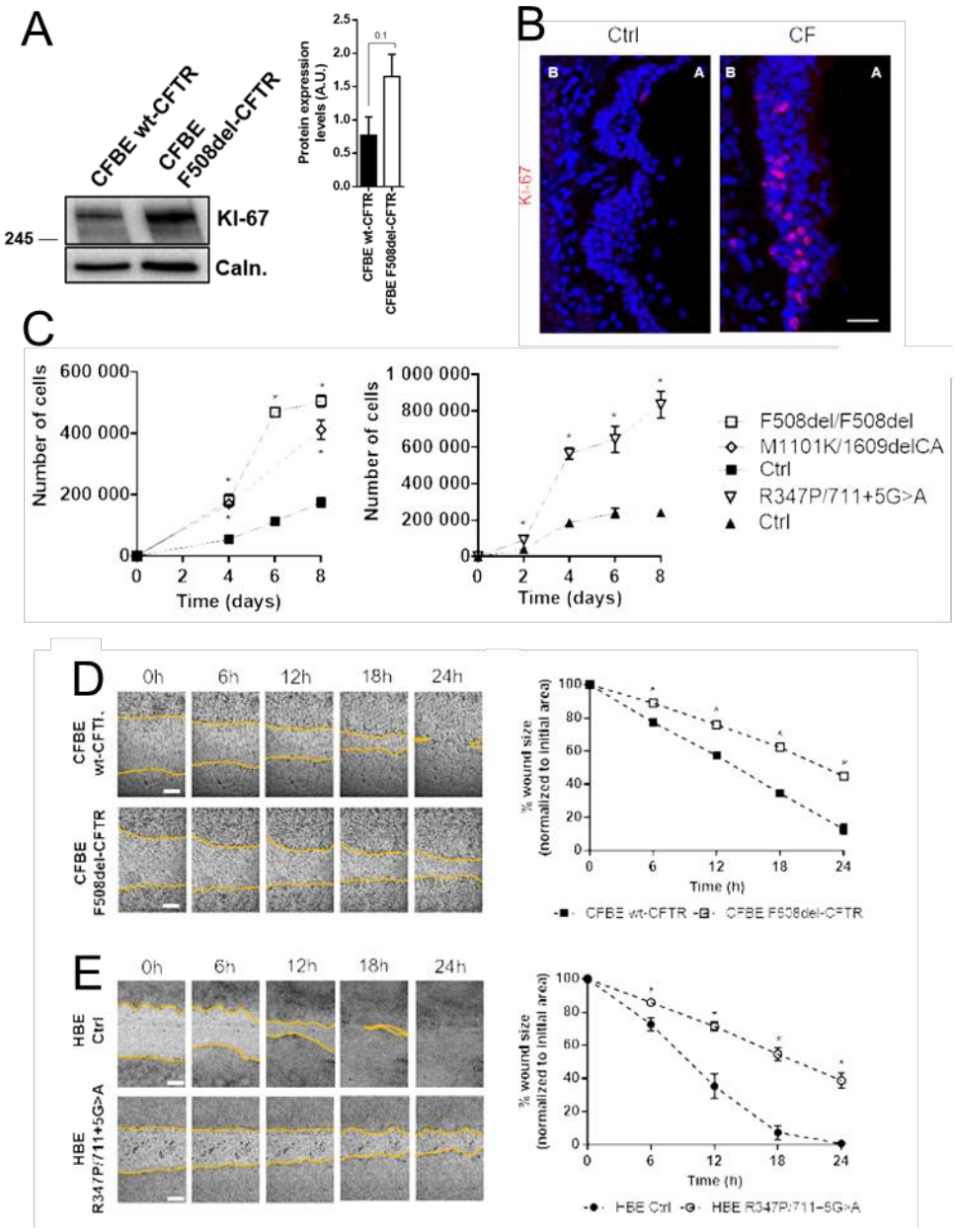

Figure S1.1 – Proliferation is upregulated in CF but wound healing is slower.
(A) *Representative WB showing Ki-67 protein levels in polarized wt- and F508del-CFTR CFBE cells. Calnexin was used as loading control. Quantification by densitometry of the protein expression detected by WB. Data are normalized to loading control and shown as arbitrary units (A.U.) (n=3, unpaired t-test, p-value < 0.5).* ***(B)*** *IF was performed in native human lung and CF lungs for Ki-67 signal (red), scale bar = 25µm. A – apical, B – basal. Images are shown as maximal image projections. The CF lung in this figure had a R347P/711+5G>A genotype, but similar findings were obtained in lungs with a F508del/F508del genotype. (n=3).* ***(C)*** *Growth curve on non-polarized pHBE cells. (genotypes F508del/F508del, R347P/711+5G>A and M1101K/1609delCA). The two different graphs represent experiments performed independently with different controls. Data are shown as cell number over 8d (n=3, unpaired t-test, p<0.05).* ***(D and E)*** *Wound healing assays were performed in polarized CFBE (D) and polarized pHBE (E). Data are represented as a percentage of the initial area of the wound. (n=5, unpaired t-test, p<0.05). [WB, growth curves and IF in this image were performed by Margarida Quaresma and wound healing assays were performed by Ines Pankonien and are reproduced here with permission].*

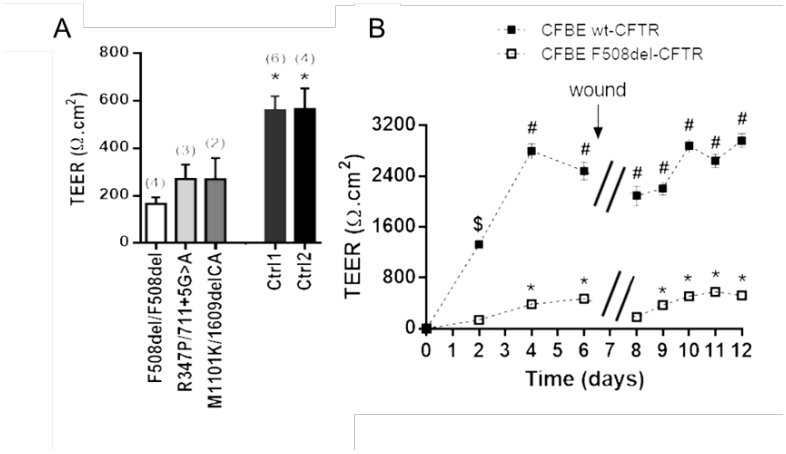

Figure S1.2 – CF cells display lower TEER in pHBE and in CFBE during wound healing.
(A) *TEER measurements of CF and controls pHBE cells after 21d in ALI. CF individuals' genotypes were F508del/F508del, R347P/711+5G>A and M1101K/1609delCA. (n used is present on each bar, * p<0.05, unpaired t-test)* ***(B)*** *TEER measurements of the polarized CFBEs before and after wound. Wound was performed on day 6 after seeding, as indicated. Data are represented as Ω.cm² over 12d. * indicates significant when compared to day 2 of same cell line, $ indicates significant when compared to F508del-CFTR, # indicates significant when compared to day 2 and F508del-CFTR (n=5-7, p<0.01, unpaired t-test). [Data were performed by Margarida Quaresma and are reproduced here with permission].*

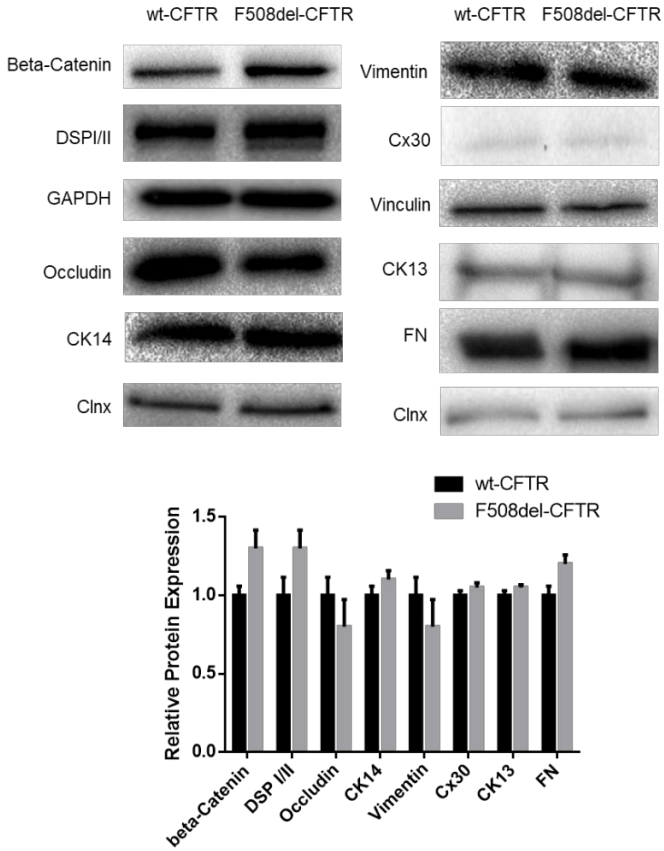

Figure S1.3 - Epithelial and differentiation markers unaltered in non-polarized CF cells.
Representative WB are shown for Vimentin, ϐ-catenin, Occludin, DSPI/II, CK14, CK13, FN1, using calnexin and GAPDH as loading controls.

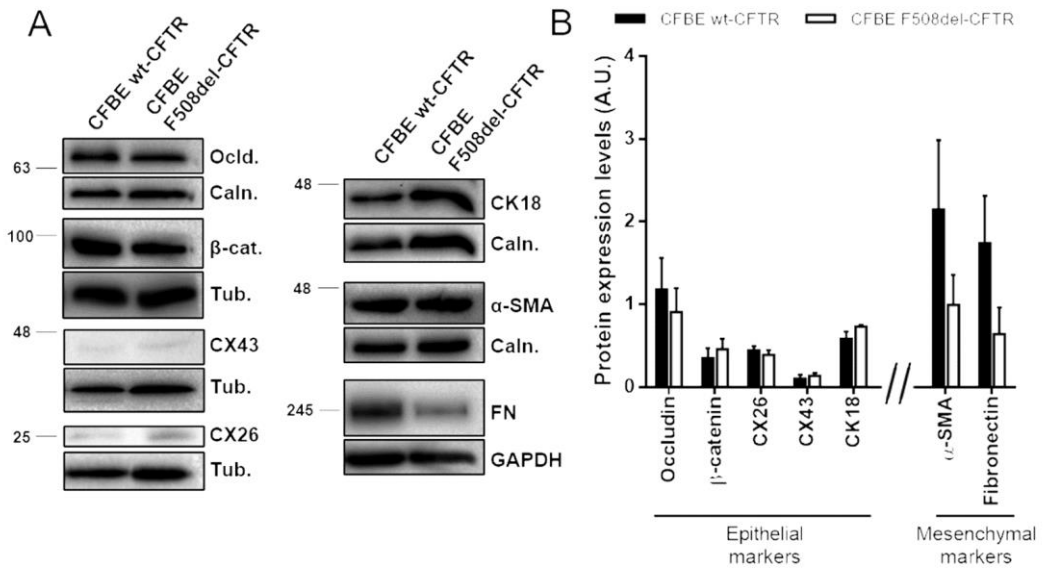

Figure S1.4 - Epithelial and differentiation markers unaltered in polarized CF cells.
(A) *Representative WBs showing several epithelial (occludin, β-catenin, CX26, CX43 and CK18) and mesenchymal (αSMA and fibronectin) protein levels. Calnexin, ß-tubulin and GAPDH were used as loading controls.* ***(B)*** *Quantification by densitometry of the proteins detected in (A). Data are normalized to loading control and shown as arbitrary units (A.U.) (n=3) [WBs for Occludin, β-catenin, CK18, α-SMA and FN were performed by Margarida Quaresma and are reproduced here with permission].*

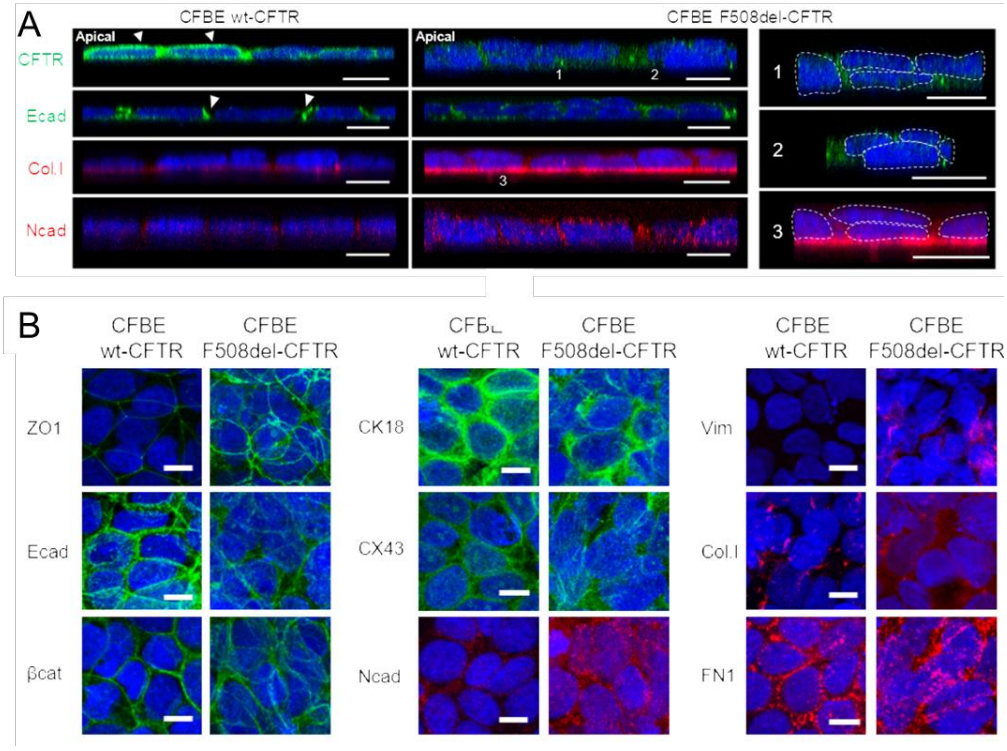

Figure S1.5 - Altered patterns of expression of several epithelial and mesenchymal markers in polarized CFBE cells.
(A) *Z-stack of a representative group of cells showing the different localization of CFTR, E-cad, collagen I and N-cad in polarized wt- and F508del-CFTR cells (xz/yz axis). Epithelial markers display the correct localization in wt-CFTR cells (arrowheads) but not F508del-CFTR cells. F508del-CFTR cells also display abolishment of a single cell monolayer with cells accumulating on top of each other (right panel, 1, 2, 3). The apical side of the epithelia is identified, and it is the same in all pictures. (scale bar 10μm, n=3)* ***(B)*** *Subcellular*

localization of epithelial (ZO-1, E-cad, β-catenin, CX43) and mesenchymal (N-cad, vimentin (Vim), collagen I and fibronectin) markers in polarized CFBE cells by IF. Nuclei are depicted in blue and immunostained proteins in green (epithelial markers) or red (mesenchymal markers). (Scale bar = 10μm, n=3). [IFs were performed by Margarida Quaresma and are reproduced here with permission].

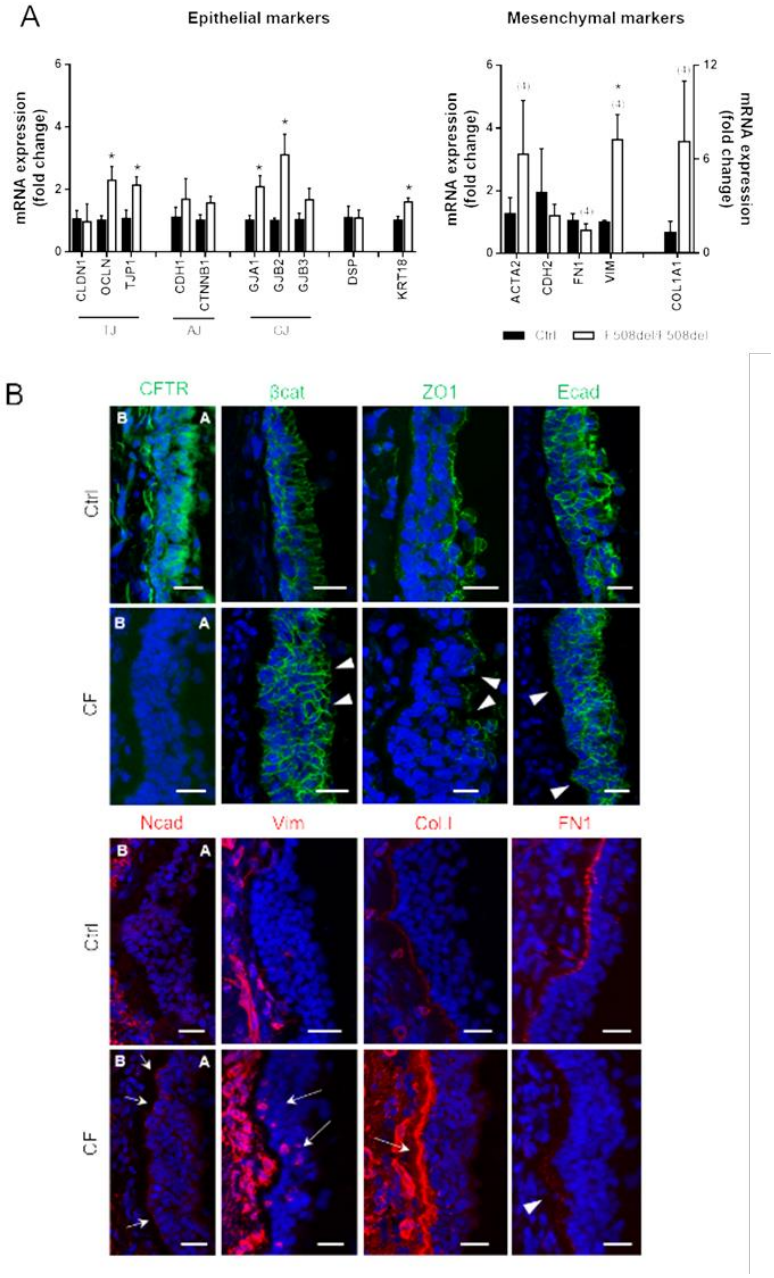

Figure S1.6 - Altered epithelial and differentiation markers in lung tissue.
F508del/F508del individuals show upregulation of several epithelial and mesenchymal genes. ***(A)*** *qRT-PCR was performed on different control and F508del homozygous lung samples. Fold-change in mRNA expression levels was assessed for different epithelial and mesenchymal markers. These comprised markers of several cell junctions as well as the cytoskeleton and the ECM. Fold-change values were calculated by applying the ΔΔCT method (n=3, unless indicated by (4), unpaired t-test, p<0.05).* ***(B)*** *Representative images of native human bronchial tissue (Ctrl and CF) immunostained for epithelial (CFTR, β-catenin, ZO-1, E-cad) and mesenchymal (N-cad, Vimentin (Vim), Collagen I, FN) markers. Nuclei are depicted in blue and proteins in green (epithelial) or red (mesenchymal). Scale bar represents 25μm. Disrupted or abnormal epithelial markers and FN1 (arrowheads) and increased mesenchymal markers (arrows) are observable in CF tissue when compared to controls. Apical and basal (A and B respectively) sides of the epithelia are identified and are the same in all pictures. CFTR, ZO-1, Vim, Collagen I and FN1 are displayed as maximum image projections (MIPs) and β-catenin, E-Cad and N-Cad as individual z-stacks.*

The CF individual in this figure had a R347P/711+5G>A genotype, but similar findings were obtained for the F508del/F508del genotype, demonstrating that the phenotype was not restricted to any genotype. Several controls were also assessed with similar results between themselves. (n=2-3 samples). [IFs in this image were performed by Margarida Quaresma and qRT-PCR were performed by Luka Clarke and are reproduced here with permission].

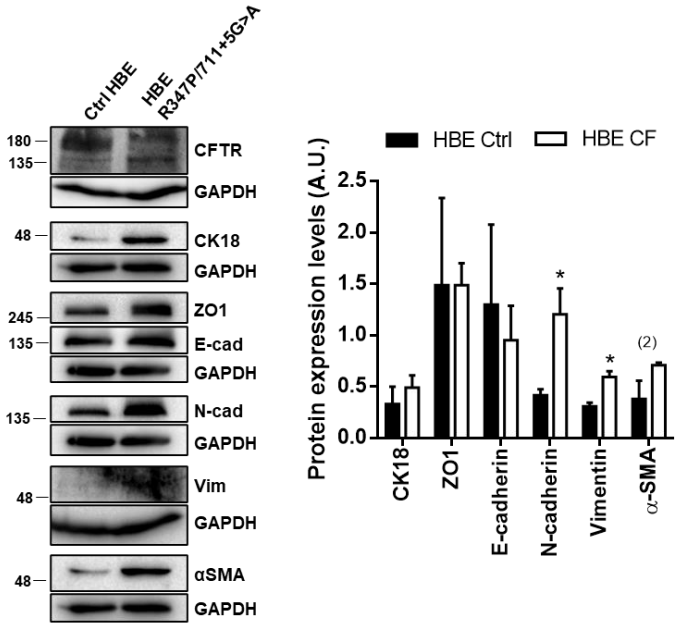

Figure S1.7 - Altered expression of differentiation markers in pHBE cells.
(A) WBs showing epithelial (CFTR, CK18, ZO-1, E-cad) and mesenchymal (N-cad, vimentin (Vim), αSMA) protein levels in fully differentiated Ctrl and CF (R347P/711+5G>A) pHBE cells (21d of differentiation). In the R347P/711+5G>A pHBE cells, a faint band C can still be seen for CFTR (arrowhead). GAPDH was used as a loading control. (B) Quantification by densitometry of the protein expression detected by WB in (A). Data are normalized to loading control and shown as arbitrary units (A.U.) (n=3 unless stated otherwise, unpaired t-test, p<0.05). The CF individuals' genotypes were F508del/F508del, R347P/711+5G>A and M1101K/1609delCA. [WB were performed by Margarida Quaresma and are reproduced here with permission].

