From the Department of Neurology, Rostock University Medical Center

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Identifying Forkhead Box Q1 as a novel regulator of ferroptosis

Dissertation to obtain the academic degree of

Doctor rerum humanarum (Dr. rer. hum.)

of the Medical Faculty at the University of Rostock

Submitted by

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Rostock, 2023

https://doi.org/10.18453/rosdok_id00004666

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Date of submission: 28 Aug 2023

Date of defense: 19 Dec 2023

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ABSTRACT

Ferroptosis, an iron-dependent form of regulated cell death, is characterized by phospholipid peroxidation and metabolic constraint. Ferroptosis has emerged to play an important role in cancer biology to contribute into many pathologies. Cells respond to ferroptotic stimuli by regulation of selenoproteins, including the key regulator of ferroptosis phospholipid hydroperoxide-reducing enzyme glutathione peroxidase 4 (GPX4). Though, the underlying mechanisms and signalling pathways of ferroptotic cell death remain relatively unknown. Current research aims at deeper understanding of the pathophysiological role of ferroptosis and how it may be exploited for the treatment of cancer and neurodegenerative diseases.

In this cell-based study, the GPX4 inhibitor (1S, 3R)-RSL3 (RAS selective lethal) and the system xc^- (a cysteine/glutamate antiporter system) inhibitor erastin were used to identify Forkhead Box Q1 (FOXQ1) as a promising factor involved in the regulation of cellular ferroptosis sensitivity. The transcription factor FOXQ1 is a member of forkhead proteins that control important functions in biological development and tumorigenesis. A very limited number of studies have investigated the role of the FOXQ1 in human cancer. However, whether FOXQ1 and FOXQ1-regulated genes have a potentially predictable role in ferroptosis remains to be further explored, since pleiotropic responses in different cell lines were observed in the present study. An overexpression cloning approach was used to elucidate the factors and cell-autonomous mechanisms that underlie the regulation of ferroptosis in the given cell line. Present data suggest increased sensitivity to ferroptosis in FOXQ1 overexpressing cells compared to control cells. The finding was expanded to a panel of human breast cancer cell lines to show that overexpression of FOXQ1 mediated the sensitivity to RSL3- and erastin-induced ferroptotic cell death. To demonstrate the effect of FOXQ1 mediated sensitivity to ferroptosis in stably overexpressing cancer cell lines, several ferroptosis regulatory genes were monitored at several time points.

In an attempt to further illuminate the mechanism of ferroptosis execution, targeted knock out (KO) of FOXQ1 in different cancer cell lines was performed using CRISPR/CAS technology, which resulted in reduced invasive ability of the cells. Furthermore, it was demonstrated that FOXQ1 deletion results in lower proliferation rates of cells and cell death. Consequently, various cancer cell lines overexpressing FOXQ1 were generated to enable investigations on

the role of FOXQ1 and the role of novel transcriptional targets of FOXQ1 in ferroptosis that may serve as a potential therapeutic target for the development of anticancer therapies.

ZUSAMMENFASSUNG

Ferroptose, eine eisenabhängige Form des regulierten Zelltods, ist durch Phospholipidperoxidation und metabolische Restriktion gekennzeichnet. Ferroptose spielt eine wichtige Rolle in der Tumorbiologie und trägt zur Entstehung vieler Pathologien bei. Zellen reagieren auf ferroptotische Reize durch die Regulation von Selenoproteinen, einschließlich des Schlüsselregulators für Ferroptose, dem Phospholipid-Hydroperoxid-reduzierenden Enzym Glutathionperoxidase 4 (GPX4). Die zugrunde liegenden Mechanismen und Signalwege des ferroptotischen Zelltods sind jedoch weitgehend unbekannt. Aktuelle Forschungen zielen darauf ab, das pathophysiologische Verständnis der Ferroptose und ihre mögliche Nutzung für die Behandlung von Krebs und neurodegenerativen Erkrankungen zu vertiefen.

In dieser zellbasierten Studie wurden der GPX4-Inhibitor (1S, 3R)-RSL3 (*RAS selective lethal*) und der Inhibitor von System Xc (einem Cystein/Glutamat-Antiporter-System), Erastin, verwendet und es konnte *Forkhead Box Q1* (FOXQ1) als vielversprechender Faktor bei der Regulation der zellulären Ferroptose-Empfindlichkeit identifiziert werden. Der Transkriptionsfaktor FOXQ1 ist ein Mitglied der *Forkhead*-Proteine, die wichtige Funktionen bei der biologischen Entwicklung und der Tumorentstehung kontrollieren. Nur eine sehr begrenzte Anzahl von Studien hat die Rolle von FOXQ1 bei menschlichem Krebs untersucht. Ob FOXQ1 und die von FOXQ1 regulierten Gene eine potenziell vorhersagbare Rolle bei der Ferroptose haben, muss jedoch weiter untersucht werden, da in der vorliegenden Studie pleiotrope Effekte in verschiedenen Zelllinien beobachtet wurden. Ein Überexpressionsklonierungsansatz wurde verwendet, um Faktoren und zellautonome Mechanismen zu identifizieren, die der Regulation der Ferroptose in der verwendeten Zelllinie zugrunde liegen. Die vorliegenden Daten legen nahe, dass FOXQ1 überexprimierende Zellen im Vergleich zu Kontrollzellen eine erhöhte Empfindlichkeit gegenüber Ferroptose aufweisen. Diese Erkenntnis wurde durch Untersuchungen an einer Reihe von humanen Brustkrebszelllinien erweitert, welche zeigen, dass die Überexpression von FOXQ1 die

Empfindlichkeit gegenüber durch RSL3 und Erastin induziertem ferroptotischen Zelltod vermittelt. Um den Effekt der durch FOXQ1 vermittelten Empfindlichkeit gegenüber Ferroptose in stabil überexprimierenden Krebszelllinien zu demonstrieren, wurden mehrere ferroptose-regulierende Gene zu verschiedenen Zeitpunkten analysiert.

In dem Bestreben, den Mechanismus des Ferroptosevorgangs weiter zu ergründen, wurde mit Hilfe der CRISPR/CAS-Technologie ein gezielter Knockout (KO) von FOXQ1 in verschiedenen Krebszelllinien durchgeführt, was zu einer verringerten Invasivität der Zellen führte. Des Weiteren wurde gezeigt, dass die Deletion von FOXQ1 zu einer geringeren Proliferationsrate der Zellen und zum Zelltod führt. Folglich wurden verschiedene Krebszelllinien erzeugt, die FOXQ1 überexprimieren, um Untersuchungen zur Rolle von FOXQ1 und den neuartigen Transkriptionszielen von FOXQ1 in der Ferroptose zu ermöglichen, die als potenzielle therapeutische Ziele für die Entwicklung von Antikrebstherapien dienen können.

LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
µM	micromolar
°C	degree Celsius
•OH	hydroxyl radical
%	percent
A	Ampere
AA	arachidonic acid
AB	antibody
ACS	acyl-CoA synthetase
ACSL	acyl-CoA synthetase long-chain family members
ADP	adenosine diphosphate
AIFM2	apoptosis inducing factor mitochondria associated 2
AKT1	AKT Serine/Threonine Kinase 1
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
Aβ	amyloid-β
bp	base pairs
BSA	bovine serum albumin
BSO	L-buthionine-sulfoximine
BAD	BCL-2 antagonist of cell death
BAK	BCL-2 homologous antagonist
BAX	BCL-2-associated X protein
C	carbon/carbamidomethyl
CCND1	cyclin D1
CDH1	cadherin-1
CDH2	cadherin-2
CDK4,5,6	cyclin-dependent kinase 4,5,6
Cas9	CRISPR associated protein 9
crRNA	single CRISPR RNA
Ctrl	control
CE	cholesteryl esters
c-Myc	MYC Proto-Oncogene
CoA	coenzyme A
CoQ_{10}	ubiquinone
CRISPR	clustered regularly interspaced short palindromic repeats
Cys	cysteine
cDNA	complementary DNA
CXCL9	chemokine ligand 9
DACH1	dachshund homolog 1
DNA	deoxyribonucleic acid
DSB	double strand breaks
dNTP	deoxynucleotides
DAPI	4’-6-diamidino-2-phenylinodole

DBD	DNA binding domain
DFO	deferoxamine
DG	diglycerols
DLL4	delta like canonical notch ligand 4
DMEM	Dubecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DISC	death-inducing signalling complex
DSC2	desmocollin 2
dNTPs	deoxynucleotides
EDTA	ethylendiaminetetraacetic acid
EDN1	endothelin 1
EGFR	epidermal growth receptor
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum/estrogen receptor
ERA	erastin
FA	fatty Acid
FCS	fetal calf serum
FDA	Food and Drug Administration
FDR	false discovery rate
Fe2+	ferrous iron
Fe3+	ferric iron
Fer-1	ferrostatin-1
FTH1	ferritin heavy chain
FTL	ferritin light chain
FIN	ferroptosis-inducing-agent
FN1	fibronectin 1
FOXA1	fork head box A1
FOXG1	fork head box G1
FOXO	fork head box O1
FOXP1	fork head box P1
FOXQ1	fork head box Q1
FSP1	ferroptosis suppressor protein 1
g	gram/gravitational force
gDNA	genomic DNA
Gb	gigabyte
GCH1	GTP cyclohydrolase 1
GCLC	γ-glutamate cysteine ligase catalytic subunit
GCLM	glutamate-cysteine ligase modifier subunit
GFP	green fluorescent protein
GOI	gene of interest
GPX	glutathione peroxidase
GPX4	glutathione peroxidase GPX4
GR	glutathione reductase
GSH	glutathione/γ-glutamylcysteineglycine
GSR	glutathione-disulfide-reductase
GSS	glutathione synthetase
GSSH	glutathione disulfide/oxidized GSH
GTP	guanosine-5'-triphosphate

h	hour
H_2O_2	hydrogen peroxide
HDR	homology-directed repair
HEK cells	human embryonic kidney cells
HNF4A	hepatocyte nuclear factor 4 alpha
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
Hz	Hertz
i.p	intraperitoneal
iFSP1	FSP1-specific inhibitor
IGF	insulin-like growth factor
IL1A	interleukin 1 alpha
IGFBP-2	insulin-like growth factor-binding protein 2
IL-6	interleukin 6
IRI	ischemia-reperfusion injuries
kDa	kilo Dalton
Keap1	Kelch-like ECH-associated protein 1
kg	kilogram
KO	knockout
L	liter
LB	lysogeny broth
LC/MS	liquid chromatography–mass spectrometry
L-H	lipid
Lip-1/3	liproxstatin-1/3
L-OH	lipid alcohol
LOOHs	lipid hydroperoxides
LOX	lipoxygenase
loxP	locus of X-over P1
LPC	lysophosphatidylcholine
LPCAT3	lysophosphatidylcholine acyltransferase 3
LPLAT	lysoPL acyltransferase
LPO	lipid peroxidation
M	molar/methionine
m/z	mass-to-charge ratio
MAM	mitochondrial associated membrane
MAP2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
MDM2	mouse double minute 2
min	minute
ml	milliliter
mM	millimolar
MN	motor neurons
MMP2	matrix metalloproteinase-2
ms	millisecond
mM	millimolar
MITF	microphthalmia-associated transcription factor
MTOR	mechanistic target of rapamycin kinase
MUFA	monounsaturated fatty acid

MYH11	myosin heavy chain 11
MYOCD	myocardin
NAC	N-acetyl-L-cysteine
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NDRG1	N-myc downstream regulated 1
NeuroD1	neurogenic differentiation 1
ng	nanogram
NMDA	N-methyl D-aspartate
NOX	NADP oxidase
NRXN3	neurexin 3-alpha
NHEJ	non-homologous end joining
ns	not significant
O_2^-	superoxide anion
OH-	hydroxide ion
p53	tumor suppressor p53
PAMP	protospacer adjacent motif
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDAC	pancreatic ductal adenocarcinoma
PE	phosphatidylethanolamine
PFA	paraformaldehyd
PFPA	perfluoropentanoic acid
PG	phosphatidylglycerol
pg	picogram
PI	phosphatidylinositol
PL	phospholipid
PL-OO●	lipid radical
PL-OOH	peroxidized phospholipid
PRRs	pattern recognition receptors
PS	phosphatidylserin
PSEN	presenilin
PTK2	protein tyrosine kinase 2
PUFA	polyunsaturated fatty acid
PV+	parvalbumin-positive
PVDF	polyvinylidene difluoride
qPCR	quantitative PCR
RAS	rat sarcoma virus
ROS	reactive oxygen species
rmp	revolutions per minute
RSL3	RAS-selective lethal compound 3
RT	room temperature
sec	seconds
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Se	selenium
Sec	selenocysteine

SeH	selenol
sgRNA	single guide RNA
shRNA	short hairpin RNA
*S*NAI1	snail family transcriptional repressor 1
SRF	serum response factor
SIRT1	silent information regulator 1
t	time
TAM	tamoxifen
TAGLN	transgelin
TE	Tris/EDTA
tet	tetracycline
Tf	transferrin
TF	transcription factor
TFR1	transferrin receptor
TNFα	tumour necrosis factor alpha
tRNA	transfer ribonucleic acid
TWIST1	twist family BHLH transcription factor 1
TXNRD1	thioredoxin reductase 1
Ub	ubiquitin
UTR	untranslated region
UV	ultraviolet
VCAN	versican
V	Volt
VEGFA	vascular endothelial growth factor A
VCP	valosin-containing protein
Vim	vimentin
WT/wt	wildtype
xCT	SLC7A11
α-Toc	α-tocopherol
γ-GCS	γ-glutamylcysteine synthetase
ZEBs	zinc finger E homeobox binding proteins

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1. INTRODUCTION

1.1. Ferroptosis, a new form of regulated cell death

Over the past decades, efforts have been made to characterise and reveal the mechanisms that underlie the process of cell death. The discovery of erastin and RAS-selective lethal compound 3 (RSL3) in a synthetically lethal compound screen led to the identification of a new form of cell death called ferroptosis (Yang and Stockwell 2008, Dixon, Lemberg et al. 2012). This new type of cell death was accompanied by iron accumulation and lipid peroxidation. The cell death appeared morphologically and biochemically distinct from already known mechanisms such as apoptosis and other forms of cell death (Dixon, Lemberg et al. 2012, Yang and Stockwell 2016). Erastin induces ferroptosis by inhibiting system xc^- (cystine/glutamate antiporter) resulting in the disruption of cystine uptake and the depletion of the main intracellular antioxidant glutathione (GSH), the synthesis of which requiring cysteine. Glutathione peroxidase 4 (GPX4), a master regulator in the ferroptosis process, uses GSH as a cofactor to reduce lipid hydroperoxides (L-OOH) to their corresponding lipid alcohols (L-OH) (Weaver and Skouta 2022).

In this enzymatic reaction, GPX4 uses two molecules of GSH which get oxidized (Conrad and Friedmann Angeli 2015). Therefore, it is important for the cells to maintain the necessary amount of the intracellular GSH. On the other hand, the cystine-glutamate antiporter, system xc^-, does also play a critical role by providing sufficient amount of cysteine for GSH biosynthesis to prevent ferroptosis in the cell (Conrad and Sato 2012). Hence, the metabolism of cellular cysteine has a major impact on the induction of ferroptosis (Badgley, Kremer et al. 2020).

Besides access amount of ferrous iron (Fe^{2+}) react with H_2O_2 in a Fenton type reaction which leads to the accumulation of ROS and ferroptosis (Dixon, Lemberg et al. 2012, Brigelius-Flohe and Maiorino 2013, Cao and Dixon 2016). After its discovery, multiple signalling pathways and molecular players have been linked to ferroptosis revealing the bigger picture of the underlying mechanisms. Biochemical reactions between oxidising radicals and the polyunsaturated fatty acid residues of the membrane lipids lead to extensive oxidation of the lipids of the cell membranes, resulting in their partial permeabilisation and finally in ferroptosis. Morphological analysis of ferroptotic cells showed shrunken mitochondria and increased mitochondrial membrane density compared to apoptosis and autophagy but no

effect on the nucleus or chromatin structure (Dixon, Lemberg et al. 2012, Santini, Cordone et al. 2018). To inhibit ferroptosis, cellular ROS–induced lipid peroxidation must be continuously detoxified through multiple pathways to prevent their accumulation (Perillo, Di Donato et al. 2020). Several metabolic and cellular processes were found to be involved in modulating the ferroptotic cell death pathway. Besides, number of pharmacological and natural compounds have been reported to regulate the processes of ferroptotic cell death (Zhang, Hu et al. 2021). The discovery of ferroptosis, a new form of iron-dependent regulated necrotic cell death has likely increased the opportunities in the field of anticancer therapy. Inhibition and induction of ferroptosis have potential roles in the treatment of various human diseases.

1.2. Key cellular process modulating ferroptosis execution

Several cellular processes have been studied to reveal mechanisms that determine a cells' sensitivity towards ferroptosis. Ferroptosis can be modulated by signals transduced via a series of metabolic processes. Glutathione depletion, GPX4 inactivation and the accumulation of lipid peroxides are key events during ferroptosis (Dixon and Stockwell 2014). System xc^- is the most upstream player of the cystine uptake pathway (Tang, Chen et al. 2021) (Fig.1). System xc^- is a heterodimeric amino acid antiporter composed of a catalytic subunit SLC7A11 (solute carrier family 7 member 1) and a regulatory subunit SLC3A2 (solute carrier family 3 member 2). It transports one extracellular molecule of cystine, the oxidized form of cysteine, into the cells in the exchange for one intracellular molecule of glutamate (Sato, Tamba et al. 1999, Conrad and Sato 2012). The SLC7A11 (also called xCT) is responsible for the transport activity, while SLC3A2 (also called 4F2) is necessary for membrane location of the heterodimer (Sato, Tamba et al. 1999, Dixon, Lemberg et al. 2012). Cystine taken up into the cell by system xc^- is immediately reduced to cysteine by either glutathione or thioredoxin (TNX) and subsequently available for the biosynthesis of GSH and cysteine containing proteins (Conrad and Sato 2012, Combs and DeNicola 2019). GSH in its reduced form represents the most abundant antioxidant molecule in the mammalian cells and is used as substrate by many redox enzymes including GPX4. GSH synthesis from its constituent amino acids involves two ATP-requiring enzymatic steps.