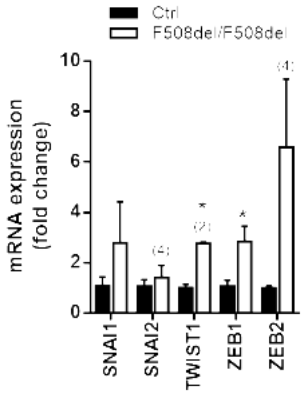

Figure S1.8 - mRNA expression levels of EMT-associated TFs in lung.
Transcript analysis of EMT-TFs levels by qRT-PCR on different control and F508del homozygous lung samples. Fold-change values were calculated by applying the $^{\Delta\Delta}CT$ method and are represented by mean

± SEM relative to the control samples (n=3, unless indicated by (2) or (4), unpaired t-test, p<0.05). [Data were obtained by Luka Clarke and Margarida Quaresma and are reproduced here with permission].

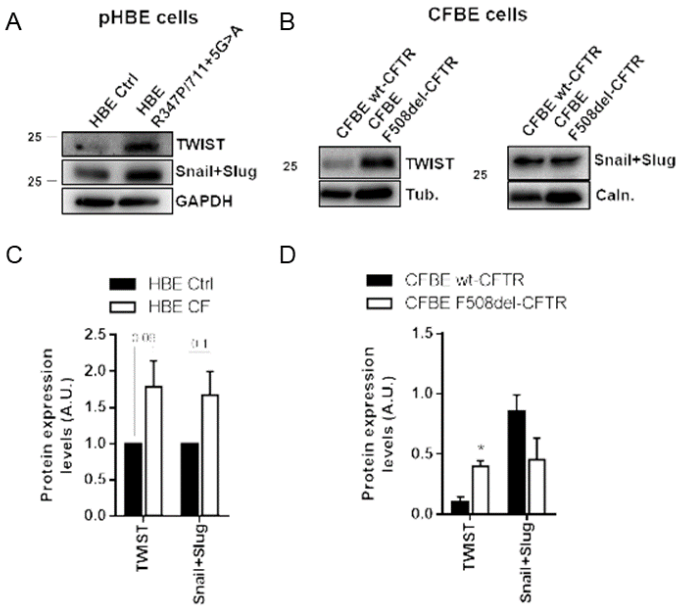

Figure S1.9 - Protein levels of EMTa-TFs in polarized pHBE and CFBE.
(A, B) *WBs showing Snail+Slug and TWIST levels on (A) fully differentiated pHBE cells and (B) polarized CFBE cells. Calnexin, tubulin or GAPDH were used as loading controls.* ***(C, D)*** *Quantification by densitometry of the protein expression detected by WB in (A) and (B), respectively. (C) Data are normalized to loading control and to Ctrl cells and shown as arbitrary units (A.U.). The CF individuals' genotypes were F508del/F508del, R347P/711+5G>A and M1101K/1609delCA. (n=3). (D) Data are normalized to loading control and shown as arbitrary units (A.U.) (n=3, unpaired t-test, p<0.05). [Data was obtained by Margarida Quaresma and are reproduced here with permission].*

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Chapter II – KLF4 acts as a wt-CFTR Suppressor Through an AKT-Mediated Pathway

Some of the data present in this chapter was included in the following work:

Sousa L, Pankonien I, Clarke LA, Silva I, Kunzelmann K, Amaral MD. KLF4 Acts as a wt-CFTR Suppressor through an AKT-Mediated Pathway. *Cells*. 2020;9(7):E1607. Published 2020 Jul 2. doi:10.3390/cells9071607 – published.

Abstract

Cystic Fibrosis (CF) is caused by >2,000 mutations in the CFTR gene, but one mutation - F508del - occurs in ~80% of patients worldwide. Besides its main function as an anion channel, CFTR protein has been implicated in epithelial differentiation, tissue regeneration and, when dysfunctional, cancer. However, the mechanisms that regulate such relationships are not fully elucidated.

Krüppel like factors (KLFs) are a family of transcription factors (TFs) playing central roles in development, stem cell differentiation, and proliferation. Herein, we hypothesized that these TFs might have an impact on CFTR expression and function, being its missing link to differentiation. Our results indicate that KLF4 (but not KLF2 nor KLF5) is upregulated in CF vs non-CF cells and that it negatively regulates wt-CFTR expression and function. Of note, F508del-CFTR expressing cells are insensitive to KLF4 modulation. Next, we investigated which KLF4-related pathways influence CFTR. Our data also show that KLF4 modulates wt-CFTR (but not F508del-CFTR) via both AKT and GSK3β signalling. While AKT acts positively, GSK3β is a negative regulator of CFTR.

This crosstalk between wt-CFTR and KLF4 via AKT/GSK3β signalling, which is disrupted in CF, constitutes a novel mechanism linking CFTR to the epithelial differentiation.

Introduction

Cystic Fibrosis (CF) is the most common lethal genetic disease among Caucasians, with a variable geographic prevalence of 1:2500-6000 in Europe according to the European Cystic Fibrosis Society registry (Zolin, Orenti et al. 2017). Over 2,000 mutations in the gene encoding the CF Transmembrane Conductance Regulator (CFTR) protein have so far been reported, but the deletion of the phenylalanine at the position 508 (F508del) is by far the most common one, present in at least one allele in ~80% of individuals with CF worldwide. The F508del mutation impairs CFTR protein folding and plasma membrane (PM) trafficking, causing CFTR retention at the level of the endoplasmic reticulum (ER), with only a minimal fraction reaching the PM (Farinha and Amaral 2005) with decreased function and stability. The association

of CFTR to epithelial differentiation has been described in several studies (reviewed recently in (Amaral, Quaresma et al. 2020)).

Being CFTR a chloride/bicarbonate channel, it is not expected to be a direct regulator of differentiation and epithelial regeneration. Therefore, such a role possibly relies on its positive/ negative effect on transcription factors (TFs) that act at the nuclear level to regulate proliferation and differentiation (Liu, Wang et al. 2019). Moreover, it was also established that CFTR expression is heavily dependent on a variety of TFs and miRNAs (Viart, Bergougnoux et al. 2015). Among candidate TFs that could link CFTR to epithelial differentiation are the Krüppel-like factors (KLF's) family members, in particular KLF's 2, 4 and 5, which are known to regulate those processes (McConnell and Yang 2010).

The Kruppel-like factors (KLFs) comprise a family of evolutionary conserved zinc finger transcription factors that regulate a variety of biological processes including proliferation, differentiation and apoptosis (McConnell, Ghaleb et al. 2007). KLFs display seemingly different and broad biological properties with each one acting as a transcription activator, repressor or both (Dang, Pevsner et al. 2000). In humans, 17 KLFs have been identified, of which KLF2, KLF4 and KLF5 have been linked to pluripotency (Farrugia, Vanderbilt et al. 2016). KLF2, KLF4 and KLF5 are coexpressed in embryonic stem cells (ESCs), their combined knockdown leads to robust differentiation (Jiang, Chan et al. 2008) and it has been shown that these KLFs are expressed in lung tissues (McConnell and Yang 2010). In ESCs, although KLF4 and KLF5 cooperate to suppress differentiation, each one appears to preferentially inhibit differentiation of specific lineages, acting respectively in the endoderm and mesoderm (Bourillot and Savatier 2010). Moreover, KLF2 and KLF4 are considered much more efficient than KLF5 in the reprogramming of somatic cells to induce pluripotency and to sustain stemness (Farrugia, Vanderbilt et al. 2016). When mutually present in epithelial tissues, KLF4 and KLF5 localize to distinct compartments. In normal adult tissues, KLF4 is commonly associated with post-mitotic differentiated cells, the ones that are more associated to CFTR activity. KLF5, on the other hand, is more highly expressed on proliferating basal cells which are not associated with CFTR activity (Chen, Couble et al. 2009, Farrugia, Vanderbilt et al. 2016). Nevertheless, KLF4 and KLF5 are often described as having cooperative relationship within tumour cells as they function in tandem to promote

tumorigenesis, in a way where pluripotency is promoted by their conserved and divergent signalling (Aksoy, Giudice et al. 2014). Both KLF4 and KLF5 are quite commonly overexpressed in tumour cells although mutations are rather uncommon, refuting the idea that these can be simply considered as tumour suppressor genes or oncogenes. In fact, their effects seem to be highly context dependent, with both tumour suppressor and tumour promoting functions being reported for these factors (Farrugia, Vanderbilt et al. 2016). Given that CF cells are somewhat more cancer-like, the effects of KLF4 and KLF5 may be of interest in this context.

KLF4 was first isolated by Shields *et al.* from a NH3T3 cDNA library (Shields, Christy et al. 1996). KLF4 gained notoriety due to its role as one of the four key factors required for the generation of pluripotent cells (Takahashi and Yamanaka 2006). KLF4 belongs to the family of SP/KLF factors characterized by three zinc finger motifs in the C-terminus. KLF4 expression is regulated at both transcriptional and post-transcriptional levels, by several mechanisms like hypermethylation and micro-RNA. Multiple signalling pathways regulate KLF4 via their effectors (Ou, Shi et al. 2015). KLF4 activity is also regulated by phosphorylation. Phosphorylation of serine 132 results in embryonic stem cell differentiation. Acetylation, SUMOylation and ubiquitination of KLF4 are also ways to regulate its activity. Noteworthy, KLF4 was shown to promote itself while being repressed by KLF5 (Dang, Zhao et al. 2002). KLF4 is a versatile transcription factor involved in regulating numerous cellular processes through the modulation of several downstream effectors (Ghaleb and Yang 2017).

Indeed, one study coupled the regulation of CFTR to KLF4 through a common regulator - miR-145 (Lutful Kabir, Ambalavanan et al. 2018). Also, KLF4 is induced transiently in response to wounding, and this phenomenon is absent in CF airway cells (Crespin, Bacchetta et al. 2014). In parallel, genomic analyses of open chromatin in human tracheal epithelial cells revealed that KLF5 is part of a transcriptional network that represses CFTR gene expression (Bischof, Ott et al. 2012, Mutolo, Leir et al. 2018) and such analyses predicted that in human intestinal organoids, KLF4 was among the top genes expected to be expressed at high levels, followed by CFTR (Yin, Ray et al. 2020). Remarkably, another study reported that KLF2 is increased by 2.5-fold in CF mouse pre-adipocytes, precluding their differentiation (Rymut, Corey et al. 2017), and yet another

described that KLF2 expression is lost in CF cells (Saavedra, Patterson et al. 2008). Moreover, KLF2 has been identified as playing a role in the inflammatory process with a possible impact within the CF context (Turpaev 2020).

Moreover, KLF4 was initially identified as a CFTR modulator in a in-house functional genomic screen performed at the lab by colleagues (data not shown). Altogether, the above studies led us to investigate whether those three KLF family members have an impact on CFTR expression and function.

Materials and methods

Cell lines and tissues

For this part of the work CFBE cell were used and cultured as described in the materials and methods section of the thesis. Explanted human lungs (wt and F508del homozygous) were collected as before (Clarke, Sousa et al. 2013) and following approval by hospital Ethics Committee (Ethical code number EK-300/15, date of approval March 4th, 2015).

Chemicals, antibodies and primers

Lists of primary and secondary antibodies used in both IF and WB are in Tables S1 and S2, respectively. Sequences for the primers used in qRT-PCR are in Table S3. siRNAs used are listed in Table S4 and inhibitors in Table S5.

KLF4-KO cells generation using CRISPR/Cas9

The knockout of KLF4 was carried out using the CRISPR/Cas9 system as previously described (Mali, Yang et al. 2013). For further details, check the materials and methods section of the thesis.

qRT-PCR

qRT-PCR was performed as previously (Simões, Quaresma et al. 2019) and as described in the materials and methods section of the thesis.

Biochemical Assays

For co-Immunoprecipitation we used a previously described protocol (Farinha, Nogueira et al. 2002). Western blot analysis of cell lysates was also as

previously (Amaral, Farinha et al. 2016). For further details, check the materials and methods section of the thesis.

Immunofluorescence staining (IF)

The IF protocol used been previously described (Simões, Quaresma et al. 2019). For further details, check the materials and methods section of the thesis.

Ussing chamber experiments

CFBE cells polarized for 7 days were mounted into a micro-Ussing chamber and analysed under open-circuit conditions at 37°C as previously described (Simões, Quaresma et al. 2019). For further details, check the materials and methods section of the thesis.

Patch-Clamp

For patch-clamping cells were grown on cover slips and transfected with KLF4-GFP and GFP only as control. The GFP signal allowed the detection of transfected cells. After 48-72 h the cells were used for patch clamp recordings in whole-cell configuration as described before (Lérias, Pinto et al. 2018). For further details, check the materials and methods section of the thesis.

Statistical analysis

Data are always presented as mean ± SEM. Student's t-test for unpaired samples was used for statistical analyses. Prism 6 software (GraphPad, Inc., San Diego, CA) was used for graph design and statistical analyses. Significant differences were defined for $p \leq 0.05$ and marked with an asterisk. Other trends or tests may be stated in the legend. N=3 unless stated otherwise in the figure or in its legend. Only WB from the same blot are analysed and compared together.

Results

1 KLF4 is upregulated in CF vs Non-CF in Native Human Lung and cell lines

To unravel the interplay between the three KLF family members under study (KLF2, 4 and 5) and CFTR in the context of CF, mRNA expression levels of these KLFs were quantified in native human lung specimens from individuals with CF and healthy controls. Data in Fig. 2.1A show that KLF4 expression levels are significantly upregulated (by 2.5-fold) in CF compared to control tissue, whereas no alteration was observed for KLF2 or KLF5 expression levels.

We then evaluated expression of KLFs in CFBE cells expressing wt- and F508del-CFTR at both RNA and protein levels (Fig. 2.1B,C). In agreement with the data from native lung tissue, both KLF4 mRNA (Fig.1B) and protein (Fig. 2.1C) were found to be significantly upregulated in F508del- vs wt-CFTR expressing cells, being the levels of KLF4 protein increased by ~5-fold in CF vs control cells. Immunofluorescence (IF) data, while also confirming higher expression levels of KLF4 in CF vs control cells, also evidenced that this TF had an almost exclusive nuclear localization in CF cells (Fig. 2.1D). Interestingly, as cell confluency increased, we observed that KLF4 levels steadily increases, coupled to a progressive decrease in the levels of CFTR (Fig.S2.1).

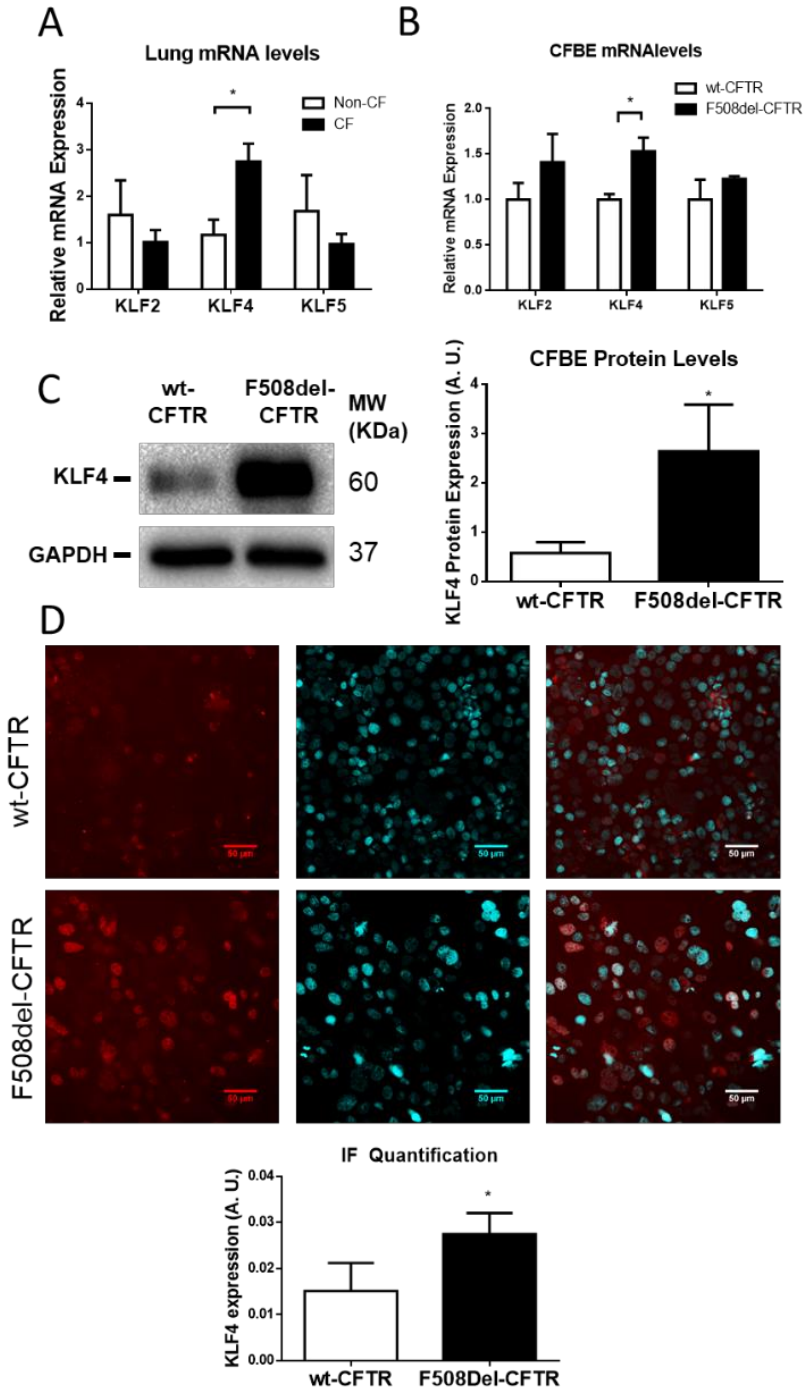

Figure 2.1 - KLF4 is upregulated in CF native human lung and cell lines.
(A) *KLF2, KLF4 and KLF5 mRNA levels were assessed by qRT-PCR in samples retrieved from lung explant specimens from individuals with CF heterozygous for F508del-CFTR or non-CF controls (n=4, unpaired t-test, p-value = 0.02).* ***(B)*** *KLF2, KLF4 and KLF5 mRNA levels in CFBE cells expressing wt- or F508del-CFTR assessed by qRT-PCR (n=3, unpaired t-test, p<0.05).* ***(C)*** *Representative WB (left) of KLF4 expression in wt- and F508del-CFTR CFBE cells, using GAPDH as loading control and (right) quantification of data in (A) in arbitrary units (A.U.) shown as relative expression vs. loading control (n=3, unpaired t-test, p<0.05).* ***(D)*** *Representative IF images showing KLF4 staining (red, left panels) in wt- and F508del-CFTR expressing CFBE cells, nuclei staining (blue, middle panels) merged images (right panel). Quantification of data below (n=4, unpaired t-test, p<0.05). [qRT-PCR on lung tissue were performed by Luka Clarke and are included here with permission].*

2 KLF4 downregulation promotes expression of wt-CFTR but not of F508del-CFTR

To determine whether there was a causal relationship between the observed differences in KLF4 and CFTR expression levels, we then assessed the impact of knocking-down (KD)/out (KO) KLF4 on CFTR expression and function. WB analyses of wt- and F508del-CFTR after KLF4 KD, shows distinct effects on wt- and F508del-CFTR: while a dramatic increase resulted for total wt-CFTR levels, no change was observed in F508del-CFTR expression (Fig. 2.2A).

To evaluate possible synergies among KLFs, we then carried out a series of experiments to assess CFTR expression upon KD of KLF2, KLF4 and KLF5 alone or combined (Fig.S2.2). Data demonstrated that only KLF4 KD (but neither KD of KLF2 nor KLF5) altered wt-CFTR expression. Noticeably, KD KLF2/5 on top of KLF4 KD seem to counteract the enhancing effect of KLF KD on CFTR expression by significantly decreasing CFTR levels.