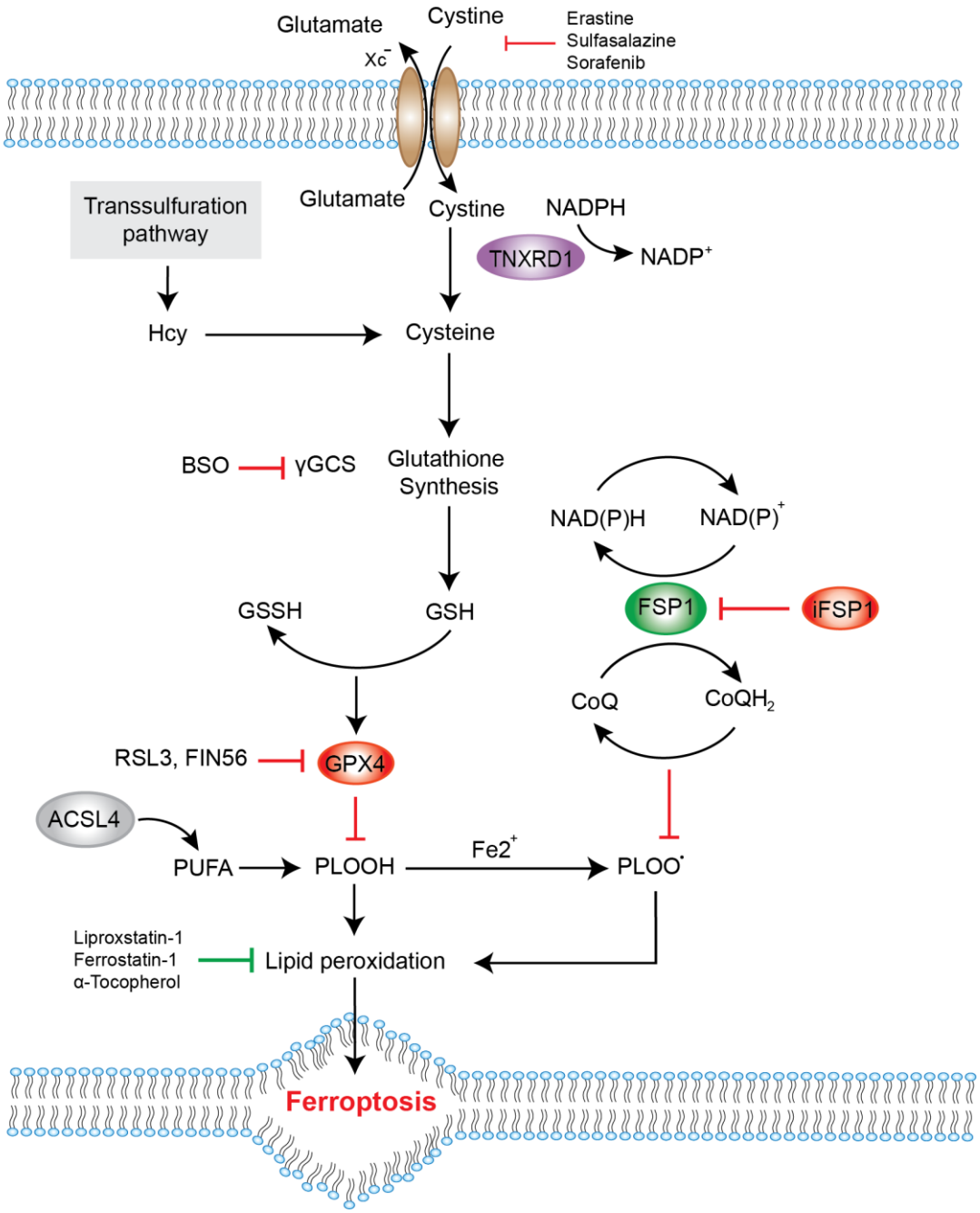


Figure 1: Molecular pathway of ferroptosis

System xc^- cystine/glutamate antiporter is the most upstream player in the ferroptotic pathway. The imported cystine is reduced to cysteine by thioredoxin which is recycled by thioredoxin reductase (TXNRD1) and used for glutathione (GSH) synthesis. Through the transsulfuration pathway homocysteine (*Hcy*) is converted to cysteine (*Cys*) via the intermediate cystathionine. GSH is synthesized by the enzymes γ-glutamylcysteine synthetase (γGGC) to γ-glutamylcysteine and by the enzyme GSH synthetase to the final molecule γ-glutamylcysteinylglycine (GSH). Glutathione peroxidase 4 (GPX4) is protecting cell from the accumulation of phospholipid hydroperoxides (PLOOH) by reducing them to their corresponding alcohols thereby preventing the escalation of lipid peroxidation and ferroptosis. Class I ferroptosis inducing compounds (FINs) erastin, the anti-rheumatic drug sulfasalazine and the tyrosine kinase inhibitor sorafenib can all inhibit system xc^-, deplete GSH, and induce ferroptosis. Class II FINs such as *(1S, 3R)*-RSL3 and FIN56, covalently bind to and inactivate GPX4. FSP1 as a ferroptosis-suppressive catalyzes the NADH-dependent reduction of extra-mitochondrial CoQ_{10}, thereby suppressing the propagation of

lipid derived radicals. Acyl-CoA synthetase long-chain family member 4 (ACSL) reshapes the lipid membrane by activating long chain polyunsaturated fatty acids (PUFAs) to be incorporated into membrane phospholipids, which then undergo lipid peroxidation, thereby causing ferroptotic cell death. Lipophilic antioxidants such as liproxstatin-1, ferrostatin-1, and α-tocopherol can be used to inhibit FINs induced ferroptosis. Figure adapted from (Conrad and Pratt 2019, Conrad, Lorenz et al. 2021).

The first step is catalysed by the enzyme γ-glutamylcysteine synthetase (γ-GCS) forming a γ-peptide bond between the γ-carboxyl group of glutamate and the amino group of cysteine. At this point buthionine sulfoximine (BSO), an inhibitor of γ-GCS, interrupts GSH synthesis and induces ferroptosis (Drew and Miners 1984, Ju, Song et al. 2021). The second step is catalysed by GSS (glutathione synthetase) converting glutamylcysteine to γ-glutamylcysteinylglycine (Dickinson and Forman 2002, Yang and Stockwell 2016). Besides, cysteine can also be provided by the transsulfuration pathway, by the conversion of homocysteine (Hcy) through the intermediate cystathionine into cysteine (Sbodio, Snyder et al. 2019) (Fig. 1). GPX4 is the sole isoform of the glutathione peroxidase family that can prevent phospholipid peroxidation by converting GSH into oxidized glutathione (GSSG) and reduce the cytotoxic phospholipid peroxides (PL-OOH) to the corresponding alcohols (PL-OH). Oxidized GSSG can be reduced back to GSH by glutathione reductase at the expense of $NADPH/H^+$ (Ribas, Garcia-Ruiz et al. 2014). Inhibition of system xc^- indirectly inactivates GPX4 activity by reducing GSH synthesis and results in loss of cellular antioxidant capacity (Ursini, Maiorino et al. 1982, Dixon, Patel et al. 2014, Yang and Stockwell 2016). However, inhibition of system xc^- and GPX4 to trigger ferroptosis varies across cancer cell lines, which suggests the existence of an alternative mechanism of resistance to ferroptosis. FSP1, previously known as apoptosis-inducing factor mitochondrial 2 (AIFM2), is a flavoprotein and NADPH-dependent oxidoreductase that acts as a glutathione-independent ferroptosis-suppressing system (Wu, Wang et al. 2021). FSP1 catalyzes the NADH-dependent reduction of extra-mitochondrial CoQ_{10} (endogenous radical trapping antioxidant), thereby suppressing the propagation of lipid derived radicals, membrane damage and finally ferroptosis (Doll, Freitas et al. 2019).

Furthermore, the discovery and development of compounds to induce or inhibit ferroptosis has enabled scientists to elucidate the underlying mechanisms. Based on the mechanism of action, ferroptosis inducing agents have been classified into three types (Jiang, Stockwell et al. 2021). Erastin (type 1 inducer) indirectly inhibits GPX4 by inhibiting system xc^-, thereby leading to cellular cysteine depletion. RSL3 (type 2 inducer) directly inhibits GPX4 activity by covalently binding to the selenium in its active centre (Yang, SriRamaratnam et al. 2014,

Vuckovic, Bosello Travain et al. 2020). RSL3 inhibits GPX4 without affecting cellular cysteine and glutathione level (Cao and Dixon 2016). Recently a third type of ferroptosis inducers has been developed (i.e., Fin56), which induce ferroptosis via promoting the degradation of the GPX4 protein. Though the underlying mechanism of Fin56-induced GPX4 degradation must be elucidated in more detail, these findings may provide new insights into ferroptosis dependent cell death (Liu, Wang et al. 2021). On the other hand, radical trapping antioxidants (RTAs) like liproxstatin-1 (Lip-1), Ferrostatin-1(Fer-1) and Vitamin E (Vit-E) inhibit ferroptosis (Zilka, Shah et al. 2017, Mishima, Ito et al. 2022). Additionally, the iron chelators DFO (deferoxamine) and DFX (deferasirox) deplete excessive intracellular iron and limit the formation of lipid peroxides and ferroptosis (Kalinowski and Richardson 2005, Kontoghiorghe and Kontoghiorghes 2016).

1.3. Regulation of iron metabolism in ferroptosis

The iron-dependent accumulation of phospholipid hydroperoxides is an important hallmark of ferroptosis (Yang and Stockwell 2016, Kuang, Liu et al. 2020). Therefore, the regulation of the cellular iron content is central to ferroptosis (Dixon, Lemberg et al. 2012). Redox active Fe^{2+} participates in the amplification of different ROS species by reacting with H_2O_2 to produce hydroxyl radicals (·OH) and hydroxyl anions (OH^-) in a Fenton type reaction (Kakhlon and Cabantchik 2002). Circulating ferric iron (Fe^{3+}) bound to the iron binding protein transferrin (Tf) can enter the cell through binding to Tf receptor 1 (TfR1) and subsequent endocytosis (Fig. 2). In the endosome Fe^{3+} is reduced to Fe^{2+} by iron oxidase reductase six-transmembrane epithelial antigen of the prostate 3 (STEAP3) and released into the cytosol by divalent metal transporter 1 (DMT1) or zinc-iron regulatory protein family (ZIP8/14) (Bu, Yu et al. 2021). The iron storage protein complex ferritin is composed of ferritin light chain (FTL) and ferritin heavy chain 1 (FTH1) subunits and stores excessive iron and hence maintains the equilibrium of the iron pool in the cell (Harrison and Arosio 1996). Studies have demonstrated that ferritin stores redox-inactive iron preventing the iron-mediated production of ROS. FTH was reported to play a proactive role by suppressing cellular ferroptosis (Hu, Zhou et al. 2021). Overexpression of FTH reduces mitochondrial ROS levels and rescues the mitochondrial homeostasis (Battaglia, Chirillo et al. 2020, Hu, Zhou et al. 2021).

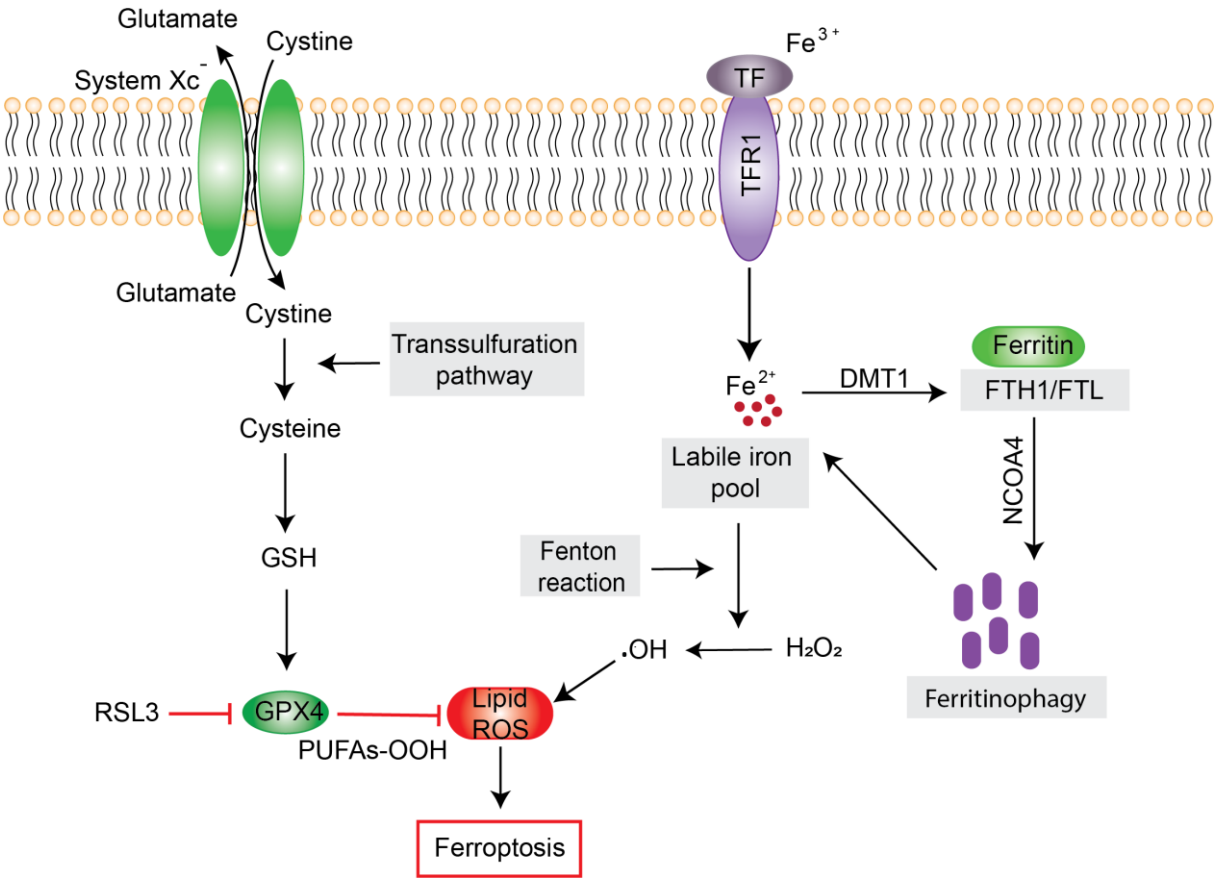


Figure 2: The role of iron in ferroptosis

Schematic representation of the mechanism of iron dependent ferroptotic cell death. System xc imports cystine in exchange of glutamate, which is reduced to cysteine within the cell, and used in biosynthesis of glutathione (GSH), a necessary substrate of glutathione peroxidase (GPX4) to eliminate lipid reactive oxygen species (ROS). Transferrin (Tf) with ferric iron (Fe^{3+}) combines with transferrin receptor 1 (TfR1) and then enters the cell through endocytosis. In the endosome, ferric iron is then reduced to ferrous iron (Fe^{2+}) and released into the cytoplasm through divalent metal transporter 1 (DMT1). Afterwards, the imported cellular iron enters the transient cytosolic labile iron pool, which is then utilized by cells for various metabolic processes or stored in ferritin (Philpott, Ryu et al. 2017). Ferritin consisting of the ferritin heavy chain (FTH1) and the ferritin light chain (FTL) has ferrous oxidase activity and oxidase Fe^{2+} to Fe^{3+}, which is then stored in ferritin. Upon the time of demand, nuclear receptor coactivator 4 (NCOA4) binds ferritin, releases iron from ferritin and mediates selective autophagy. Dysregulation of NCOA4 alters ferritinophagy and modulates susceptibility to ferroptosis (Gao, Monian et al. 2016, Hou, Xie et al. 2016).

Subsequent studies have revealed that the expression of TfR1 was found to be upregulated in the cells sensitive to ferroptosis, specifically in RAS oncogenic expressing cells. In parallel, the ferritin (iron storage protein) was downregulated, indicating the contributions of iron to ferroptosis (Yang and Stockwell 2008). Furthermore, phosphorylation of heat shock protein beta-1 (HSPB1) acts as a negative regulator of ferroptosis by reducing the intracellular iron uptake via inhibition of TRF1 expression (Sun, Ou et al. 2015). Iron response element binding protein 2 (IREB2), an intra-cellular iron metabolism RNA-binding protein, is one of the main

regulators of the cellular iron metabolism that can influence the expression of most proteins involved in iron uptake, storage and export depending on the availability of iron TFR1, FTH1 and FTL and resulted in suppression of erastin-induced ferroptosis (Dixon, Lemberg et al. 2012, Gammella, Recalcati et al. 2015, Li, Cao et al. 2020). Although, lipid peroxidation and intracellular metabolic disorders are related to iron metabolism, the underlying mechanisms that lead to ferroptosis have still not been depicted in detail.

1.4. Transcription factors

Transcription factors (TFs) are a very large and divergent protein family involved in the regulation of gene expression in all living organisms (Lambert, Jolma et al. 2018). TFs can be classified in two classes based on their mechanism of action. (i) General transcription factors, also known as basal transcriptional factors, assist RNA polymerase II to activate the process of transcribing DNA into RNA by binding to core promoter regions of genes. (ii) Specific transcription factors that stimulate or repress the transcription of genes in response to various biological signals (Thomas and Chiang 2006, Blattler and Farnham 2013). TFs establish direct contact with the upstream regulatory region of genes (specific DNA motives) through their DNA binding domain (DBD) to modulate gene expression and protein synthesis. In eukaryotes, many genes can be regulated by the same TF in different types of cells and vice versa. The regulation of a particular gene expression is the fundamental process in response to the intra- and extracellular stimuli (Molina, Suter et al. 2013, Pascual-Ahuir, Fita-Torro et al. 2020). However, complex networks of TFs interact to regulate the expression of targeted genes that can vary in different cellular contexts (Kribelbauer, Loker et al. 2020, Overton, Sims et al. 2020). These combinatorial regulations of gene expression indicate that, even within the same organism, regulatory networks TFs are dynamic (Gertz, Reddy et al. 2012).