For further validation of the KLF4 effects on CFTR expression, we then evaluated CFTR protein levels in newly generated KLF4 knockout (KLF4-KO) cells lines (clone validation in Fig.S2.3). Re-introduction of KLF4 KO was able to partially decrease CFTR levels (Fig.S2.4). Consistent with KLF4 KD data, KLF4-KO resulted in significantly higher levels of wt-CFTR, but no marked alteration of F508del-CFTR (Fig. 2.2B). Somewhat surprisingly the increase in CFTR expression resulting from KLF4-KO did not produce a significant effect on CFTR function as analysed in Ussing chamber (Fig. 2.2C).

Moreover, another approach was undertaken in which we downregulated CFTR and showed that it produced alterations in the KLF4 levels. In the case of wt-CFTR, upregulation is observed, and in case of F508del-CFTR, a downregulation is evident (Fig.S2.5). On the other hand, acute modulation of CFTR (using inhibitor-172) showed a slight decrease in KLF4 levels, while F508del-CFTR rescue using VX-809 also lead to a trend of lower levels of KLF4 (Fig.S2.6). These data indicate that not only KLF4 modulates the levels of CFTR but that the opposite also takes place in a balanced feedback mechanism, although the impact of the downregulation of CFTR in KLF4 levels is much more modest.

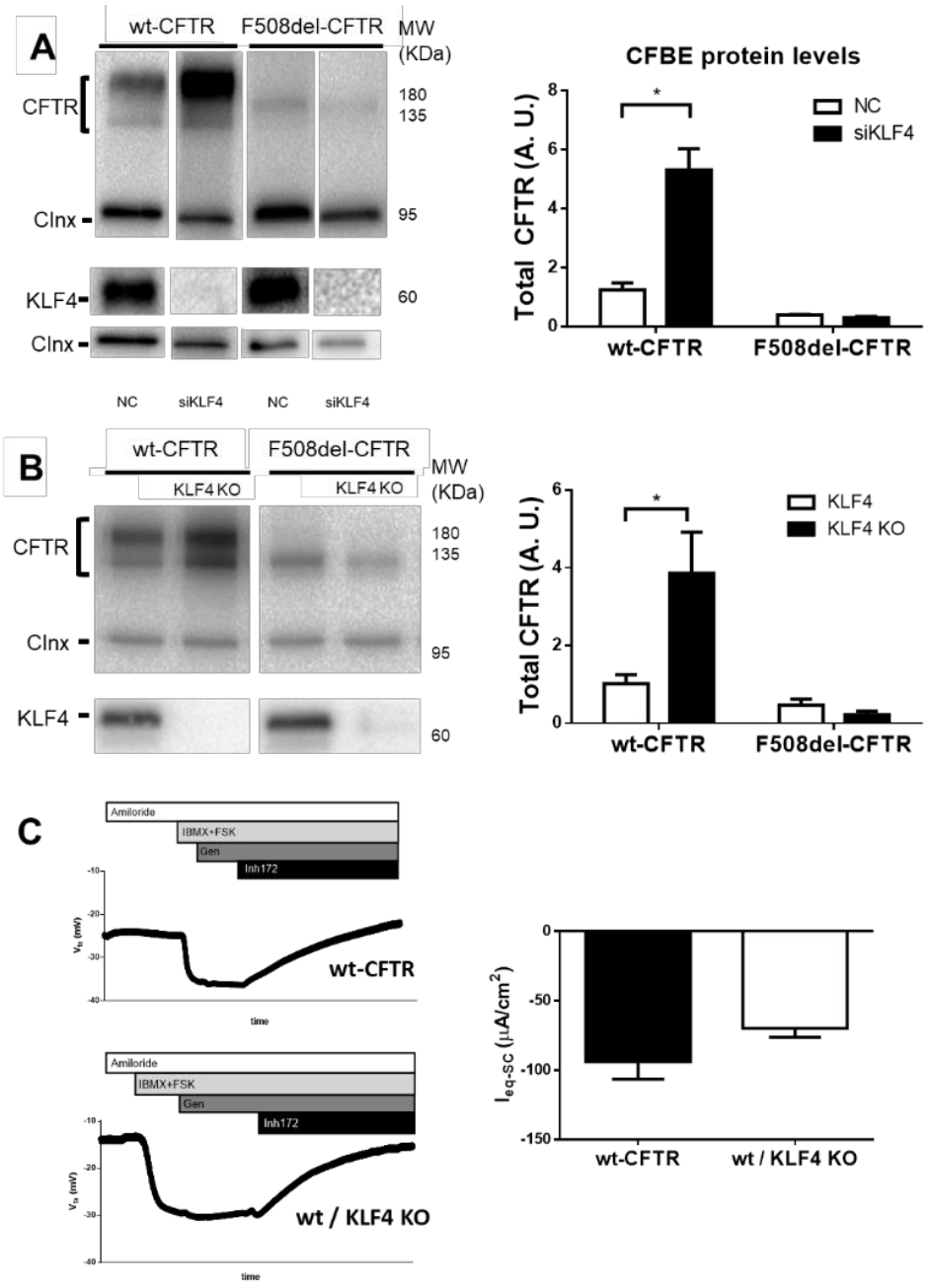

Figure 2.2 - KLF4 Knockdown/-out upregulates wt- but not F508del-CFTR.
(A) *Representative WB of KLF4 and CFTR expression in CFBE cells expressing wt- or F508del-CFTR and transfected with either siKLF4 or negative control (NC). Calnexin was used as loading control. Data are normalized to loading control and showed as arbitrary units (A. U.) (n=3, unpaired t-test, p<0.05).* ***(B)*** *Representative WB of KLF4 and CFTR expression in wt- and F508del-CFTR CFBE cells and their respective KLF4 KO (KLF4$^{-/-}$). Calnexin was used as loading control. Data are normalized to loading control and showed as arbitrary units (A. U.) (n=4, unpaired t-test, p<0.05).* ***(C)*** *Ussing chamber experiments comparing WT-CFTR cells and their KLF4 KO counterparts. Comparable resistances were observed (wt – 1400 ohm.cm^2 and KLF4 KO cells - 1280 ohm.cm^2) (n=3, unpaired t-test, p<0.05). [Ussing was performed with Iris Silva and is included here with permission].*

3 KLF4 overexpression decreases expression and function of wt-CFTR

To further explore the possible KLF4-CFTR functional relationship, we assessed how KLF4 overexpression affected CFTR expression and function. To this end, we transfected KLF4-GFP or GFP cDNA containing vectors into CFBE cells expressing either wt- or F508del-CFTR. WB data in Fig. 2.3A show that KLF4 overexpression led to a significant decrease in wt-CFTR expression, while no significant impact was observed for F508del-CFTR.

To determine whether KLF4 overexpression also had an impact in wt-CFTR function, we performed patch-clamp experiments (Fig. 2.3B). Data show that although basal currents are not affected by KLF4 overexpression, CFTR currents resulting from IBMX/Forskolin stimulation are significantly lower under KLF4 overexpression in comparison to GFP-transfected cells. By determining the I/V curve under stimulating conditions, a difference on outward currents (Cl^- influx) at voltage steps 60, 80 and 100 is also observed, being consistently lower in KLF4 overexpressing vs control cells.

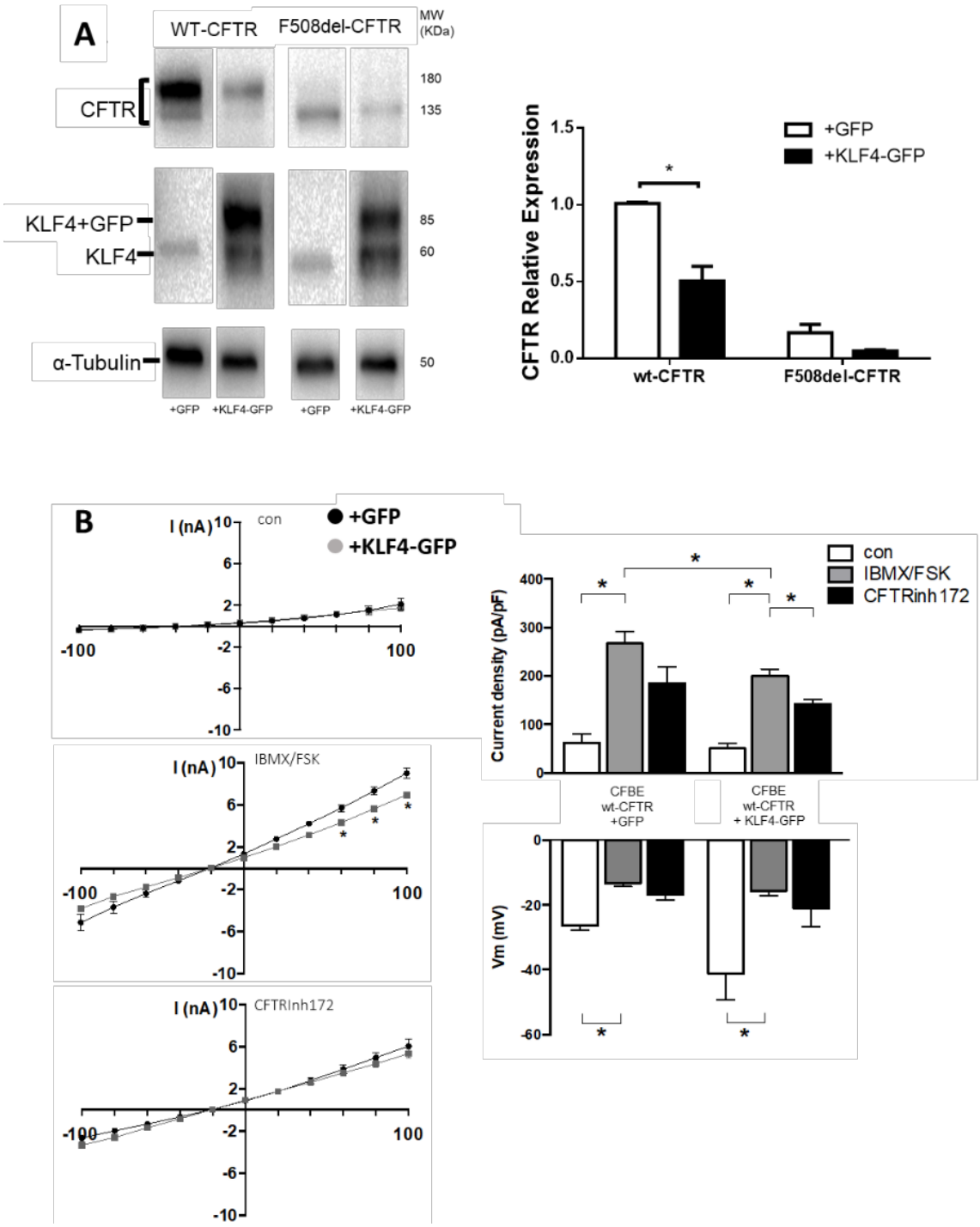

Figure 2.3 - KLF4 overexpression causes a downregulation of wt-CFTR expression and function. ***(A)*** *Transfection with KLF4-GFP was performed and the effects of KLF4 overexpression on CFTR expression were assessed by WB. Representative WB of KLF4 and CFTR expression in wt- and F508del-CFTR CFBE cells transfected with either negative control (+GFP) or KLF4-GFP (+KLF4-GFP). B-tubulin was used as loading control. Data is normalized to loading control and showed as relative expression (vs. wt-CFTR (+GFP)). (n=3, unpaired t-test, p<0.05).* ***(B)*** *CFTR chloride currents in CFBE WT cells transfected with GFP (WT) or KLF4-GFP (+KLF4). On the left, current-voltage relationship obtained in CFBE wt-CFTR and CFBE wt-CFTR + KLF4, and effects of IBMX/Fsk (center) and CFTRinh172 (lower). On the right, analysis of CFTR current density (upper) and membrane voltage (lower) before (con), and after application of IBMX/Fsk and CFTRinh172. [Patch clamp was performed by Ines Pankonien and data are included here with permission].*

4 Characterization of the KLF4-CFTR pathway crosstalk

Since CFTR is an apical PM protein and KLF4 has mostly a nuclear localization, it is not likely that a direct physical interaction occurs between these two proteins. Still, co-immunoprecipitation (co-IP) was performed to investigate this possibility and, as expected, it showed no evidence of a CFTR-KLF4 interaction (Fig.S2.7).

We thus searched in the proximal CFTR and KLF4 networks for the existence of overlapping pathways. This bioinformatic analysis revealed that EGFR-AKT and β-catenin (CTNNB) signalling actually link both networks (Fig.S2.8), evidencing 4 common key nodes, namely: AKT (serine/threonine kinase 1, also PKB), EGFR (epidermal growth factor receptor), β-catenin (β-cat), and GSK3β (glycogen synthase kinase 3 beta) (Fig.S2.8).

Accordingly, next we investigated these 4 key proteins (AKT, EGFR, β-cat and GSK3β) in CFBE cells expressing wt- or F508del-CFTR as well as their modulation through KLF4 (Fig.2.4). AKT was investigated in its phosphorylated (active) form (pAKT). Our results show that pAKT is downregulated (Fig.2.4A) and GSK3β is upregulated (Fig.2.4B) in F508del- vs wt-CFTR expressing CFBE cells, while the levels of EGFR and β-cat are slightly, albeit not significantly, lower in CF cells (Fig.2.4A,B, respectively).

To determine the KFL4-dependence of these effects, we investigated these 4 proteins in KLF4-KO cells expressing wt- or F508del-CFTR (Fig.2.4). Our data show that in the absence of KLF4, levels of pAKT, EGFR and GSK3β (but not β-cat) dramatically increase in both wt- and F508del-CFTR cells. Regarding the differences in CF vs non-CF cells, while the trend is maintained (lower AKT and EGFR and higher GSK3β in CF cells), only EGFR levels are significantly different between wt- and F508del-CFTR KLF4-KO cells. For GSK3β we note the appearance of a second band which was particularly increased in F508del-CFTR KLF4-KO cells (Fig.2.4B).

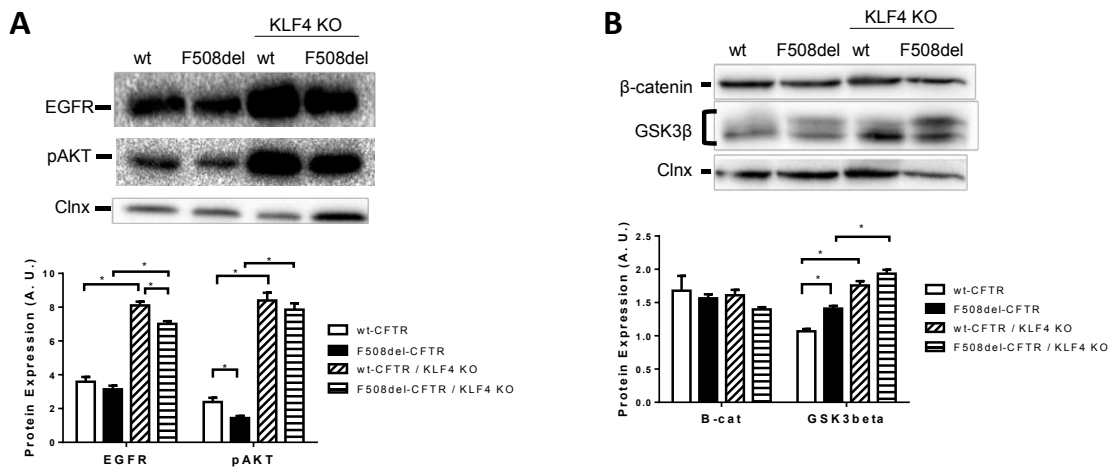

Figure 2.4 - Marked alterations in EGFR/pAKT and GSK3β signalling pathways were observed. (A,B) *Representative WB of **(A)** EGFR and pAKT, **(B)** β-catenin and GSK3β expression in wt- and F508del-CFTR CFBE and their respective KLF4 knockouts. Calnexin was used as loading control. Data is normalized to loading control and showed as arbitrary units below (A. U.) (n=3, unpaired t-test, p<0.05).*

We then tested how modulation of these pathways and their KLF4-dependency affects CFTR expression. To this end, firstly we blocked AKT using the chemical inhibitor MK-2206 (Reilly, Mroz et al. 2017). Our data from AKT inhibition in wt- and F508del-CFTR expressing cells led to differential effects, both significant (Fig.2.5): levels of wt-CFTR decreased (Fig.2.5A), while those of F508del-CFTR (immature form, band B) increased (Fig.5B). Noticeably, by assessing the processing of wt-CFTR (as measured by band C/total CFTR), a decrease in processing of ~10% was found under AKT_{inh}. This is suggestive that besides processing itself, other processes like recycling, degradation and PM stability may be affected by AKT inhibition. In parallel, we observed that under MK-2206, KLF4 levels increased in both wt-CFTR (Fig.2.5A) and CF cells (Fig.2.5B). These data suggest that while KLF4 can be the cause of wt-CFTR downregulation, its effect is not exerted on F508del-CFTR (Fig.2.2A,B).

Next, we blocked GSK3β with chemical inhibitor TWS119 (Vijay, Zhao et al. 2019). In contrast to AKT inhibition, GSK3β inhibition caused the same effect in wt- and F508del-CFTR expressing CFBE cells, leading to increased levels of both normal (Fig.2.5A) and mutant CFTR (Fig.2.5B). In parallel, there was no significant change in KLF4 levels in both cell types (Fig.2.5A,B). Other inhibitors were tested (table S5).

To address whether those observed differences were KLF4-dependent, we then tested the effects of these two chemical inhibitors in KLF4-KO cells

expressing either wt- or F508del-CFTR (Fig.2.5C). Data show that AKT inhibitor no longer decreases wt-CFTR expression in KLF4-KO cells (Fig.2.5C, left). Similarly, GSK3β inhibitor no longer increases wt-CFTR expression in KLF4-KO cells (Fig.2.5C, left). These results confirm that the AKT and GSK3β effects on wt-CFTR are KLF4-dependent.

In contrast, the previously observed increases in F508del-CFTR cells under either AKT or GSK3β inhibitors are still present even in the absence of KLF4 (Fig.2.5C, right), confirming that KLF4 effects are not exerted on F508del-CFTR.

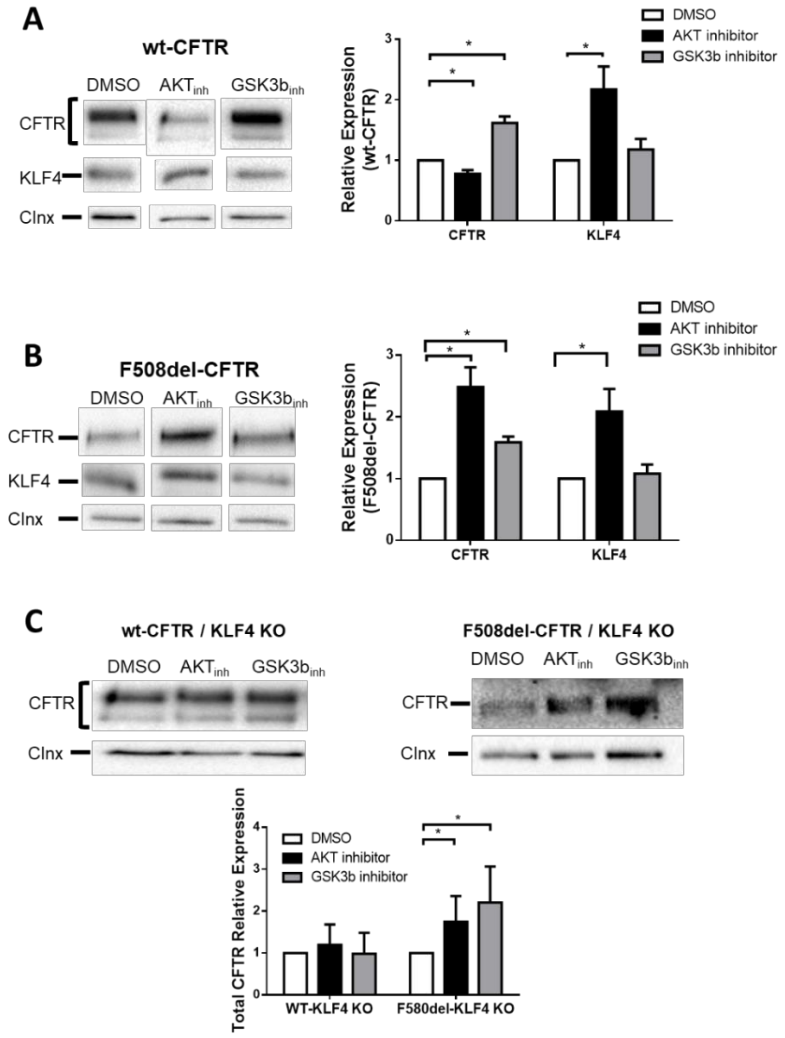

Figure 2.5 - AKT inhibition impacts negatively on wt-CFTR expression and positively on F508del-CFTR, while GSK3β inhibition impacts positively on both wt- and F508del-CFTR. *Representative WB of CFTR and KLF4 expression in wt-* ***(A)*** *and F508del-CFTR CFBE* ***(B)*** *and their respective KO counterparts* ***(C)*** *under DMSO or the inhibitors treatments. Calnexin was used as loading control. Data are normalized to loading control and showed as relative expression (vs. DMSO) (n=3, unpaired t-test, p<0.05). A time-course experiment to assess the best incubation time for these inhibitors is shown in Fig.S2.9.*

Discussion

Besides its function as a chloride/bicarbonate channel, CFTR has also been implicated in epithelial differentiation and regeneration (Amaral, Quaresma et al. 2020), being however, unclear how this occurs mechanistically. Here, we investigated whether the Krüppel-like factors (KLFs), a family of evolutionary conserved zinc finger transcription factors that regulate a variety of biological processes including proliferation, differentiation and apoptosis (McConnell and Yang 2010), have an impact on CFTR expression and function. Among these TFs, we selected KLF2, KLF4, and KLF5 because of previous reports relating these TFs to CFTR (Saavedra, Patterson et al. 2008, Bischof, Ott et al. 2012, Crespin, Bacchetta et al. 2014, Rymut, Corey et al. 2017, Lutful Kabir, Ambalavanan et al. 2018, Mutolo, Leir et al. 2018, Turpaev 2020, Yin, Ray et al. 2020) and for their reported role on differentiation (McConnell and Yang 2010).

Our data (Fig. 2.1) indicate that among the three KLFs tested, only KLF4 is altered (upregulated) in CF, both in lung tissue and in CF cell lines vs controls. Furthermore, downregulation of the three KLF's (Fig.S2.2) showed that only KLF4 KD by itself was able to exert a significant decrease on wt-CFTR (but not F508del) expression, indicating a specific impact of KLF4 on normal CFTR. However, we also observed that different KD combinations (namely siKLF2/siKLF4, siKLF4/siKLF5 and siKLF2/siKLF4/siKLF5) promoted a significant decrease in CFTR expression. This may indicate that there is some compensatory mechanism between KLFs, as well as some degree of redundancy. In fact, KLF2 and KLF4 partial redundancy has been previously reported (Villarreal, Zhang et al. 2010), although our data did not confirm this. However, KLF5 and KLF2 concomitant knockdown display no major impact on wt-CFTR levels, suggesting that KLF5 or KLF2 KD require KLF4 repression to produce the observed wt-CFTR downregulation. Interestingly, previous studies have indicated KLF5 as a repressor of CFTR (Mutolo, Leir et al. 2018), an effect which we could not observe here. Since KLF4 and KLF5 regulate each other (Ghaleb and Yang 2017), it is possible that the observed effect results from the interplay of these two TFs .

Moreover, our data also show that KLF4 modulation impacts CFTR expression levels and activity (Fig. 2.2 and 2.3). The KLF4 KD/KO experiments

lead to increased levels of wt-CFTR. However, this is not accompanied by a corresponding increase in CFTR activity as assessed by Ussing chamber experiments, which we attribute to a technical limitation possibly because CFTR levels activity measured are already close to their maximum. It is also possible that Fsk-stimulated activity of wt-CFTR can still be maximally activated, namely by phosphorylation (Jih and Hwang 2013, Cui, Stauffer et al. 2019). Thus, depending on the phosphorylation state of the cell, there is still room to further maximize Fsk-stimulated activity of wt-CFTR. However, in converse experiments (i.e., under KLF4 overexpression) we did observe a marked decrease both in the wt-CFTR expression and activity. This is further validated by our confluency assays, in which increased levels of confluency were coupled to increased KLF4 and decreased wt-CFTR expression.

Interestingly, the concept that KLF4 activity is highly context dependent (Park, Lewis et al. 2019) also emerges from our data. In fact, F508del-CFTR CFBE cells appear to be refractory to KLF4 modulation since the ability of the latter to negatively regulate CFTR is lost. It is possible that the intrinsic instability of F508del-CFTR protein (Riordan 2005) could mask the enhancement of immature F508del-CFTR protein by KLF4. In parallel, since it is established that F508del- and wt-CFTR have different interactomes (Wang, Venable et al. 2006, Pankow, Bamberger et al. 2015, Lim, Legere et al. 2017, Canato, Santos et al. 2018, Santos, Canato et al. 2019), F508del-CFTR as an immature/unstable protein does not establish the required interactions for KLF4 to exert its signalling as a F508del-CFTR modulator. Moreover, alterations in intracellular pH caused by defective CFTR may also have an impact in the signalling exerted by KLF4 (Strubberg, Liu et al. 2018). In fact, KLF4 KD/KO and overexpression experiments show no major impact on F508del-CFTR expression. Dysfunctional KLF4 in CF cells may be the cause of its observed overexpression in CF, possibly via compensatory mechanisms.

We speculate that this may be due to the fact that CF cells display a partial EMT/cancer-like phenotype (Amaral, Quaresma et al. 2020) which leads to altered signalling pathways namely those linking KLF4 to CFTR. Indeed, KLF4 regulates gene expression through transcriptional activation or repression via either DNA binding or protein-to-protein interactions, and thus the outcome of

KLF4-mediated regulation largely depends on the cellular context, e.g., the presence of oncogenic drivers among other factors (Park, Lewis et al. 2019).

The negative regulation of wt-CFTR (but not F508del-CFTR) by KLF4 led us to consider that KLF4 may play a particularly interesting role in non-CF cells and prompted further mechanistic investigation.

Although the CFTR gene possesses several major enhancer/promoter regions potentially binding several TFs (Fishilevich, Nudel et al. 2017), KLF4 (nor KLF2, KLF5) is not predicted to bind these regions. In fact, CFTR is not among its KLF4-transcribed genes (Villarreal, Zhang et al. 2010). We also did not find a direct interaction between these two proteins. Therefore, the KLF4-CFTR crosstalk must be mediated by signalling pathways and/or interactors.

Bioinformatic network analysis suggested two plausible pathways linking KLF4 and CFTR, namely those involving GSK3β/β-catenin and EGFR/AKT.

Our data (Fig. 2.4) indicate that pAKT is downregulated and GSK3β is upregulated in F508del-CFTR CFBE cells, while EGFR and total β-cat levels are unchanged. Although observed in previous studies, our data do not indicate alterations in the β-catenin signalling in the CF context. We speculate that this may be due to the fact that those studies have used different cell models/organisms (Le Henaff, Mansouri et al. 2015, Liu, Zhang et al. 2016, Zhang, Wang et al. 2017, Strubberg, Liu et al. 2018). It is also possible that the levels of active β-catenin are altered because we only measured total β-catenin. Interestingly, GSK3β upregulation in CF cells occurs with concomitant appearance of a second band which may correspond to an isoform implicated in other diseases (Castano, Gordon-Weeks et al. 2010). Moreover, our results also indicate that KLF4 appears to act as a repressor of pAKT, EGFR and GSK3β (but not β-cat), as in KLF4-KO cells there was a marked increase in the levels of these proteins. To pinpoint the effects of Akt and GSK3β on CFTR we chemically inhibited these two proteins (Fig. 5). Under Akt inhibition (MK-2206), we observed again a differential effect on wt- and F508del-CFTR: while wt-CFTR levels markedly decreased, those of F508del-CFTR significantly increased. Interestingly, KLF4 levels increased in the wt-CFTR expressing cells but remained unchanged in CF cells.

Altogether, these data strongly suggest that the effect of AKT on wt-CFTR is mediated by KLF4 (Fig.6, left): when AKT is active, KLF4 levels are kept low

and wt-CFTR is normally expressed. However, upon Akt inhibition, KLF4 is derepressed downregulating CFTR. In contrast, AKT seems to act as an active repressor of F508del-CFTR regardless of KLF4 (Fig.6, right). Indeed, despite the increase in KLF4 by AKT inhibition, F508del-CFTR is still upregulated. This effect of AKT modulation on F508del-CFTR has been previously shown (Trzcinska-Daneluti, Chen et al. 2015). Moreover, another study targeting the PI3K/Akt/mTOR signalling pathway in CF also showed that inhibition of Akt using MK-2206 increased stability and expression of mutant CFTR and that this effect may be possibly mediated by BAG3 (Reilly, Mroz et al. 2017). Another interesting report establishes the connection between AKT and CFTR through ezrin (Quan, Sun et al. 2019), a known stabilizer of CFTR at the PM (Loureiro, Matos et al. 2015).

The data shown here for KLF4-KO cells, showing that the AKT inhibitor no longer affects wt-CFTR but still affects F508del-CFTR, imply that the AKT signalling impact on wt-CFTR is KLF4-dependent, but this dependency is lost in CF cells (Fig.6). Noteworthy, KLF4 may have opposing effects on AKT activity depending on the malignancy levels (Xiong, Schober et al. 2018). This finding may be relevant for the present data, if we consider that CF cells display a more cancer-like phenotype (Amaral, Quaresma et al. 2020).

Using the GSK3β inhibitor we observed an increase in both wt- and F508del-CFTR expression, but with no change in KLF4 levels. Other authors found that GSK3β inhibition rescues F508del-CFTR (Trzcinska-Daneluti, Nguyen et al. 2012), a finding that we did not detect here despite the increased levels of band B in F508del-CFTR. Our data suggest that GSK3β acts as a repressor of CFTR (both normal and mutant) and that this effect is not KLF4-independent in wt-CFTR cells while this seems to be the case in F508del-CFTR cells. However, in KLF4-KO cells, the effect disappeared on wt-CFTR (while remaining on F508del-CFTR) leading to envisage that GSK3β is also impacted by KLF4, but not vice-versa (Fig.6). Interestingly, KLF4 is a known promotor of Cadherin 3 (CDH3) and GSK3β is a downstream effector of CHD3 (Li, Yu et al. 2019). Accordingly, it is plausible that GSK3β levels are affected by KLF4 but not the opposite. In certain particular contexts like differentiation, however, GSK3β is required for transient KLF4 expression (Cervantes-Camacho, Beltran-Langarica et al. 2015). For the β-catenin, previously described as a positive regulator of

CFTR, we could not find a dependence on KLF4 for its effects on CFTR. One possibility is that NF-kB is a mediator of this GSK3β signalling in CFTR (Liu, Zhang et al. 2016).

Taken our data globally, we propose that AKT is a positive regulator of wt-CFTR, dependent on KLF4 and that KLF4 may negatively affect wt-CFTR via Akt repression (Fig.6). Possible mediators of this pathway include Hsp90, Ezrin and BAG3. However, KLF4 has this regulatory role disrupted towards mutant CFTR.

GSK3β in turn, is a negative regulator of CFTR (both wt and mutant), but only dependent on KLF4 in non-CF cells (Fig.6). Multiple connections between KLF4, AKT and GSK3β are, however, possible (Fig.S8).

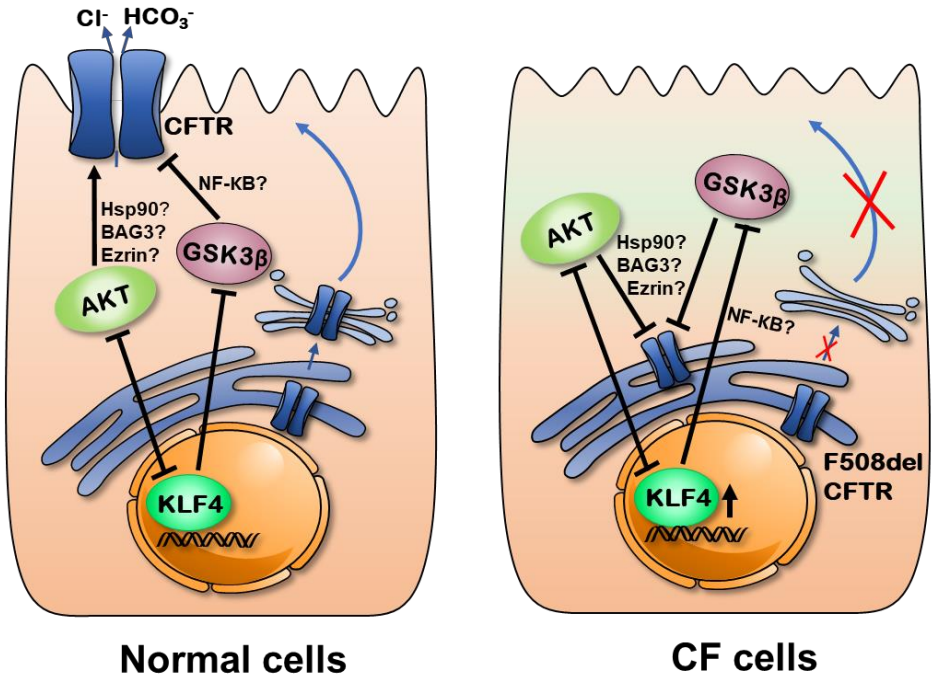

Figure 2.6 - KLF4 role within the CF context. KLF4 acts as a negative regulator of wt-CFTR but has its function disrupted in the F508del-CFTR cells.
Our data suggest that the AKT pathway is a positive regulator of wt-CFTR while being a negative regulator of F508del-CFTR in a way that is possibly at least partially mediated by KLF4, which in turn seems to be negatively regulated by AKT signaling in wt-CFTR cells. GSK3β, on the other hand, seems to be a negative regulator of CFTR which is KLF4-dependent in wt-CFTR and KLF4-independent in F508del-CFTR expressing cells.

Altogether, these data show that KLF4 acts as a negative regulator of wt-CFTR expression and function, but its effects are not exerted on F508del-CFTR. This may be due to the fact that CF cells display a partial EMT/cancer-like phenotype (Amaral, Quaresma et al. 2020) and that CFTR has been proposed to act as a tumour suppressor (Than, Linnekamp et al. 2016). This should lead to

altered signalling pathways namely those linking KLF4 to CFTR. Thus, further studies are required to completely unravel the interplay between these factors and CFTR.

By establishing a relationship between CFTR and the AKT/GSK3β pathways and KLF4, all related to differentiation, this work also open new avenues for CF therapy. Since our data suggest that targeting AKT and GSK3β may increase levels of immature F508del-CFTR, this may be a way to enhance the effect of other therapies, i.e., corrector drugs, that rescue F508del-CFTR. Noticeably, the therapeutic potential of several inhibitors of the AKT and GSK3β signalling pathways have been extensively studied in the context of cancer (Faes and Dormond 2015, Duda, Akula et al. 2020). Interestingly, individuals with CF were also shown to have increased risk of cancer (Maisonneuve, Marshall et al. 2013, Slae and Wilschanski 2018, Yamada, Komaki et al. 2018). So, these may be safe options worth exploring in further detail with KLF4 arising both as potential indirect therapeutic target or biomarker (Fadous-Khalifé, Aloulou et al. 2016), especially because kinases are often regarded as preferential therapeutic targets (Quan, Xiao et al. 2017). Moreover, considering KLF4 a key factor in differentiation (Ghaleb and Yang 2017), modulation of its downstream effectors may be a way to partially correct the underlying differentiation defect observed in CF (Hajj, Lesimple et al. 2007).

Supplementary Data

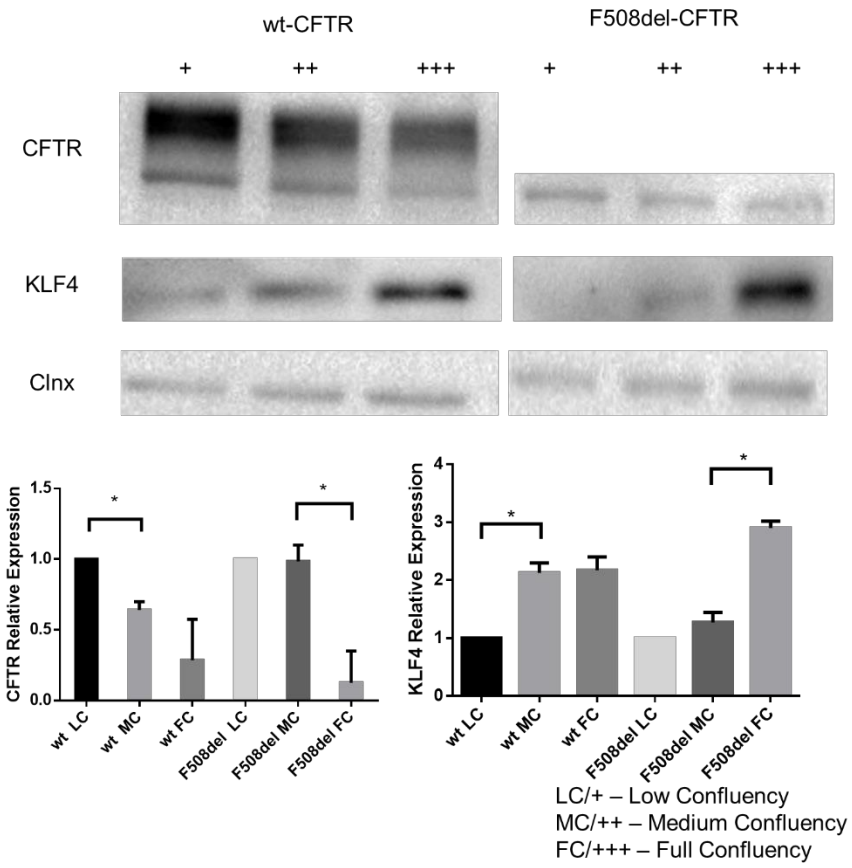

Figure S2.1 – Confluency levels influence the expression levels of CFTR and KLF4.
Representative WB of CFTR and KLF4 are shown for both wt- and F508del-CFTR CFBE under different confluency. Calnexin was used loading control. (n=3, unpaired t-test, p-value < 0.05).

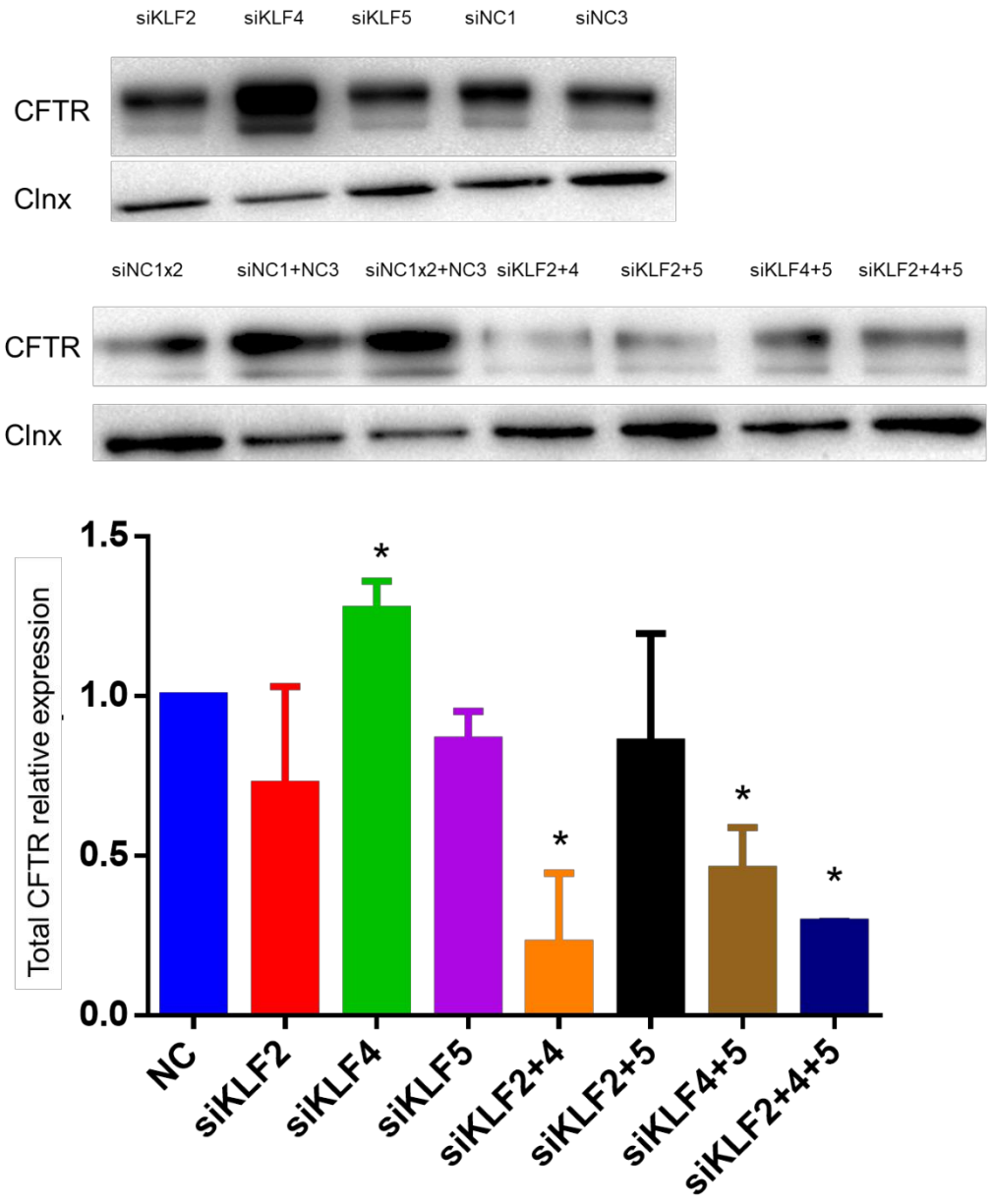

Figure S2.2 - Different combinations of siRNAs for KLF2, KLF4 and KLF5 were tested.
Representative WB for CFTR under the different conditions. Calnexin used as loading contro. Data shown as relative expression (vs. negative control). (N=2, unpaired t-test, p-value < 0.05). NC1 is the negative control for KLF2 and KLF5, while NC3 is the negative control for KLF4. Different combinations of siKLFs were compared to their respective combinations of negative controls.