Furthermore, DBDs are one of the distinct features of transcription factors that enable them to recognise and bind enhancer or promoter sequences of the DNA (Vandenbon, Kumagai et al. 2012). The specific DNA motifs engaged by TFs are called transcription factor binding sites (TFBS). TFs can bind to variable binding motifs, some can bind to a DNA promotor region and others can bind to enhancer/silencer region either to stimulate or repress transcription of the related gene (Siggers and Gordan 2014, Boeva 2016, Inukai, Kock et al. 2017).This combination

forms the so called transcription initiation complex. Some TFs function in a combinatorial manner, as TFs bind to short recognition sites within an enhancer sequence in order to read the essential regulatory information and call up cofactors and mediate RNA poly II recruitment and activation at the core-promotor of the particular gene and enable transcription (Spitz and Furlong 2012, Shlyueva, Stampfel et al. 2014, Reiter, Wienerroither et al. 2017). While in some cases, either based on local sequence context within enhancer or due to the clustering of TF with some cofactors, it may result in opposite effects on the function of TFs and can produce switch like effects. This suggests that cofactors might be regulated by housekeeping genes, or that TFs might interact with the mediator complex through distinct subunits (Frietze and Farnham 2011, Spitz and Furlong 2012, Stampfel, Kazmar et al. 2015). The actions of transcription factors are essential to coordinate differential gene expression in cells containing the same genome. Multiple lines of evidence suggest that a wide range of human diseases are associated with either mutations in the TFs' own recognition region or in the recognition sequence in its binding sites. As previously said TFs function in combinatorial manner, so the mutation in the TF can cause loss of function or disrupt the binding to its partner TF to a specific site of enhancer (Heinz, Benner et al. 2010, Kasowski, Grubert et al. 2010, Deplancke, Alpern et al. 2016). Besides, the variability of a TF in its binding to the recognition sequence is not only driven by mutation of the corresponding TF or by sequence alterations in the motif of the tissue specific partner TF but also via cell type-specific transcription factor binding sites. From multiple studies, TFs can specify and bind to the recognized DNA sequence sites. In combinatorial mode of function, a TF is redirected to bind at different DNA sequence site in different cell line and results a cell type specified response to the input signals (Halfon, Carmena et al. 2000, Sandmann, Jensen et al. 2006, Reiter, Wienerroither et al. 2017). TFs play an essential role in cancer by regulating gene activity involved in cellular division. However, TFs are vital for most cellular functions, and adjust gene expression in diseases but also in the normal development of an organism (de Mendoza, Sebe-Pedros et al. 2013).

1.5. Role of transcription factors in ferroptosis

Studies have demonstrated that various conditions can change the sensitivity of individual cell to ferroptosis (Nishizawa, Yamanaka et al. 2023). It is still unclear how and where the defence system of body fails against lipid peroxidation leading to ferroptosis. Recent progress has

shown that transcription factors play a crucial role in the regulation of genes of the defence system (Sun, Ou et al. 2016, Nishizawa, Matsumoto et al. 2020). Several transcription factors (e.g., TP53, NRF2, BACH1, YAP1, TAZ, SP1, HIF2A, and) play multiple roles in regulating ferroptosis sensitivity through either transcription dependent or independent mechanisms. T53 is an important player in stress response not only to promote ferroptosis but also to inhibit ferroptosis. T53 transcriptionally represses the expression of SLCA11 gene by binding to its promotor region and induces ferroptosis. In contrary, T53 suppresses erastin-induced ferroptosis in HT1080 cells via mediating the expression of p21 (Tarangelo, Magtanong et al. 2018).

The transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) controls the basal and inducible expression of over 200 genes that regulate iron metabolism, cell proliferation, DNA repair and mitochondrial physiology. In particularly, NRF2 regulates the expression of ferroportin and ferritin (FTH1, FTL1) to prevent free iron accumulation and prevent ferroptosis (Rojo de la Vega, Chapman et al. 2018). BTB and CNC homology 1 (BACH1) binding transcription factor regulates iron- and heme-related genes and oxidative stress in normal cells (Igarashi and Watanabe-Matsui 2014). Studies have demonstrated that BACH1 promotes ferroptosis via repressing genes involved in the synthesis of glutathione and sequestration of free labile iron (Nishizawa, Matsumoto et al. 2020). Specificity protein (Sp1) is a member of the SP/KLF transcription factor family widely involved in regulating the expression of many genes in mammalian cells (Suske, Bruford et al. 2005). From previous studies, Sp1 binds to the promoter region of ACSL4 to promote expression and induce ferroptosis (Li, Feng et al. 2019). YAP/TAZ are well-characterized transcriptional effectors of Hippo signalling involved in a variety of physio-pathological processes, including tumorigenesis and tissue regeneration (Boopathy and Hong 2019). The target genes of YAP/TAZ regulate ferroptosis in various ways, including regulating PUFA-PL synthesis (*ACSL4*), intracellular iron availability (*TFRC*: transferrin receptor), and ROS production (*NOX2/NOX4*) (Magesh and Cai 2022). The complex nature of YAP/TAZ signalling and its role in ferroptosis can be attributed to cell-type specificity and the various mechanisms involved. The molecules associated with YAP/TAZ play central roles in driving or inhibiting ferroptosis. YAP/TAZ targeted therapy is an attractive option that could improve the clinical outcomes of cancer patients (Pobbati and Hong 2020, Magesh and Cai 2022). Hypoxia-inducible factor 2 alpha (HIF2A) members of the basic helix–loop-helix-PER-ARNT-SIM (bHLH–PAS) family is known to regulate multiple pathways involved in cell

proliferation, apoptosis, and metabolism. It has been shown that HIF2A activation increases cellular iron to promote ferroptosis (Selman and Jaquette 1977).

1.6. Forkhead box transcription factors family

The Forkhead box (FOX) containing protein family was discovered by the German biologists Detlef Weigel and Herbert Jäckle in a random mutagenesis screen performed in *Drosophila melanogaster* over 20 years ago (Weigel and Jackle 1990). This family consists of an evolutionarily conserved group of transcriptional regulators that share a common 110-amino acid DNA-binding domain (Myatt and Lam 2007). The FOX genes are widely distributed in eukaryotes, from unicellular organisms to mammals and share an evolutionarily conserved DNA-binding core consisting of a winged-helix domain. These winged helices comprise a subset of the so-called helix-turn-helix motif and are made up of three α helices, three β-strands, and two wing-like loops (W1 and W2). The β2-strand and the wings W1 and W2 serve critical roles in DNA binding (Kaestner, Knochel et al. 2000, Cirillo and Zaret 2007, Harami, Gyimesi et al. 2013). Based on sequence conservation about 50 members have been identified and classified into nineteen subgroups (FOXA to FOXS) in humans and 44 members into subgroups FOXA to FOXS in mice (Jackson, Carpenter et al. 2010, Dai, Qu et al. 2021).