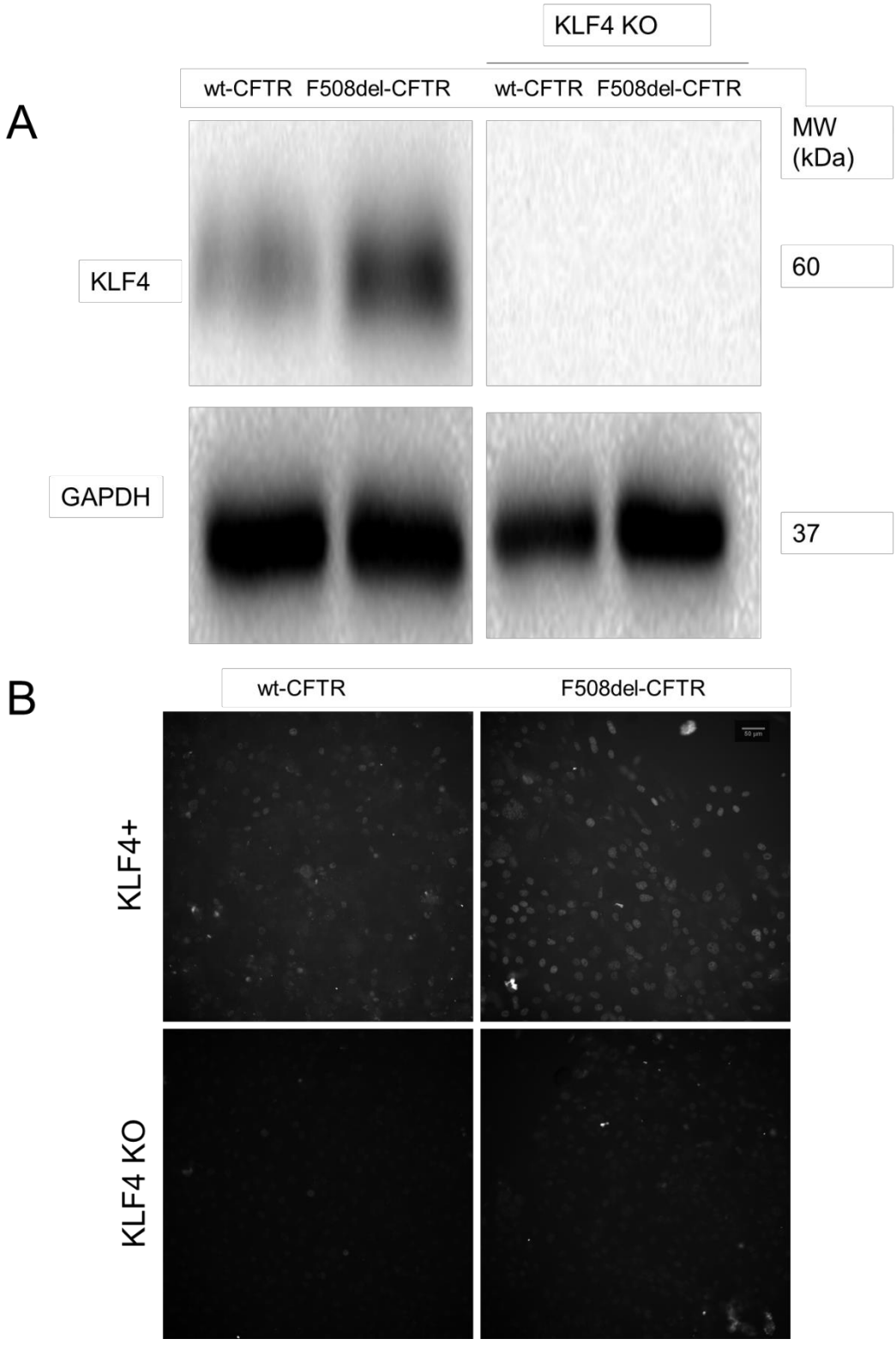

Figure S2.3 - KLF4 KO validation.
KLF4 Knockout was performed using a CRISPR/Cas9 approach and confirmed by gene sequencing (data not shown) and at the protein level by Western Blotting (A) and Immunocytochemistry (B). Representative images are shown for both western blot and immunofluorescence. GAPDH used as loading control.

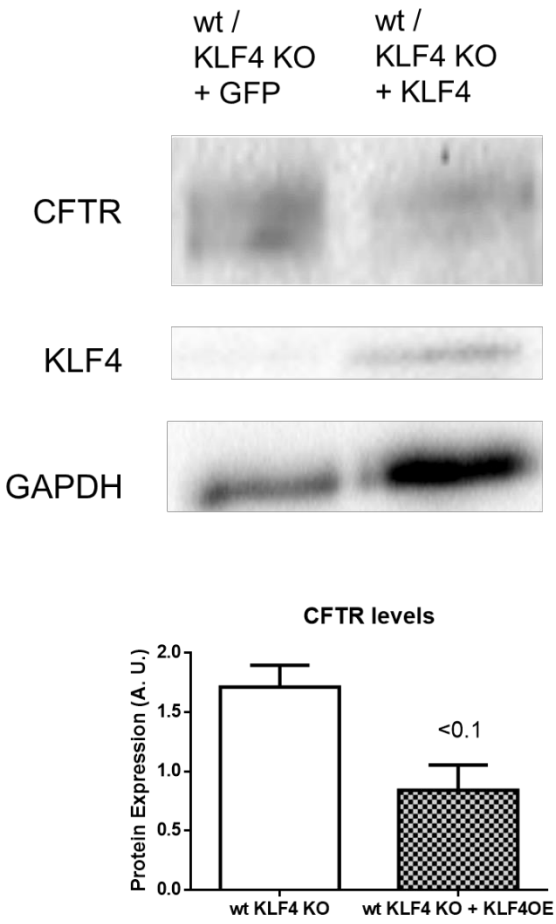

Figure S2.4 - KLF4 Re-introduction in KLF4 KO cells.
Representative images of the WB for CFTR and KLF4 are shown, using GADPH as a control. (n=2, unpaired t-test, p=0.08).

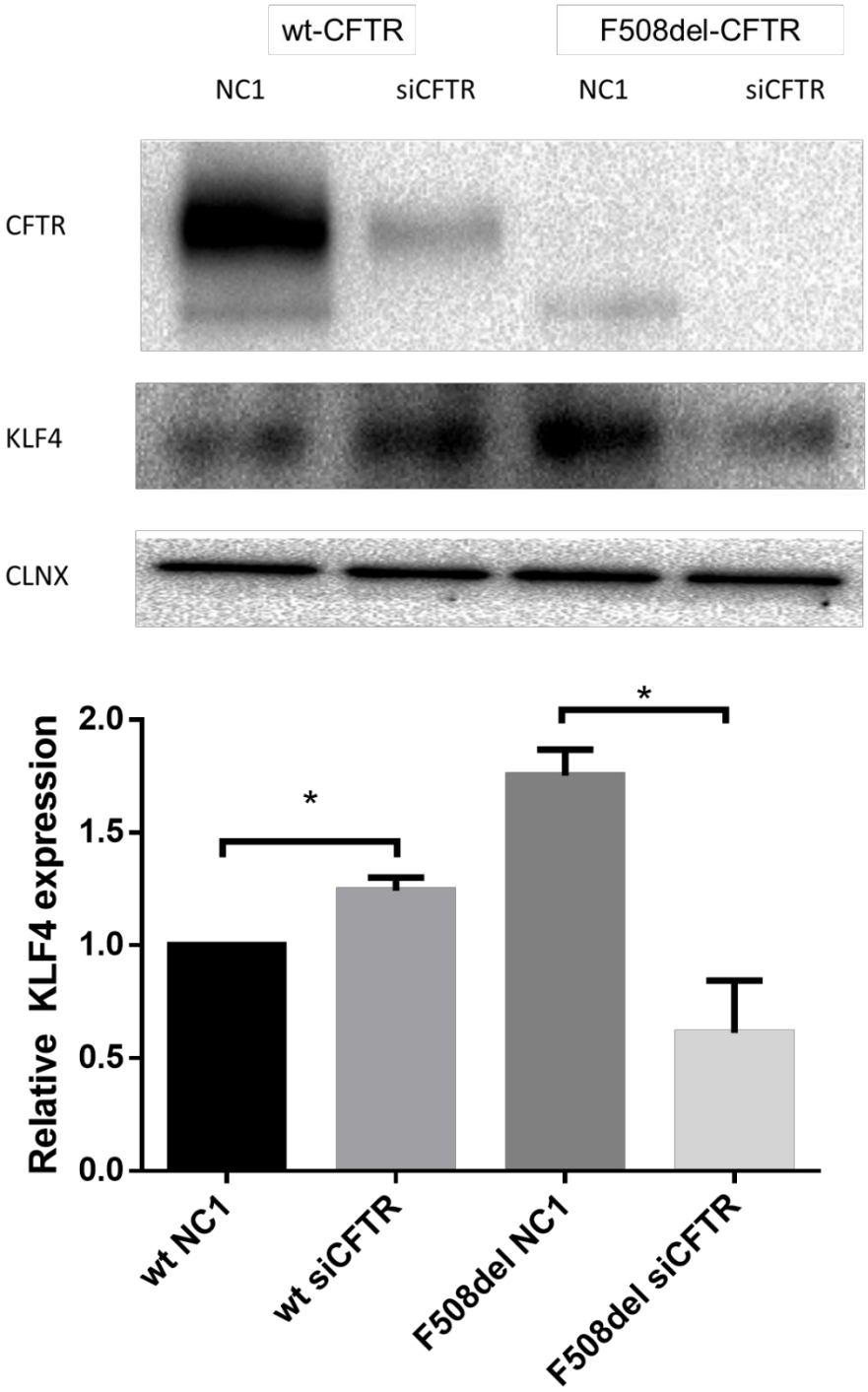

Figure S2.5 - impact of CFTR knockdown in KLF4 levels.
Representative images of the WB for CFTR and KLF4 in both wt- and F508del-CFTR under CFTR siRNA or Negative control (NC1). Data shown as relative KLF4 expression (vs. wt-CFTR NC1). (n=3, unpaired t-test, $p<0.05$).

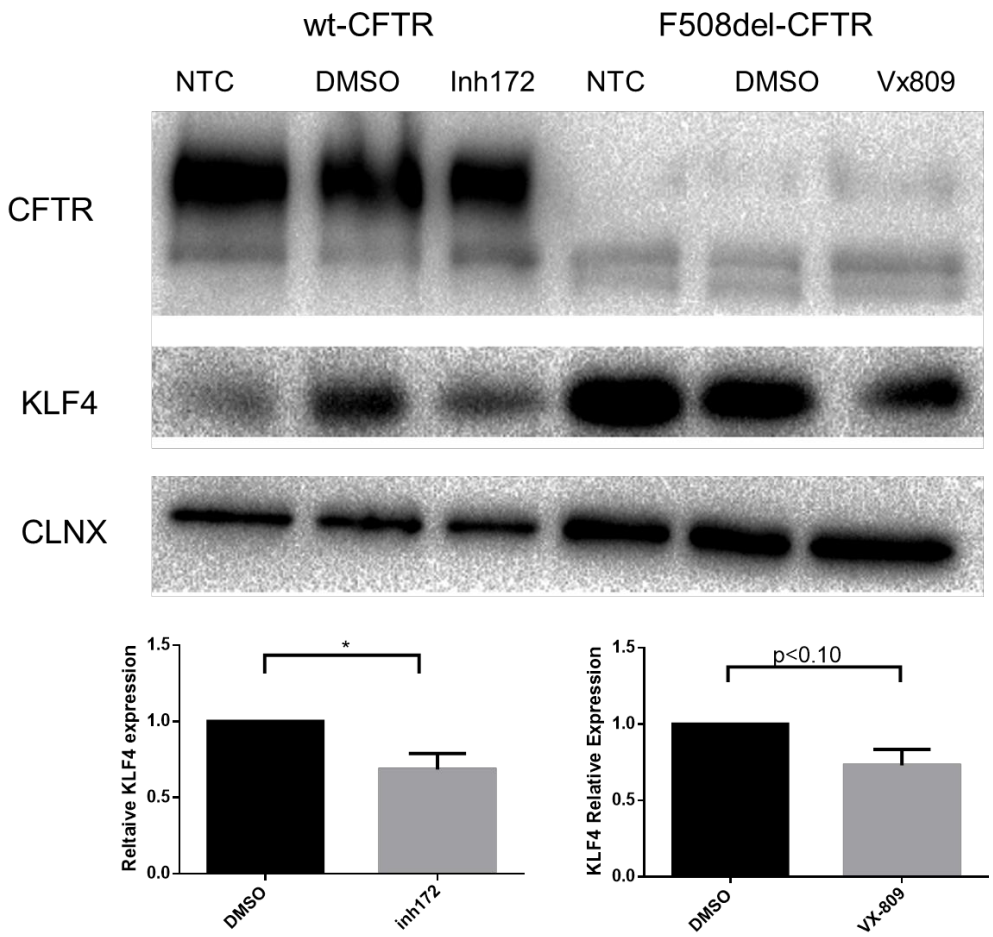

Figure S2.6 - Impact of CFTR modulation on KLF4 levels.
Representative WB of CFTR and KLF4 on wt- and F508del-CFTR CFBE cells, under treatment with inh172 (inhibitor 172), VX809, DMSO and NTC (Non-treated control). Data shown as relative expression of KLF4 (vs. DMSO). (n=3, unpaired t-test, p<0.05).

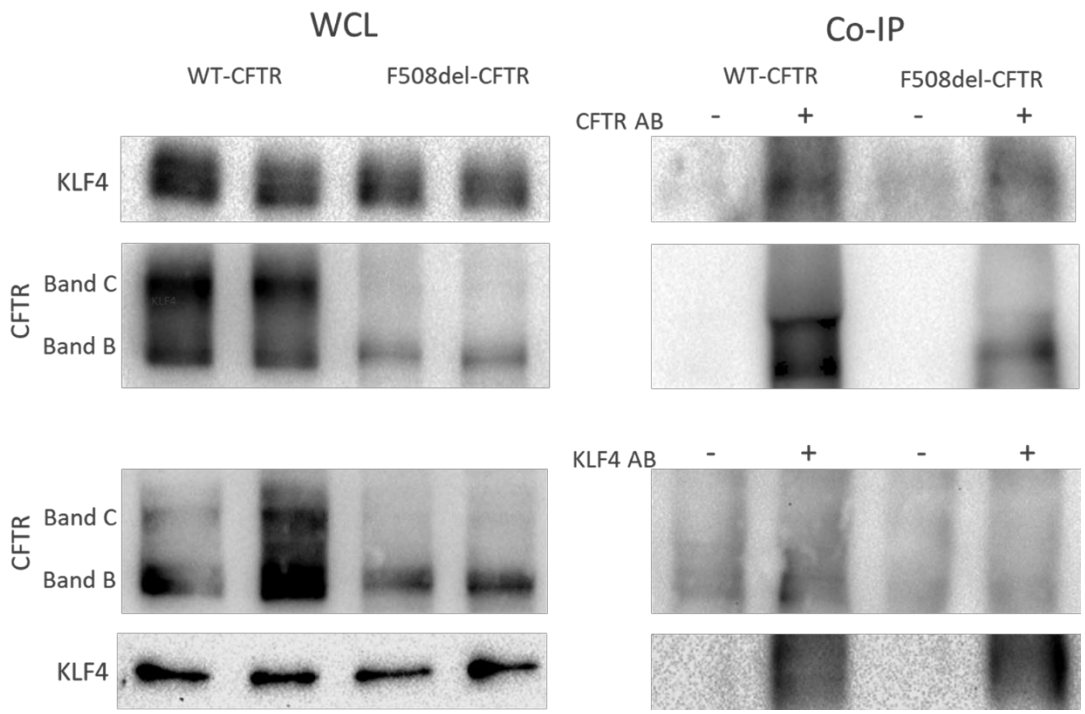

Figure S2.7 - Co-immunoprecipitation results. *No clear evidence of interaction between KLF4 and CFTR is shown. Representative images of 3 Co-IP experiments with pull down of KLF4 and CFTR. [Co-IP experiments were performed with Ines Pankonien and included here with permission].*

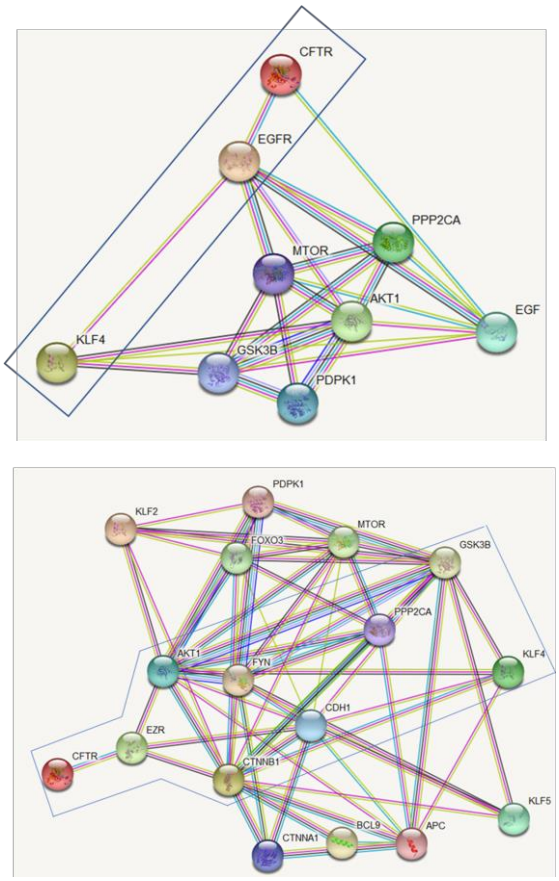

Figure S2.8 - Major signalling pathways connecting CFTR and KLF4. *These were generated using String-db and by searching and expanding the relevant pathways connecting KLF4 and CFTR.*

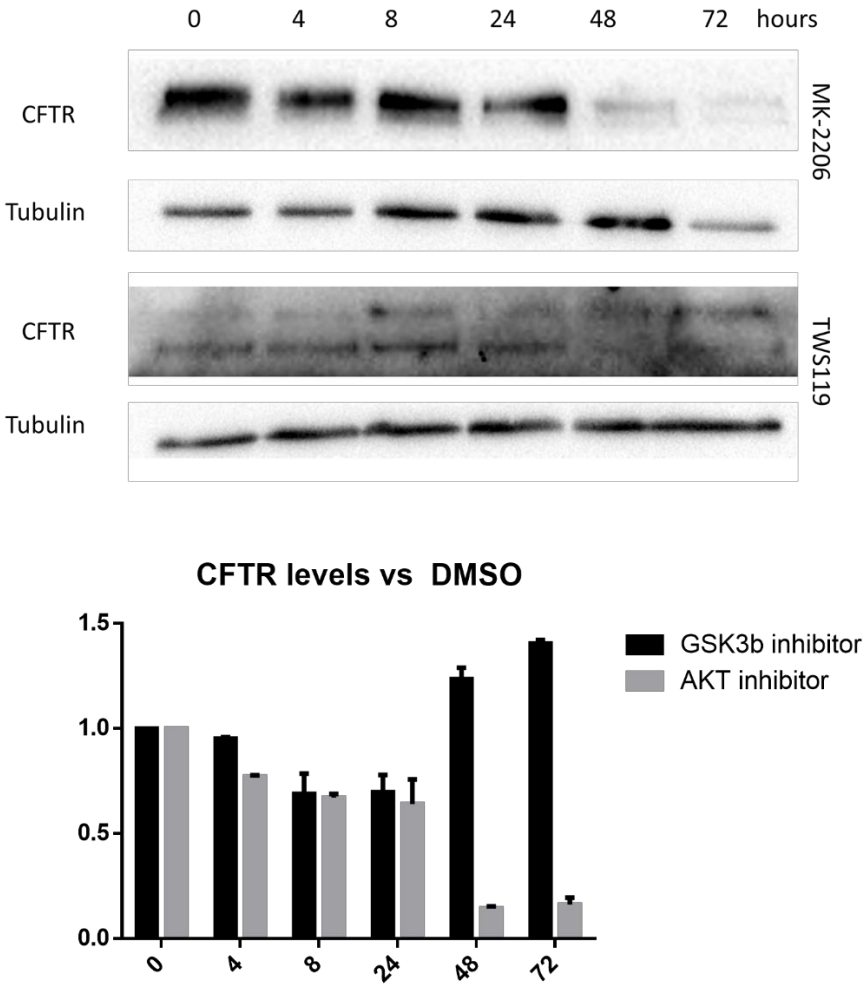

Figure S2.9 - Time-course experiment of treatment with AKT and GSK3β inhibitors.
Time-course experiment of treatment with both AKT (MK-2206) and GSK3β (TWS119) performed to identify the time of inhibition that produces the most striking results in terms of the output of interest (CFTR levels). Representative WB for CFTR are shown using Tubulin as loading control. N=2.

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Chapter III – Characterization of KLF4 KO impact on proliferation and epithelial differentiation in the CF context

Some of the data present in this chapter is included in the following work:

Sousa L, Pankonien I, Amaral MD. Impact of KLF4 on cell proliferation and epithelial differentiation in the context of Cystic Fibrosis. – in preparation.

Abstract

As we have shown (Chapters 1 and 2), CF cells present a more cancer-like phenotype and exhibit KLF4 upregulation. We have also demonstrated that this transcriptional factor acts as a negative regulator of wt-CFTR. KLF4 is described as exerting its effects in a cell-context dependent fashion, but it is generally considered a major regulator of proliferation, differentiation and wound healing, all processes also altered in CF.

Therefore, it is relevant to characterize the differential role of KLF4 in these processes in CF vs non-CF cells. To this end, we used wt- and F508del-CFTR CFBE cells and their respective KLF4 knock-out (KO) counterparts to compare the expression of several epithelial differentiation markers as well as to evaluate processes like cell proliferation and wound healing rates.

Our data indicate a minor impact of KLF4 KO in proliferation rate even though KLF4 is commonly regarded as a promoter of growth arrest. We observed a differential impact of KLF4 KO in TEER acquisition and wound healing rate, with KLF4 reducing TEER acquisition and wound healing rate in wt-CFTR cells while increasing both processes in F508del-CFTR cells. In parallel, we also observed a differential impact on the levels of some differentiation markers and EMT-associated transcription factors. In broad terms, KLF4 seems to generally promote the expression of mesenchymal factors, although differences between wt- and F508del-CFTR cells are evident.

In conclusion, KLF4 impacts TEER acquisition, wound healing rate and the expression of differentiation markers, in a way that is partially dependent on the CFTR-status of the cell.

Introduction

Our work shown before (Chapters 1 and 2), demonstrated that CF cells present a more cancer-like phenotype and that KLF4 is upregulated in CF vs non-CF cells. Moreover, our work also indicates that KLF4 acts as a negative regulator of wt-CFTR expression and function and that this regulation is lost in CF cells. Additionally, we have shown that the regulation of wt-CFTR by KLF4 is mediated by AKT and GSK3β signalling.

KLF4 transcriptional profiling reveals its key role in cell cycle regulation and epithelial differentiation (Chen, Whitney et al. 2003). Therefore, here we aimed at understanding the KLF4 role on cell proliferation, wound healing, Epithelial-Mesenchymal transition (EMT) and differentiation in the context of CF, since these are processes that are described as being disrupted in CF (Leigh, Kylander et al. 1995, Larson, Delcarpio et al. 2000, Hajj, Lesimple et al. 2007, Amaral, Quaresma et al. 2020).

It has been demonstrated that KLF4 may exert very distinctive effects, depending on the cell context. For instance, KLF4 is often regarded as an inhibitor of cell proliferation. In fact, it was first identified as a promoter of growth arrest (Shields, Christy et al. 1996). Consistently, KLF4 has been associated with p53 in growth arrest (Zhang, Geiman et al. 2000), inhibiting proliferation by blocking the G1-to-S progression (Chen, Johns et al. 2001, Yoon, Chen et al. 2003). Accordingly, loss of KLF4 has been shown to promote cell proliferation (Katz, Perreault et al. 2005). KLF4 function in proliferation has been associated with both GSK3β (Li, Yu et al. 2019) and AKT signalling pathways (Tang, Zhong et al. 2018). However, in certain contexts, KLF4 has also been shown to promote proliferation (Wang, Yang et al. 2015, Mai, Zhong et al. 2019).

Taking into account its effectors, like E-cadherin (E-Cad) (Villarreal, Zhang et al. 2010), we can expect a possible role of KLF4 in epithelial differentiation and wound healing which is of potential interest in the CF context.

Therefore, our aim here was to assess the impact of KLF4 knock-out (KO) in those processes both in wt- and F508del-CFTR expressing human bronchial cells.

Materials and Methods

Cells and tissues

CFBE and BCi cells were used in this part of the work as described in the Materials and Methods section of the thesis.

Chemicals, antibodies and primers

Lists of primary and secondary antibodies used in WB are in Tables S1 and S2.

TEER measurements

Transepithelial electrical resistance (TEER) measurements were carried out as described in the Materials and Methods section of the thesis.

Western Blot (WB)

WB were performed as previously described in (Amaral, Farinha et al. 2016). For further details, check the Materials and Methods section of the thesis.

Growth curves

Growth curves were performed as previously described in the Materials and Methods section of the thesis.

Proliferation assays

Proliferation assays based on Ki-67^{+} staining were performed as described in the Materials and Methods section of the thesis.

Wound healing

Wound healing assays were performed as previously described in the Materials and Methods section of the thesis.

Statistical analyses

Data are presented as mean ± SEM. Student's t-test for unpaired samples was used for statistical analyses. Prism 6 software (GraphPad, Inc., San Diego, CA) was used for graph design and statistical analyses. Significant differences were defined for $p \leq 0.05$ and marked with an asterisk. Other trends or tests may be stated in the legend. N=3 unless stated otherwise in the figures or in legends. Comparisons are only made between from the same WB blot.

Results

1 KLF4 KO impact on proliferation

KLF4 KO has no major impact on cell proliferation (Table 3.1, Fig.3.1) as shown by both growth curves (Fig.3.1A) and Ki-67+ cells data (Fig.3.1B). A trend, albeit non-significant is observed for KLF4 KO being associated with higher

proliferation in F508del-CFTR CFBE cells while having the opposite effect on wt-CFTR cells (a marginal decrease is observed in these cells). However, since these differences are not statistically significant, we conclude that KLF4 KO has no major impact on cell proliferation in our system. Accordingly, no major impact of KLF4 KO was observed in the expression of Ki-67 as assessed by WB (Fig.S3.1).

Table 3.1 - Average number of cells on a time-course.

Time (h)	wt-CFTR	wt-CFTR KLF4 KO	F508del-CFTR	F508del-CFTR KLF4 KO
0	42.3	42	76.3	77.7
3	57.0	37	80.0	79.7
6	52.0	38	76.0	81.7
8	52.0	38	78.7	86.7
22	47.0	44	101.0	102.3
24	50.3	45.7	96.3	105.7
30	67.3	46.3	100.7	109.3

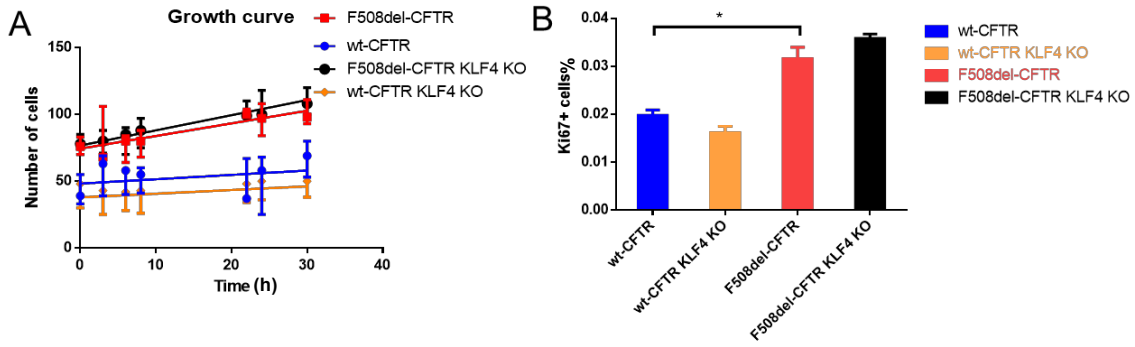

Figure 3.1 - Impact of KLF4 KO on cell proliferation.
(A) *Growth curves of wt- and F508del-CFTR CFBE cells and their KLF4 KO counterparts (n=3).* ***(B)*** *Ki-67⁺ staining data. (n=3, unpaired t-test, p<0.05)*

2 KLF4 KO impact on TEER and wound healing

We then assessed the impact of KLF4 KO in TEER acquisition and wound healing rate. KLF4 KO increased the TEER of F508del-CFTR cells (indicating a less leaky epithelium) whilst decreasing the TEER of wt-CFTR (indicating a leakier epithelium). Therefore, we observe once again a differential effect of KLF4 KO in the two cell types (Table 3.2,Fig.3.2A). Preliminary data of wound healing assays also showed a consistent behaviour. Wound healing rate was increased in F508del-CFTR and slowed in wt-CFTR CFBE cells upon KLF4 KO (Fig.3.2B).

KLF4 KO may also alter the morphology of the wound recovering cells by possibly promoting "sharper" edges at the closing front with less lamellipodia formed (Fig.3.3).

Table 3.2 – Average TEER (ohm.cm²) over time – KLF4 KO.

Time (d)	wt-CFTR	wt-KLF4 KO	F508del-CFTR	F508del-KLF4 KO
3	1876.3	1063.7	527.0	732
4	2001.3	1226.3	583.0	1206.3
5	2088.0	1375	528.0	1386.7
6	2423.3	1787.7	542.7	1582
7	2700.7	2172	796.0	2969

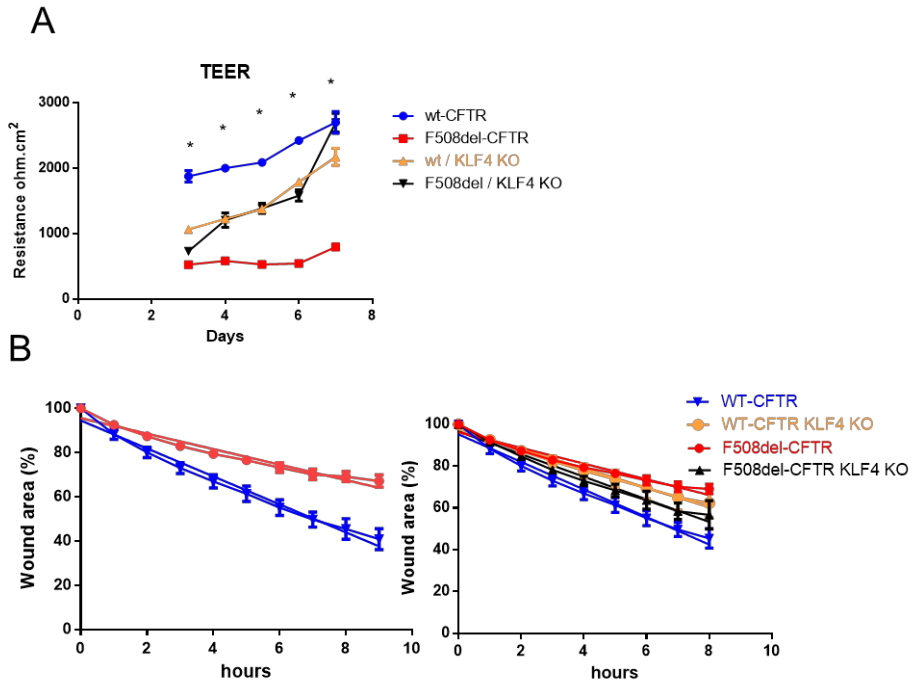

Figure 3.2 - Impact of KLF4 KO on TEER and wound healing,
TEER and wound healing. ***(A)*** *TEER in WT- and F508del-CFTR CFBE cells and their KLF4 KO counterparts over time. (n=3, unpaired t-test, p<0.05)* ***(B)*** *Wound healing kinetics in wt- and F508del-CFTR CFBE cells and their respective KLF4 KO counterparts (n=2)*

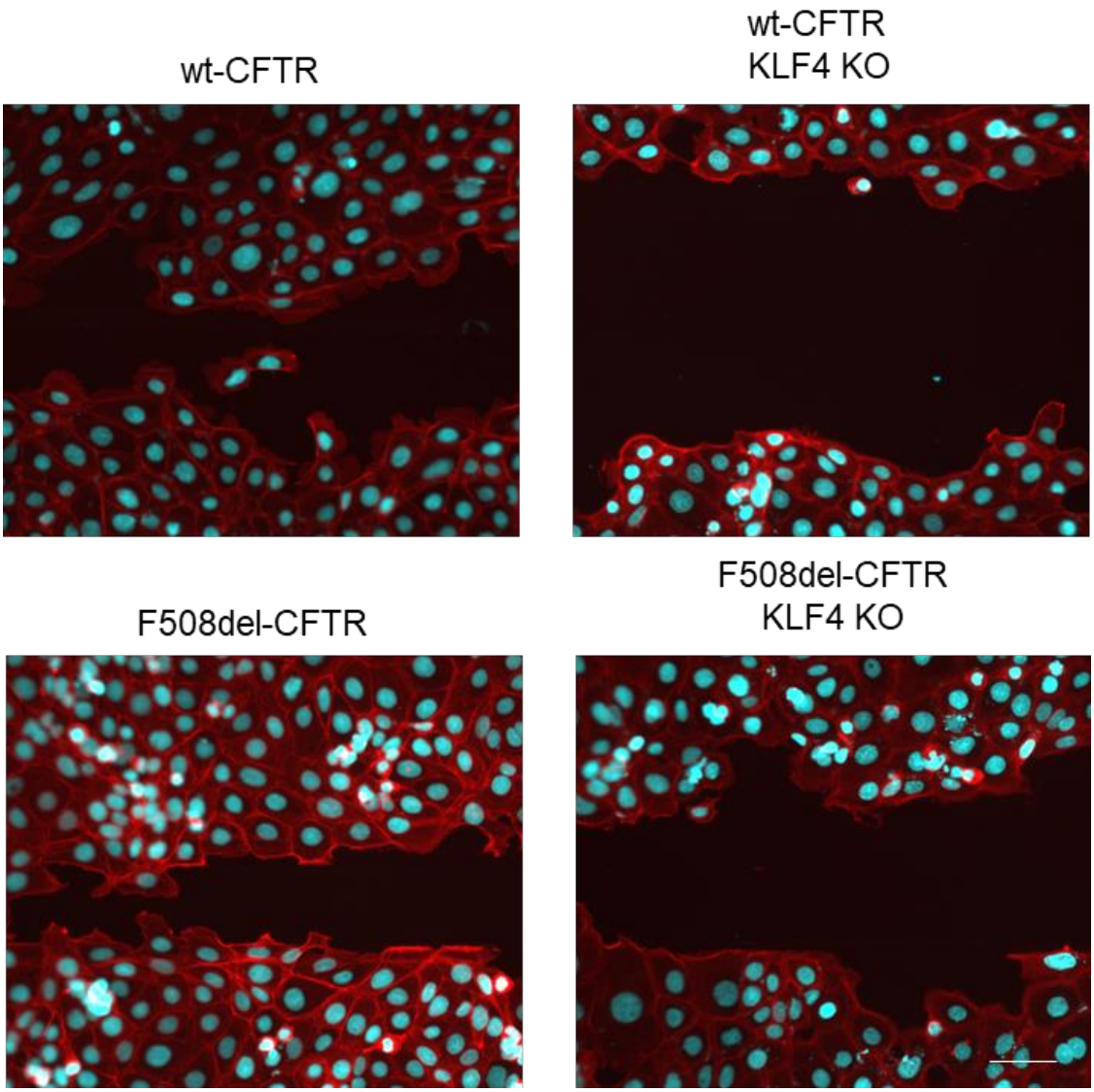

Figure 3.3 – Impact of KLF4 KO in the morphology of wound recovery.
Wound healing staining in wt- and F508del-CFTR CFBE cells and their respective KLF4 KO counterparts. Cyan is nuclei staining, red is SiR-Actin staining. Bar= 25µm. {Data obtained with Ines Pankonien and included here with permission].

3 KLF4 KO impact on differentiation markers

We then investigated the impact of KLF4 KO on the levels of several epithelial differentiation markers and of other KLF4-related proteins.

Initially, we evaluated the effects of KLF4 KO on the expression levels of KLF2 and KLF5 (Fig.3.4). Our data show that KLF4 KO leads to KLF5 upregulation. Interestingly, the effects of KLF4 KO on KLF2 expression levels, are differential in CF vs non-CF cells: being increased in wt-CFTR cells and decreased in F508del-CFTR cells.

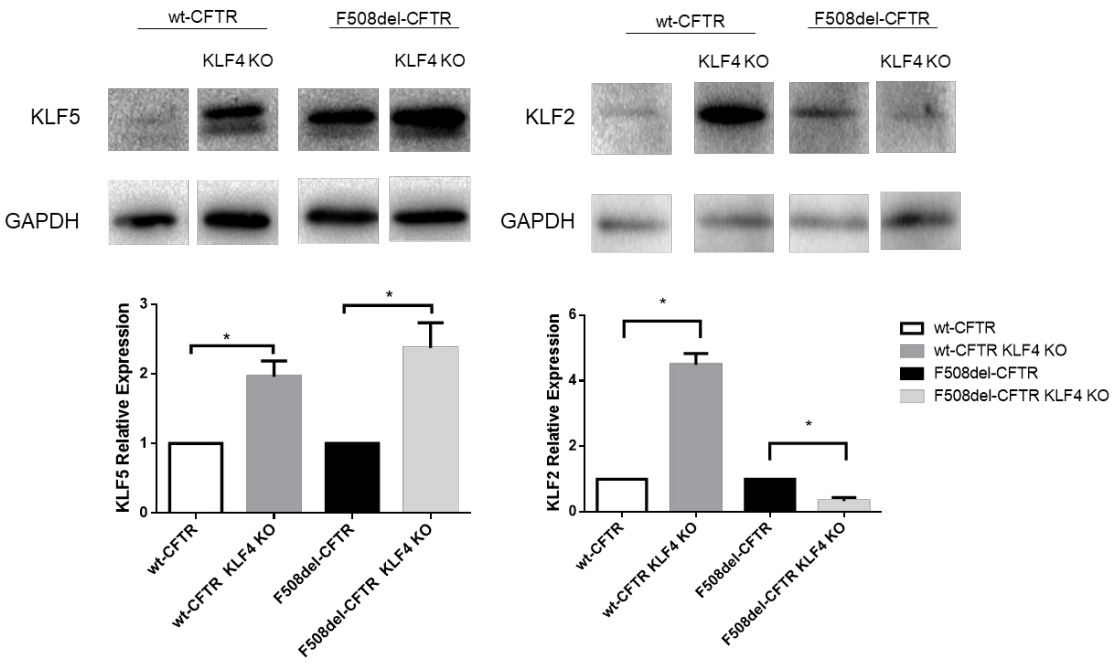

Figure 3.4 - Impact of KLF4 KO on KLF2 and KLF5 levels.
Protein levels on wt- and F508del-CFTR CFBE and their respective KLF4 KO counterparts. Representative WB of KLF2 and KLF5. GAPDH was used as loading control. Data are normalized to loading control and showed as relative expression (KO vs non-KO) (A) (n=3, unpaired t-test, p<0.05).

KLF4 KO had major impact on the levels of several differentiation markers (Fig.3.5). Indeed, KLF4 KO decreased the levels of E-Cad and Cytokeratin 18 (epithelial markers) whilst increasing the levels of Vimentin and N-Cad (mesenchymal markers). Therefore, KLF4 seems to be a suppressor of Epithelial to-Mesenchymal transition (EMT). KLF4 KO on the other hand, showed a differential impact on the expression levels of Fibronectin (mesenchymal marker) and ZO-1 (epithelial marker) between wt- and F508del-CFTR CFBE cells. While KLF4 KO increased the expression levels of Fibronectin in wt-CFTR cells, it led to its decrease in F508del-CFTR cells. Regarding ZO-1, KLF4 KO decreased its expression in F508del-CFTR CFBE cells, having no major impact on wt-CFTR CFBE cells.