FOX transcripts exhibit functional diversity in key biological processes, including proliferation, differentiation, oxidative stress, apoptosis, immune function, and development. However, each member possesses its own tissue- and cell type-specific roles (Brown and Webb 2018, Herman, Todeschini et al. 2021). Genetic mutation of FOX genes are closely associated with human diseases including metabolic disorders, autoimmune diseases and particularly cancers (Bach, Long et al. 2018, Ramezani, Nikravesh et al. 2019, Herman, Todeschini et al. 2021). Under physiological and pathological conditions, FOX factors like FOXOs and FOXP3 regulate phosphatidylinositol 3-kinase/protein kinase B (PI3K-AKT), transforming growth factor beta (TGF-β) and WNT/β-catenin signalling pathways (Benayoun, Caburet et al. 2011, Laissue 2019). As described, FOX proteins share a highly conserved DBD, most pathogenic missense mutations occur in this region. The winged helix structure representing the DBD consists of about 100 amino acids and shows considerable differences between the FOX subfamilies, while the flanking N- and C-terminal sequences are more or less conserved (Gao, Chen et al.

2019, Dai, Qu et al. 2021). Although the winged helix domain is considered the main DNA interaction motif of FOX TFs, other domains outside of this core site interact with flanking DNA regions. Additionally, post-translational modifications such as acetylation and phosphorylation affect the transcriptional activity of Fox transcripts, as well as the DNA binding affinity of these transcripts (Vander Heiden, Chandel et al. 1999, Hoekman, Jacobs et al. 2006, Jolma, Yan et al. 2013). The diverse role of the FOX family is well-documented in the literature, including a wide array of general and tissue specific functions. Among the regulated biological processes are cell-cycle arrest, DNA repair and cell death. In order to better understand the importance and complex role of FOX transcription factors, FOX proteins were classified into families and subfamilies (Jackson, Carpenter et al. 2010). The involvement of a selected number of forkhead members and their role in cancer are shown in figure 3. FOXA1 and FOXA2 are highly conserved and bind to a similar *cis*-acting element. Both TFs share about 95% homology in the winged helix DNA binding domains between mice and humans (Jackson, Carpenter et al. 2010). FOXA1-TF play a critical role in the progression of breast and prostate cancer via functioning as an interacting partner and functional mediator of androgen receptor and estrogen receptor- α (Kaestner 2010, Zaret and Carroll 2011, Robinson and Carroll 2012). Additionally, FOXA1 regulates the expression of the cell cycle regulator cyclin D1 and promotes glioma cellular proliferation via easing G1/S transition (Zhang, Yang et al. 2018). FOXA1/2 also regulate the normal development of the bile duct in mice by depressing *IL-6* expression. The loss of both FOXA1/2 leads to the abnormal expansion of the bile duct (Strazzabosco 2010, Zhang, Yang et al. 2018). FOXG1, formerly named forebrain restricted transcription factor, plays an important role in the development of the telencephalon. Increased expression of FOXG1 in ovarian cancer cells suppresses cyclin-dependent kinase (CDK) inhibitor p21 $^{WAF1/CIP1}$ and acts as a negative regulator of TGF-*ß* signalling (Rodriguez, Huang et al. 2001, Chan, Liu et al. 2009). Further studies have revealed that FOXG1 is essential for cell cycle regulation and cortical progenitor cell proliferation (Hou, hAilin et al. 2020). The O subclass of the mammalian forkhead transcription factors consists of four members (FOXO1, FOXO3, FOXO4, and FOXO6). FOXOs are implicated in a wide range of cellular function, together with oxidative stress, autophagy, regulation of metabolism, cellular proliferation, DNA damage and apoptosis (Gomes, Brosens et al. 2008).

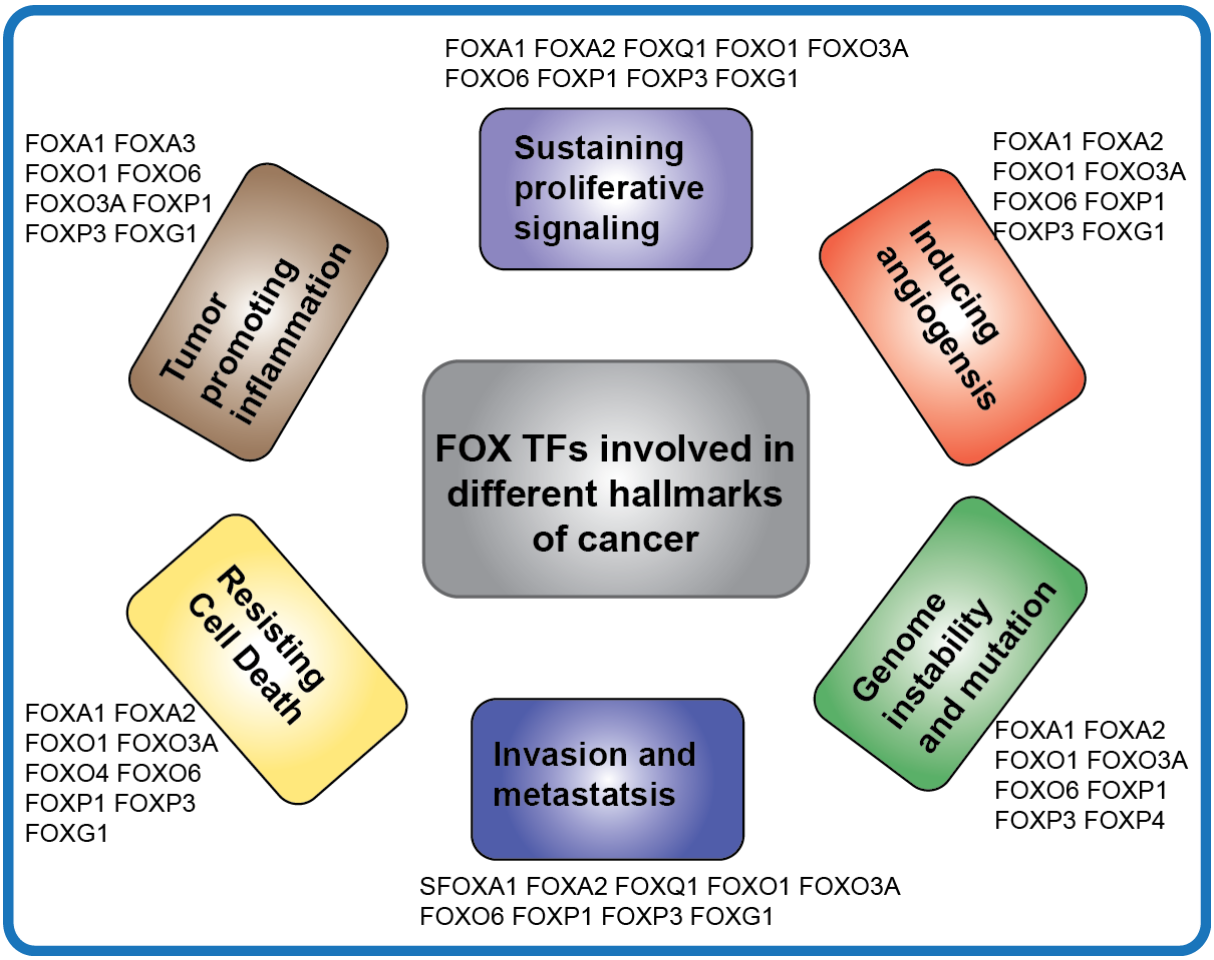


Figure 3: The Forkhead transcription factor family

A schematic representation of the human FOX family of transcription factors and their role in different hallmarks of cancer. The indicated members are involved directly or indirectly in cancers and genetic diseases. The arrangement of the transcription factor families is independent from functional or evolution considerations. Family of FOXA and FOXO appears to be associated with every hallmark of cancer. FOXP4 is only related to genome instability and mutation. FOXQ1 relates to proliferative signalling and invasion. Figure adapted from (Bach, Long et al. 2018).