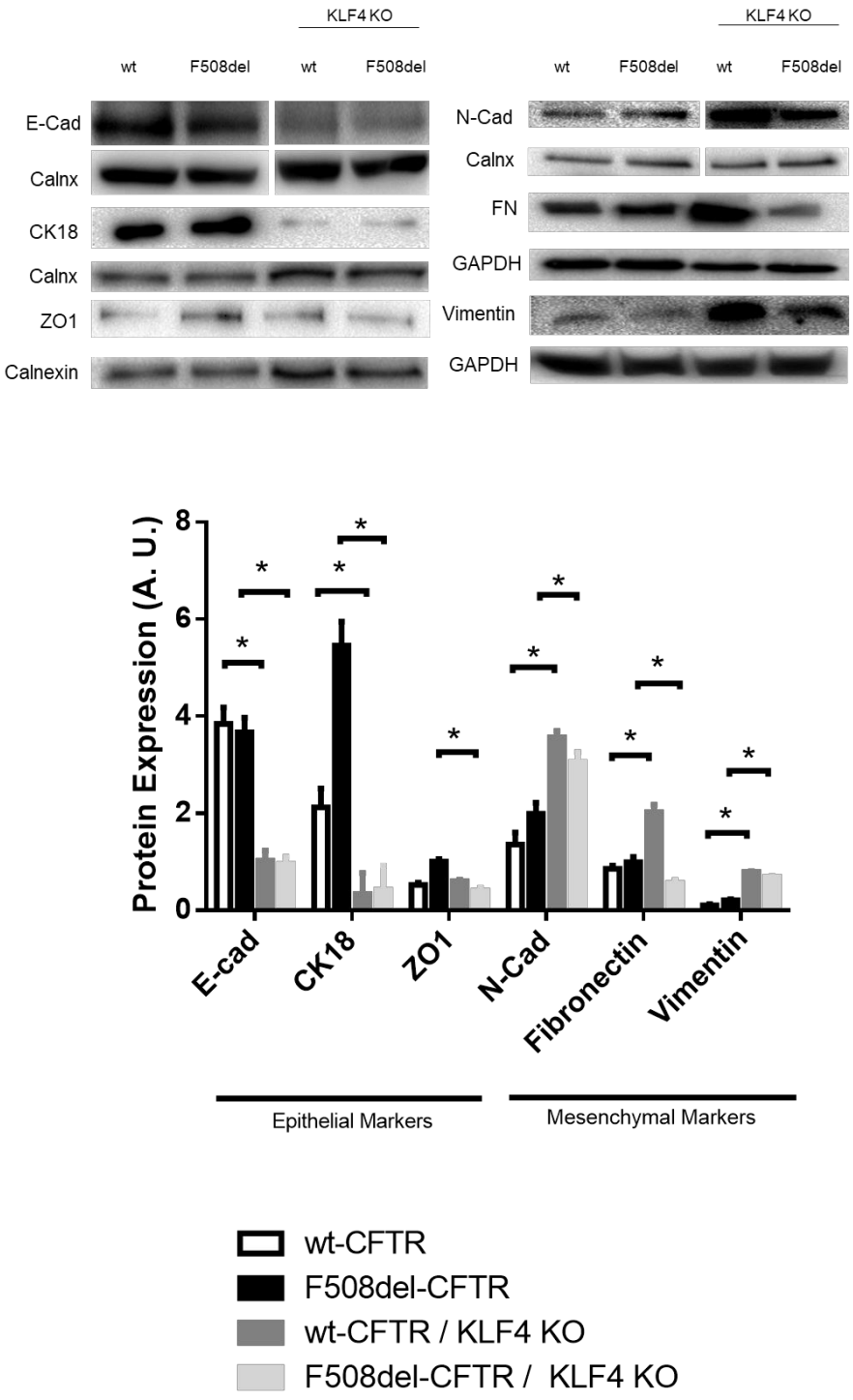

Figure 3.5 - KLF4 KO impact on differentiation markers.
Protein levels on wt- and F508del-CFTR CFBE and their respective KLF4 KO counterparts. Representative WB of E-Cad, N-Cad, CK18, ZO-1, Fibronectin (FN) and Vimentin. Calnexin, GAPDH and Vinculin were used as loading control. Data are normalized to loading control and showed as arbitrary units (A. U.) (n=3, unpaired t-test, p<0.05).

In terms of the impact of KLF4 KO on the levels of EMT-associated transcription factor (TF) Twist1 (Fig.3.6), we observed that besides being up-regulated in CF cells, KLF4 KO has a differential impact on wt- vs F508del-CFTR CFBE cells. Indeed, while KLF4 KO had no major impact on the Twist1 levels in

wt-CFTR CFBE cells, it produced a marked decrease of this TF in F508del-CFTR CFBE cells. KLF4 KO also promotes the expression of TGFβ-related proteins (TGFβ Receptors I and II) whose signalling is often associated with EMT in wt-CFTR CFBE cells (although a trend may also be observed in F508del-CFTR albeit non-significant). Nevertheless, when we look at SMADs, phosphorylated (active) SMAD2 (pSMAD2) is differentially modulated by KLF4 KO (upregulated in wt- and downregulated in F508del-CFTR cells) and a significant decrease of SMAD7 was observed and only in F508del-CFTR CFBE cells (Fig.3.6).

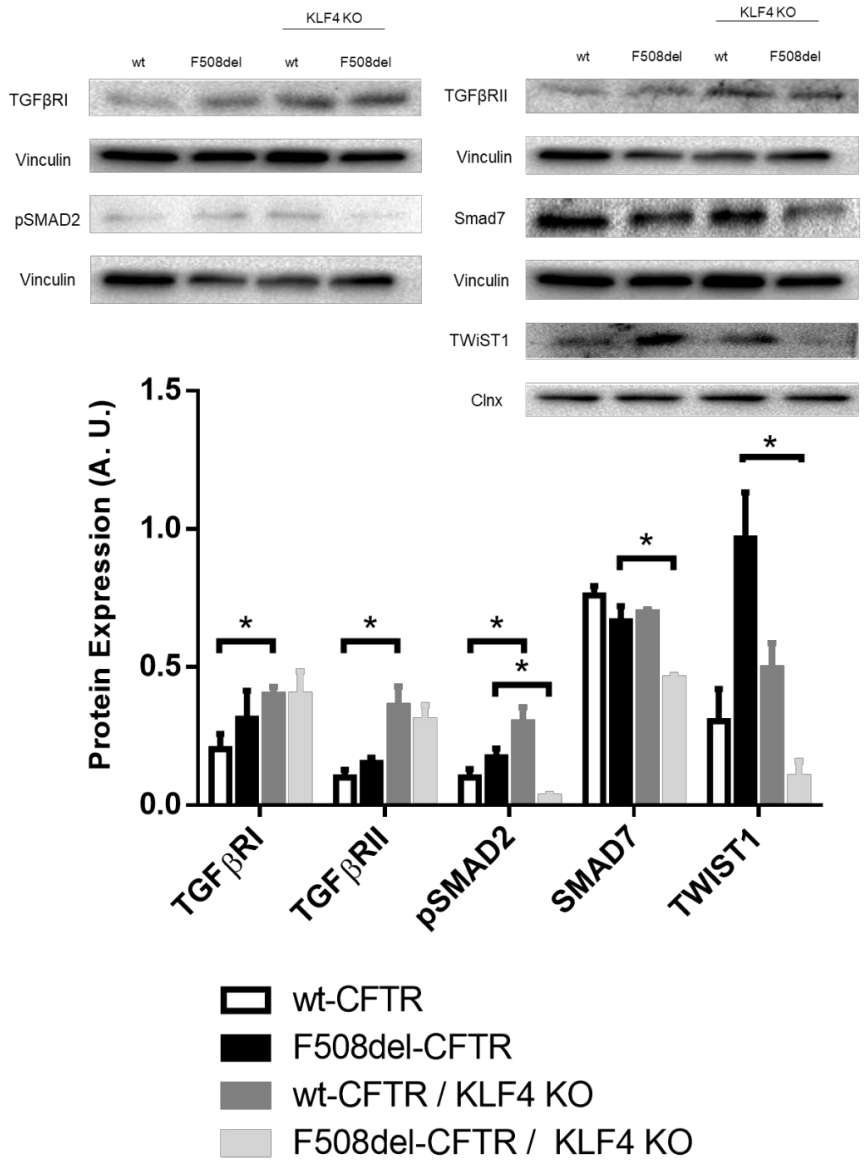

Figure 3.6 - KLF4 impact on the TGFβ pathway proteins and TWIST1 levels.
Protein levels on wt- and F508del-CFTR CFBE and their respective KLF4 KO counterparts. Representative WB of TGFβRI, TGFβRII, pSMAD2, SMAD7 and TWIST1. Calnexin and Vinculin were used as loading control. Data are normalized to loading control and showed as arbitrary units (A. U.) (n=3, unpaired t-test, p<0.05).

KLF4 KO showed no marked impact on the levels of other differentiation markers, such as connexin (Cx) 31, Occludin and CK5, as well as other TFs such as SNAIL+SLUG (Fig.S3.2).

Recently, immortalized human airway basal cells that retain multipotent differentiation capacity over long-term culture were developed (Basal Cell immortalized non-smoking 1.1 – Bci NS1.1). These cell lines display a tremendous potential to study the processes underlying epithelial differentiation in the CF context. (Walters, Gomi et al. 2013, Wang, Lou et al. 2019). Having access to these cells, we decided to ascertain the expression of KLFs during BCi differentiation.

Our data seem to indicate that KLF4 expression follows a pattern of expression that increases from day 0 to day 15/20 and a slight decrease from day 20 onwards. Noticeably, IL4 treatment promoted KLF4 overexpression (Fig.3.7A).

We then decided to characterize the pattern of expression of KLF2 and KLF5 (Fig.3.7B). The data shows that KLF5 expression followed a pattern of expression somewhat like the one observed for KFL4. However, KLF2 expression decreased with differentiation. We have also observed that CFTR expression increases steadily with differentiation (maximum at day 30) (Fig.3.7C). qPCR data also confirmed this pattern of expression for KLF4 and KLF5, although having contrasting results in terms of the KLF2 mRNA levels (Fig.3.7D).

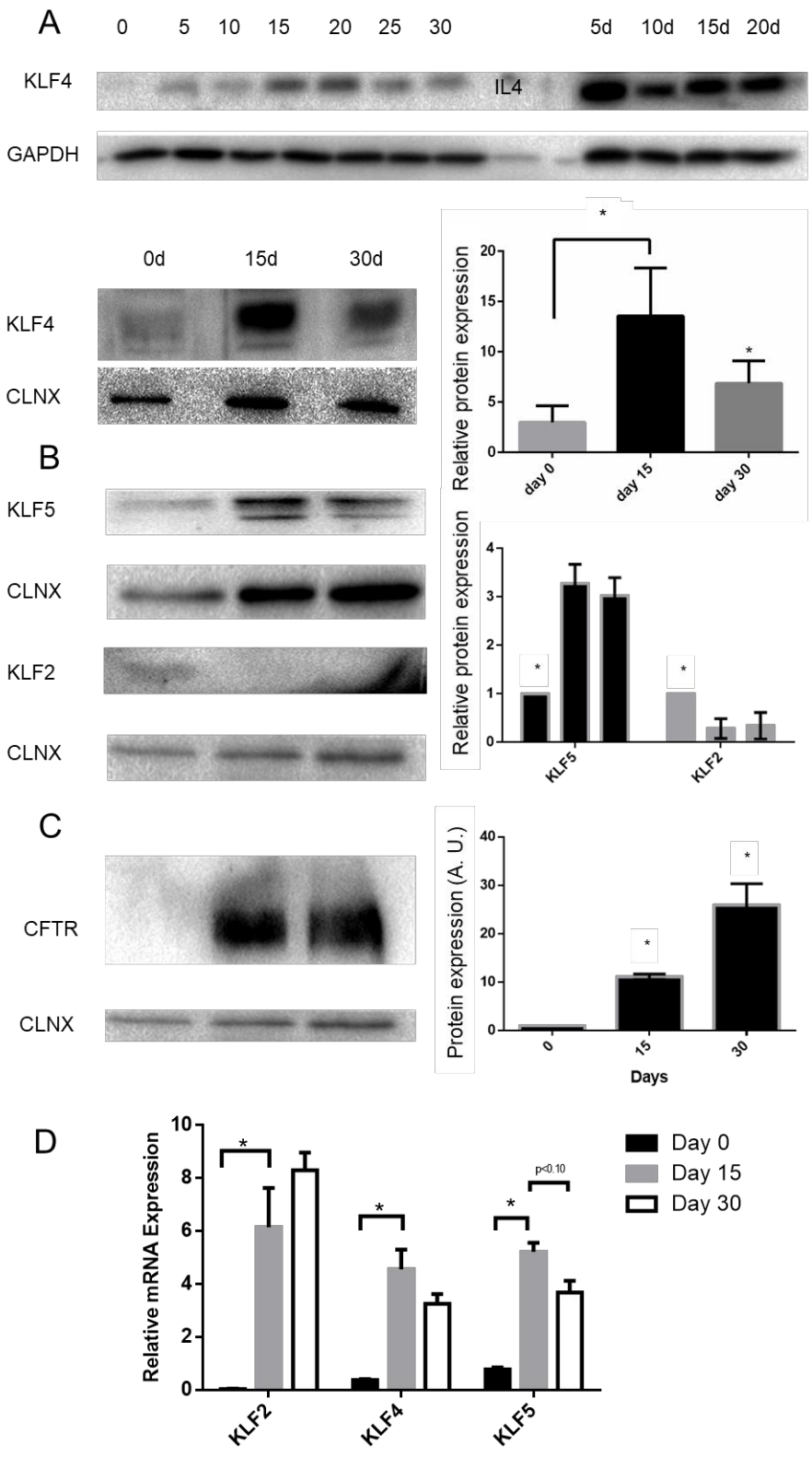

Figure 3.7 - KLF2, KLF4, KLF5 and CFTR expression levels during BCi differentiation.
KLF4 ***(A)****, KLF5 and KLF2* ***(B)*** *and CFTR* ***(C)*** *expression during BCi differentiation. Representative KLF4, KLF2, KLF5 and CFTR representative WB images are shown. Calnexin was used as loading control. Data is normalized to loading control and showed as relative expression (vs. D0) (B) or as arbitrary units (A. U.) (A and C) (n=3, unpaired t-test, p<0.05).* ***(D)*** *mRNA levels of KLF2, KLF4 and KLF5 were assessed by qRT-PCR. Samples were retrieved during BCi differentiation (n=3, unpaired t-test, p-value<0.05).*

Analysis of the levels of signalling pathways related to differentation in BCi cells show that β-Catenin, EGFR, GSK3β and pAKT levels mimic KLF4 pattern of expression. Ki-67 and Desmoplakin I/II (DSPI/II) on the other hand seem to decrease their expression during differentiation (Fig.3.8).

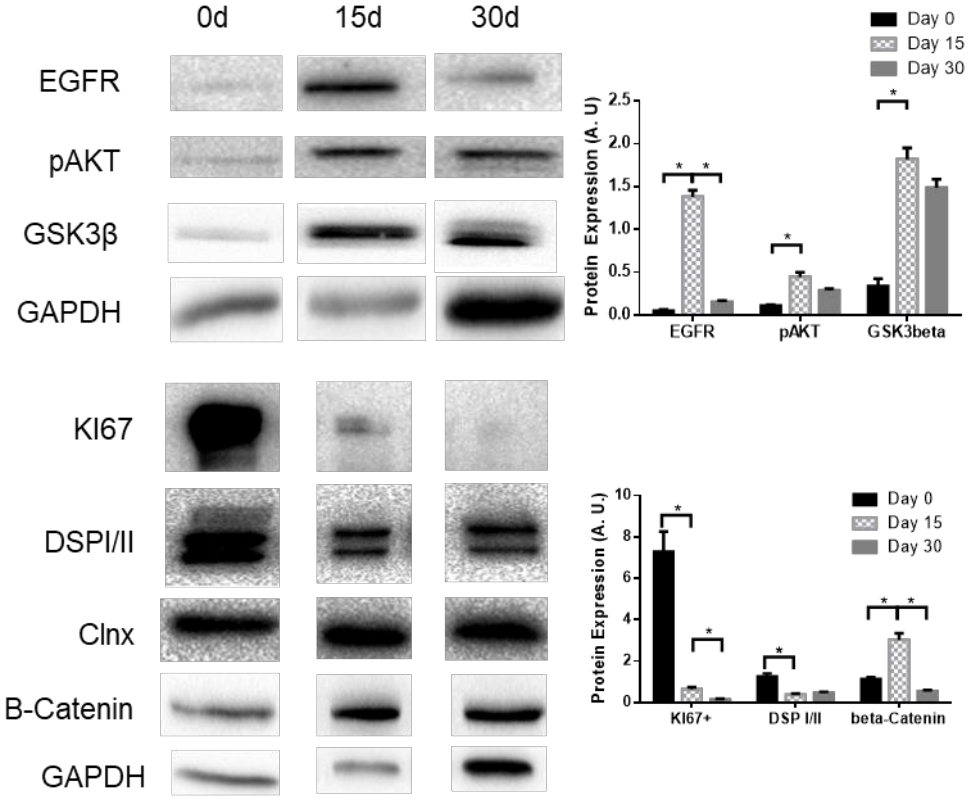

Figure 3.8 - Expression levels of EGFR, pAKT and GSK3β during BCi differentiation.
Representative WB of EGFR, pAKT, GSK3β, Ki-67, DSPI/II and β-catenin expression in BCi cells during differentiation. Calnexin and GAPDH were used as loading control. Data is normalized to loading control and showed as arbitrary units (A. U.) (n=3, unpaired t-test, p<0.05).

Discussion

KLF4 has been reported to transactivate promoters of epithelial genes like laminin 1 (Higaki, Schullery et al. 2002), Cytokeratin (CK) 19 (Brembeck and Rustgi 2000) and CK4 (Okano, Opitz et al. 2000). KLF4 roles in differentiation have been reported in a variety of tissues. For example, KLF4 is required for Goblet cell differentiation (Katz, Perreault et al. 2002), lung differentiation (Jean, George et al. 2013), epithelial barrier formation (Segre, Bauer et al. 1999), monocyte differentiation (Feinberg, Wara et al. 2007), testis (Behr and Kaestner 2002) and T-cells (Panigada, Porcellini et al. 1999). Moreover, KLF4 has been described as facilitating cutaneous wound healing by promoting fibrocyte

generation (Ou, Shi et al. 2015). Another study has shown that Cx 26 overexpression as a consequence of KLF4 KO delayed epidermal barrier recovery (Djalilian, McGaughey et al. 2006).

This work aimed at characterizing the possible impact of KLF4 KO on proliferation, differentiation, and wound healing rate in the context of CF.

Our data (Fig.3.1 and Fig.S3.1) show that KLF4 KO does not have a major impact on proliferation rate of CFBE cells, as assessed by its impact on the growth curves, Ki-67^{+} staining and Ki-67 levels assessed by WB. We expected that, considering its role as a growth inhibitor (Chen, Johns et al. 2001, Yoon, Chen et al. 2003), KLF4 KO would induce a higher proliferation rate (Katz, Perreault et al. 2005). Nevertheless, our data, show minimal changes in Ki-67 levels and growth rates, indicating that KLF4 KO does not affect the proliferation rate in our system. We speculate that this may be due to cell-type specificity or due to activation of compensatory mechanisms that maintain the proliferation levels steady. In fact, we have seen that KLF4 KO induced overexpression of KLF5 (Fig.3.4), which may counteract the effects of KLF4 abrogation, as KLF5 is often considered a promoter of proliferation (Ghaleb, Nandan et al. 2005).

We then evaluated the impact of KLF4 levels on epithelial integrity as assessed by the acquisition of TEER and wound healing (Fig.3.2). Taken together, these data show once again that, for a variety of cellular processes, KLF4 had differential effects depending on the CFTR state of the cells. Indeed, KLF KO had opposing effects on TEER acquisition and wound healing rate in wt- vs F508del-CFTR CFBE cells. In fact, in wt-CFTR CFBE cells, KLF4 KO decreases the wound healing rate and promotes a “leakier” epithelium, phenotypes often associated with a more mesenchymal state. Such an impact has been described previously (Agbo, Huang et al. 2019). Surprisingly, in F508del-CFTR cells, which are already more mesenchymal (Rout-Pitt, Farrow et al. 2018, Amaral, Quaresma et al. 2020), KLF4 KO leads to the opposite effect, with cells acquiring higher levels of TEER. We speculate that since CF cells start from a more mesenchymal state, this may influence the outcome of KLF4 KO. Noticeably, KLF4 KO impact on KLF2 expression is also dependent on the CFTR status (Fig. 3.4) and KLF2 has been show to mediate EMT (Xia, Bhandari et al. 2018). Consistently, KLF4 has been shown to prevent EMT in human corneal epithelia (Tiwari, Loughner et al. 2017) as well as in some types of lung (Wu, Lin

et al. 2019) and gastrointestinal cancers (Cui, Shi et al. 2013). In fact, in some contexts, it has been shown that KLF4 actively promotes Mesenchymal-to-Epithelial transition, i.e., MET (Li, Zhao et al. 2017).

We also observed here that KLF4 KO seems to promote the presence of "sharper" wound edges (Fig.3.3), thus providing 'direction' to the wound closure and thus with a likely impact on the wound healing kinetics. It has been shown in other cell lines (mouse fibroblasts) that KLF4 may have an impact on lamellipodia and on actin organization. Therefore, the observed impact of KLF4 in these processes is not totally unexpected (Brauer, Kim et al. 2018).

Our data (Fig.3.4,3.5) indicate that KLF4 KO has a profound impact on the levels of several differentiation markers and related transcription factors. Overall, these tend to indicate a shift towards a more mesenchymal phenotype in CF cells. Considering that (as shown in Chapter 1) F508del-CFTR cells are already more mesenchymal/cancer-like, we can then speculate that this transformation may have a higher relative impact on wt-CFTR cells. This may explain why KLF4 KO has a differential impact on the overall cellular processes in CF vs non-CF cells. Since KLF4 has been shown to prevent EMT (Tiwari, Loughner et al. 2017), the effect of its KO goes in accordance with what was expected, especially taking into account that KLF4 is a transcriptional factor of some of the evaluated genes, namely E-cad, N-cad and vimentin. (Tiwari, Meyer-Schaller et al. 2013). For instance, E-cad downregulation by decreasing levels of KLF4 has been previously reported (Yori, Johnson et al. 2010), in parallel with the upregulation of vimentin and N-cad (Tiwari, Meyer-Schaller et al. 2013). Also, KLF4 has been shown to promote CK18 expression (Chen, Whitney et al. 2003). Considering that KLF4 has a differential impact on CFTR levels and on CFTR-dependent wound healing, mediators that are differentially regulated in wt- and F508del-CFTR may be of particular importance. Among the ones tested, ZO-1 and Fibronectin are differentially modulated by KLF4 KO. In fact, ZO-1 regulation and signalling has been associated with CFTR, having been demonstrated that ZO-1 directly interacts with CFTR to regulate epithelial differentiation (Ruan, Wang et al. 2014). Consistently, there it was reported that ZO-1 mislocalization and loss of function occur in the absence of CFTR, albeit with no impact on the ZO-1 expression levels (Castellani, Guerra et al. 2012). In fact, KLF4 KO has been previously shown to promote ZO-1 upregulation and mislocalization (Yu, Chen et

al. 2012). Regarding Fibronectin, it was previously reported to be upregulated in the secretome of CF bronchial epithelia (Peters-Hall, Brown et al. 2015) and its levels have been reported to be modulated by KLF4 (Jean, George et al. 2013). Moreover, it may have an impact in CF pathology as it may have an impact in Pseudomonas aeruginosa colonization. (Badaoui, Zoso, et al. 2020) Therefore, we can generally say that KLF4 KO induced a clear-cut EMT in wt-CFTR cells, but in F508del-CFTR cells the KLF4 KO seems to promote EMT but has somewhat contradictory effects in certain mesenchymal markers/promoters like upregulation of fibronectin and downregulation of SMAD7 and TWIST1. Interestingly, in Chapter 2 KLF4 KO was shown to promote CFTR levels and in wt-CFTR CFBE cells, so we speculate whether this upregulation of CFTR expression may be a way of compensating the emerging EMT that KLF4 KO causes, possible through a feedback mechanism to retain epithelial function.

Some of the impact of KLF4 on differentiation and EMT have been associated with TGFβ signalling (He, Zheng et al. 2015, Sun, Peng et al. 2017, Fujimoto, Hayashi et al. 2019, Tiwari, Swamynathan et al. 2019), which in turn can be linked to CFTR through NEDD4L (Bossmann, Ackermann et al. 2017, Yan, Du et al. 2018) (Fig.S3.3). In fact, CFTR has been previously linked to the TGFβ pathway, as TGFβ inhibits CFTR expression and individuals with CF have increased TGFβ signalling (Prulière-Escabasse, Fanen et al. 2005, Snodgrass, Cihil et al. 2013, Nicola, Kabir et al. 2019). Therefore, we also characterized here the impact of KLF4 KO on the expression levels of some TGFβ signalling associated proteins (Fig.3.6). Overall, KLF4 KO seems to promote the expression of TGFβRI and RII, although this effect is only statistically significant in wt-CFTR cells. Analyses of the SMADs levels, show that phosphorylated (active) SMAD2 is increased in wt-CFTR cells and decreased in F508del-CFTR cells, while the TGFβ-inhibitory SMAD7 is decreased in F508del-CFTR cells. Altogether, these seem to indicate that KLF4 KO promotes TGFβ signalling in wt-CFTR cells both at the receptor and pSMAD2 levels, which may promote EMT. On the other hand, KLF4 KO has somewhat contrasting impacts on F508del-CFTR cells, as it downregulates the expression of opposing factors (pSmad2 and Smad7), with a more unpredictable result in which non-canonical TGFβ signalling may also have an impact (Zhao, Crowe et al. 2000, Korrodi-Gregório, Silva et al. 2014).

We then evaluated the levels of TWIST1, transcription factor associated with EMT. In fact, TWIST1 was upregulated in CF cells but the impact of KLF4 KO on TWIST1 levels is differential in CF vs non-CF cells as they decrease in F508del- but not in wt-CFTR cells. Given its role as a regulator of EMT (Meng, Chen et al. 2018), we speculate that this differential impact on TWIST1 levels may at least partially explain the differential responses of wt- and F508del-CFTR CFBE cells to KLF4 KO. Figure 3.9 summarizes the different effects of KLF4 KO in wt- and F508del-CFTR cells.

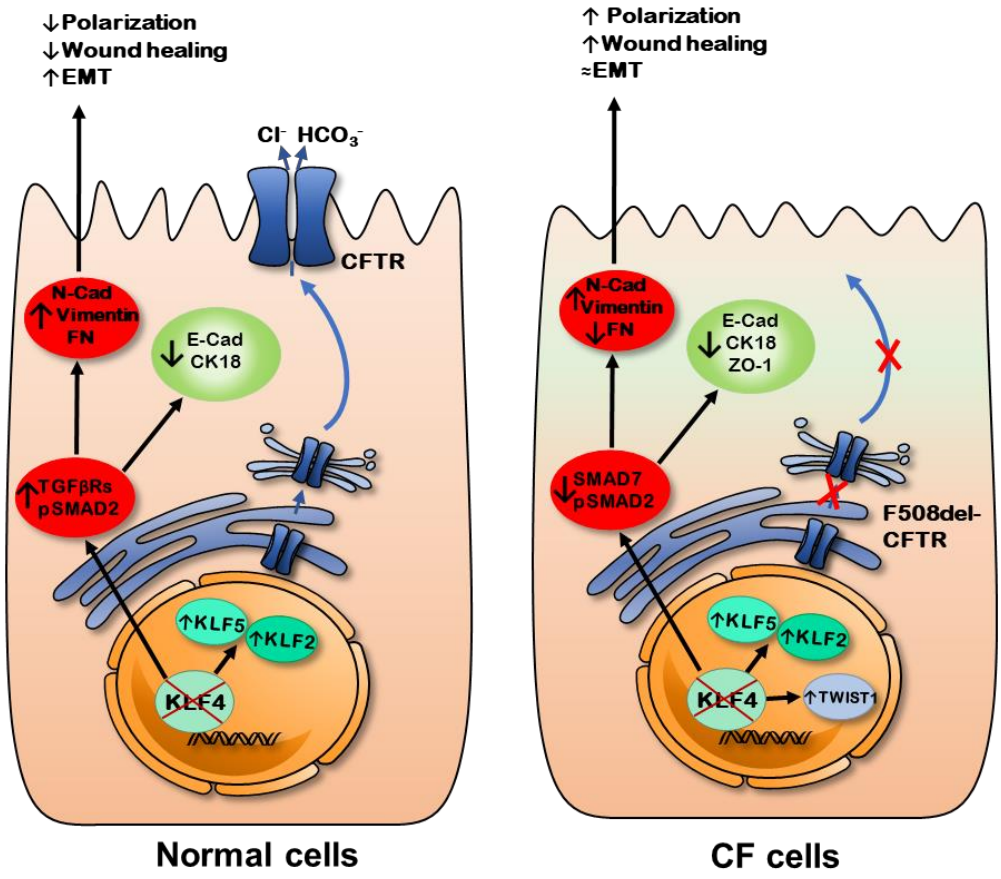

Figure 3.9 – Effects of KLF4 KO on wt- vs F508del-CFTR cells.
KLF4 KO seems to modulate the expression of several differentiation markers and promote a somewhat more mesenchymal phenotype. Nevertheless, there are markers that are differentially regulated in wt- vs F508del-CFTR cells. Ultimately, the KLF4 KO seemed to modulate polarization and wound healing depending on the CFTR status.

Using a model for airway epithelial differentiation (BCi cells, Fig.3.7,3.8), we observed that KLF2 expression decreases with BCi differentiation, being replaced by KLF4 and KLF5 expression which peaks at 15 days of differentiation. We observed that GSK3β, EGFR and pAKT display similar patterns of expression. Interestingly, their maximal expression predates the maximum expression of CFTR which is reached only at day 30, raising the hypothesis that

the expression of these proteins is an upstream event to CFTR expression. Also, the pro-inflammatory IL4, which may play a role in CF pathophysiology (Nunes, Becker et al. 2009, Bergin, Hurley et al. 2013), promotes the expression of KLF4, as previously reported (Kapoor, Niu et al. 2015).

Altogether, these results shed some light on the impact of KLF4 in the cellular processes of differentiation, proliferation wound healing and EMT that are important in the context of CF. Further studies however are required to fully address the full role that KLF4 (or its downstream effectors) plays in those processes to propose it as a potential therapeutic target in the context of CF. This may be of particular interest in the correction of the developmental and differentiation/regeneration aspects that are disrupted in CF.

Supplementary Data

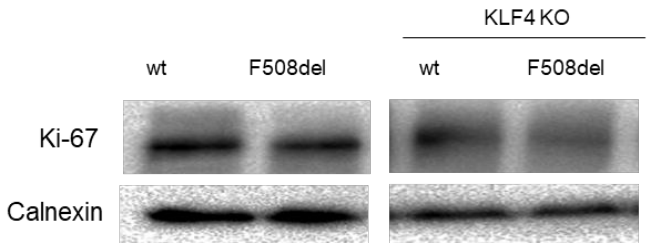

Figure S3.1 - KLF4 KO does not have major impact on the expression levels of Ki-67.
Representative images of WB of Ki-67 in wt- and F508del-CFTR CFBE cells and their KLF4 KO counterparts, Calnexin was used as a loading control (n=3).

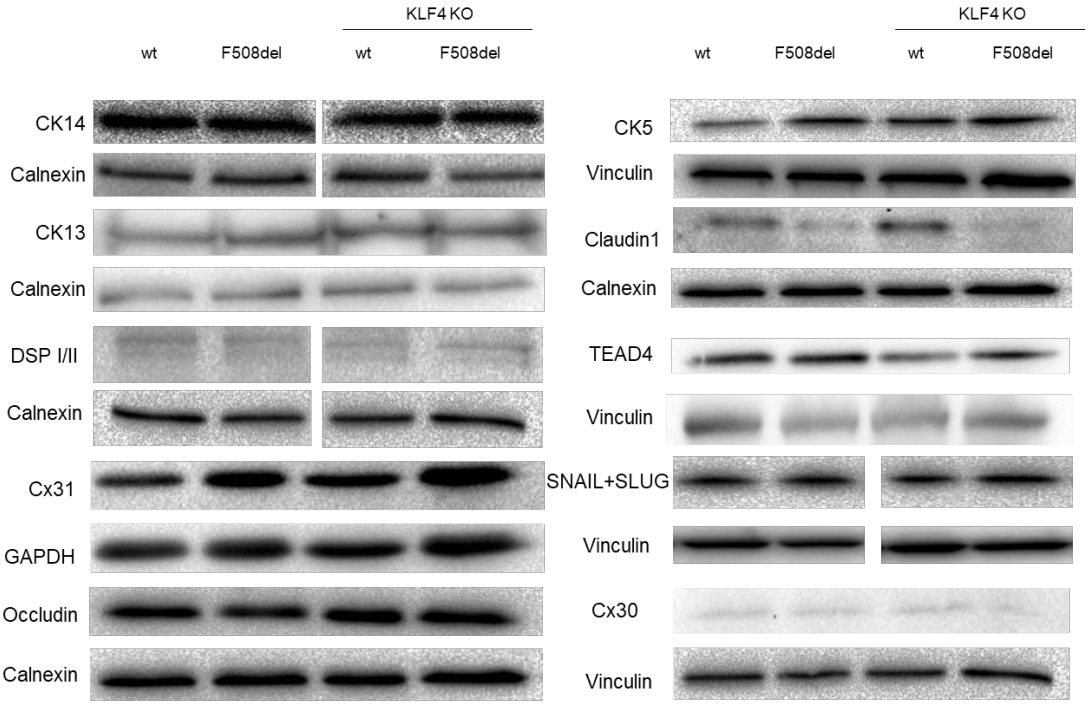

Figure S3.2 - KLF4 KO showed no major impact on the levels of several differentiation markers and transcription factors.
Representative images of WB of CK14, CK13, DSPI/II, Occludin, Cx30, Cx31, CK5, Claudin1, TEAD4 and SNAIL+SLUG on wt- and F508del-CFTR CFBE cell and their KLF4 KO counterparts. Calnexin, GAPDH and Vinculin were used as loading controls (n=2-4).

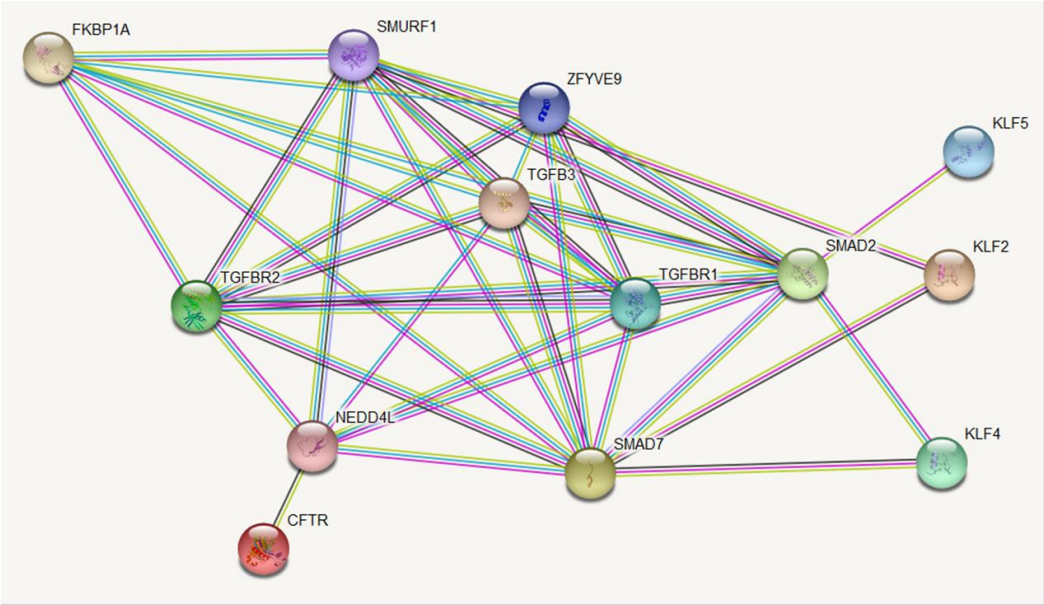

Figure S3.3 -Signalling pathways connecting TGFβ to CFTR.

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Section 4 – Concluding remarks

Despite its relatively simple aetiology, the physiopathology underlying Cystic Fibrosis still remains not fully understood. Indeed, increasing evidence points to CFTR being more than just an ion channel and the basic defect in ion homeostasis does not appear to account for the pleiotropy of processes occurring in CF epithelial disease.

The classical way of considering CF as a basic Cl^-/HCO_3^- transport defect, does not account for the extensive alterations that are observed in CF epithelia, namely in the airways. Underlying alterations in the processes of epithelial differentiation, regeneration, wound healing, proliferation, and inflammation seem to play a decisive role in CF disease progression. CFTR, a large multi-domain protein with a wide variety of interactors seems to play, directly or indirectly, a central role in the processes of embryonic development and epithelial differentiation.

This doctoral work aimed to contribute to increase the knowledge in the topic of epithelial differentiation in the context of CF by clarifying three major aspects, namely:

Firstly, and based on previous reports, we evaluated the occurrence of alterations in the key cellular processes of proliferation, polarization, wound healing and epithelial differentiation in CF.

Secondly, we tested whether some key transcriptional factors in these processes - the Krüppel-like factors (KLFs) – previously related to CF (KLF2, 4 and 5) were differentially expressed in human native lung and cells line and further explored the significant impact that one of them (KLF4) had on wt- (but not F508del-) CFTR expression levels and function by evaluating the possible signalling pathways involved.

Our last goal was to ascertain the impact of KLF4 knock-out (KO) on proliferation, polarization, wound healing, and differentiation in the context of CF.

The first part of our work led us to confirm that CF cells display higher proliferation and have inherent defects in the polarization as expressed by their marked inability to acquire high levels of transepithelial resistance. These effects were coupled to a slower wound healing rate and marked alterations in epithelial differentiation. Noticeably, in a collaborative work with other lab members, we found that a partial EMT signature is present in CF tissues/cells, as defined by a clear-cut increase in mesenchymal markers with no repression of epithelial

markers. Moreover, the EMT-specific transcriptional factor TWIST1 emerged as the most interesting EMT transcription factor in the CF context. Therefore, this work led to the conclusion that CF cells display a phenotype that is somewhat cancer-like.

The second part of this work focused on KLFs and their possible impact in the context of CF. An initial assessment indicated KLF4 as a promising factor in the CF-associated differentiation defect and thus we further characterized its putative role in the modulation of CFTR expression levels and function. Our data show that KLF4 is upregulated in CF vs non-CF cells thus deserving further mechanistic insight. Using KD/KO and overexpression approaches we found that KLF4 has a dual role that is dependent on the CFTR status of the cells. Our data show that KLF4 acts as a negative regulator of wt-CFTR expression levels and function, but F508del-CFTR seems to be refractory to this effect, as modulation of KLF4 levels being without significant impact on F508del-CFTR expression levels or function. Since CFTR and KLF4 do not directly interact and because the CFTR gene is not transcribed by KLF4, this functional interaction between the two proteins must be mediated through signalling pathways. Therefore, using a Bioinformatics approach, we identified the EGFR-AKT and β-Catenin-GSK3β pathways as possibly involved in the KLF4 impact on CFTR. By assessing the effects of inhibitors we were able to identify that GSK3β is a KLF4-independent modulator of both wt- and F508del-CFTR, while AKT acts as a negative regulator of wt-CFTR that is at least partially dependent on KLF4, while positively regulating F508del-CFTR. Ultimately, these data led to the identification of a novel pathway modulating CFTR. Given that this pathway has distinct effects on CF vs non-CF cells it has the potential of being the first mechanistic link of CF to the differentiation defect. In fact, KLF4 is known for having distinct effects in cancer vs non-cancer cells and, bearing in mind the concept established in part I that CF cells are more cancer-like, we can now better understand how KLF4 effects are context-dependent, i.e., CF vs non-CF.

The last part of this doctoral work had the goal of characterizing the impact of KLF4 KO on the cellular processes of proliferation, polarization (as TEER acquisition), wound healing and epithelial differentiation. Once again, KLF4 displayed an impact that is context-dependent, as it evidenced distinct effects on wt-CFTR vs F508del-CFTR expressing cells. KLF4 KO led to increased

polarization and wound healing rates in F508del-CFTR cells, while displaying opposite effects in wt-CFTR. Regarding epithelial differentiation in the non-CF context, KLF4 seems to contribute to a more mesenchymal phenotype. Yet, in collaboration with other members of the Amaral lab (Margarida Quaresma, namely), we were able to identify some players that are differentially regulated in a CF context-dependent manner such as the EMT- specific transcription factor TWIST1,as well as KLF2 and Fibronectin. Considering that CF cells are already more mesenchymal in nature, we hypothesize that these other factors can contribute to the distinct impact of KLF4 KO in the general landscape of the cell and CFTR modulation.

As a conclusion, we believe that this work contributed to a better understanding of the mechanistic relationship between epithelial differentiation and CFTR, namely by elucidating the role of KLF4 and some relevant signalling pathways. Notwithstanding, as it happens in science, it also raised new questions.

Thus, several aspects deserve future clarification for continuation of this work, namely:

1) Further clarification of the EMT-associated Transcription Factors that drive the partial EMT observed in CF cells, as well as the pathways that are modulated.
2) Elucidation of the intermediates that link KFL4 to CFTR, as these may constitute potential therapeutic targets.
3) Clarify the impact of the modulation of other proteins in the GSK3β and AKT signalling pathways and their impact in CFTR activity and levels.
4) Evaluate the impact of the modulation of GSK3β and AKT in the levels of epithelial and mesenchymal markers, proliferation and wound healing.
5) Unravelling additional pathways that modulate CFTR may provide further insight into the mechanistic link between epithelial differentiation and CFTR in the CF context.
6) Evaluate the impact of KLF4 KD and CFTR mutant expression in the differentiation of BCi cells.

Albeit limited, our contributions to the field are done with the objective of providing a background and a platform for new studies. Ultimately, increasing our global knowledge on this subject may open novel avenues for the treatment of CF but also of other ‘epithelial disorders’ like cancer.