Studies have revealed that FOXOs regulate the expression of genes involved in cell cycle arrest (p21, p27) (Nowak, Killmer et al. 2007). In addition, oxidative stress regulates the activity of FOXO proteins which in turn induces the expression of genes that are involved in cell cycle arrest (Furukawa-Hibi, Kobayashi et al. 2005, Storz 2011). In contrast, downregulation of FOXO increases intracellular oxidative stress and ROS-induced cell death. However, FOXO deficiency leads to a depletion of neural stem cells in the brain associated with neuronal cell death and an increase in ROS levels (Renault, Rafalski et al. 2009, Yeo, Lyssiotis et al. 2013).

The P subclass consists of 4 members that have been functionally characterized (FOXP1-4). It has been reported that depletion of FOXP1 inhibits cell proliferation via cell cycle arrest (Wang, Sun et al. 2016, Kim, Hwang et al. 2019). Others groups have demonstrated that FOXP1 negatively regulates anti-immune responses via controlling the expression of chemokine (De Silva, Garaud et al. 2019). FOXP4 is highly expressed in non-small lung cancer cells where its loss markedly reduces cell proliferation and also facilitates epithelial-mesenchymal transition (Ma and Zhang 2019).

1.7. FOXQ1

FOXQ1 appeared in a CRISPR/Cas9 screen to find factors that influence the ferroptosis sensitivity (unpublished data of Dr. Sebastian Doll, Helmholtz Institute Munich, Germany) and is of special interest in this thesis. FOXQ1, previously known as hepatocyte nuclear factor 3 forkhead homolog 1 (HFH1), was first isolated by Bieller et al. in 2001. FOXQ1, a member of the super family of FOX proteins, is coded by a single exon gene located on chromosomal region 6p25.3 and characterized by a distinctive conserved DBD. Functionally, FOXQ1 regulates the expression of genes mandatory for cell proliferation, differentiation and embryonic development (Elian, Are et al. 2021). In comparison to the other TFs, the coding region of human FOXQ1 exhibits only 82% homology to mouse and rat FOXQ1 (Bieller, Pasche et al. 2001). Studies have indicated that FoxQ1 is highly expressed in different solid tumours including breast cancer, lung cancer, liver cancer, ovarian cancer and kidney cancer, but the mechanisms of its putative oncogenic potential still remains unclear (Qiao, Jiang et al. 2011, Feng, Zhang et al. 2012, Gao, Shih Ie et al. 2012). Several studies have revealed that a myriad of genes downstream of FOXQ1 may play a role in tumorigenesis and cell proliferation. In addition, involvement of FOXQ1 in controlling metabolic processes (glucose and lipid metabolism), T cell activation, angiogenesis, mucin secretion and epithelial differentiation has been demonstrated (Jonsson and Peng 2005, Li, Zhang et al. 2016). Altogether, numerous physiological functions of FOXQ1 have been reported in various studies but the link to ferroptosis has not been evident so far.

1.8. Regulation of FOXQ1

In recent years, a number of pathways have been illustrated that regulate expression of FOXQ1 in tumour cells. The Wnt/β-catenin signalling pathway is an evolutionary conserved mechanism that facilitates cell proliferation and differentiation and cancer stem cell maintenance (Clevers 2006, Steinhart and Angers 2018). Wnt/β-catenin signalling has an important role in initiation and progression of human carcinoma (Tarapore, Siddiqui et al. 2012). FOXQ1 is a direct Wnt target and a downstream mediator in the Wnt/β-catenin signalling pathway. Upon activation, the Wnt-pathway enhances the transcriptional activity of FOXQ1, although the underlying mechanism is incompletely understood (Pizzolato, Moparthi et al. 2022). It has been reported that, FOXQ1 is highly expressed in colorectal cancer cell lines and has been linked directly to metastasis and tumour growth (Christensen, Bentz et al. 2013, Pizzolato, Moparthi et al. 2022).

Similarly, it has been reported that a number of miRNAs function as tumour regulators targeting FOXQ1 (Li, Zhang et al. 2016). Other studies have demonstrated that miRNAs targeting FOXQ1 are associated with certain types of human cancer. In line with this, Peng et al. 2015, demonstrated that miR-124 represses FOXQ1 expression via directly targeting its 3'-UTR region which inhibited the proliferation of nasopharyngeal carcinoma cells. Additionally, miR-506, miR-422a and miR-1271 also suppressed cell proliferation and tumour invasion via regulating the expression of FOXQ1. On the contrary, FOXQ1 overexpression could partially recover the repression of miRNA-124 (Valencia-Sanchez, Liu et al. 2006, Peng, Huang et al. 2014, Xiang, Deng et al. 2015, Zhang, Ma et al. 2015).

1.9. Downstream targets of FOXQ1

The epithelial–mesenchymal transition (EMT) is a dynamic biological process through which tightly packed and non-motile epithelial cells acquire a loosely organized mesenchymal phenotype. EMT transition results in loss intercellular adhesion, cellular polarity and a gain in motility, invasive property and resistance to apoptosis. In some cases, EMT is also reversible and cells undergo a mesenchymal to epithelial transition (MET) to become more stationary and gain an epithelial phenotype (Larue and Bellacosa 2005). Both EMT and MET are dependent on the cell adhesion molecules E-cadherin encoded by the *Cdh1*gene and vimentin

encoded by the *VIM* gene. Recently, studies have also shown that FOXQ1 transcriptionally regulates E-cadherin. FOXQ1 ablation markedly reduced the invasive ability in breast cancer cells and induced conversion from spindle-like mesenchymal morphology into epithelial-like morphology (Larue and Bellacosa 2005, Ray, Ryusaki et al. 2021). Many studies have demonstrated that genes from the FOX family are involved in G1/S and G2/M phase transitions in cell cycle. Likewise, FOXQ1 overexpression promote tumour cell proliferation, while depletion of FOXQ1 inhibited cell division (Kaneda, Arao et al. 2010, Zhang, Yang et al. 2015). For instance FOXQ1 overexpression was found to regulate neurexin, a family of polymorphic neuronal-specific cell surface proteins that serve critical roles in connecting neurons at the synapse (Ushkaryov, Petrenko et al. 1992). Sun et al. demonstrated that FOXQ1 regulates the activity of neurexin 3, which enhanced cancer cell proliferation and migration (Rowen, Young et al. 2002, Sun, Cheng et al. 2013). Another downstream target of FOXQ1 is the cyclin-dependent kinase inhibitor p21 (Kaneda, Arao et al. 2010). *p21* fulfils a crucial function in modulating DNA repair and is an important tumour suppressor (Xiong, Hannon et al. 1993, Abbas and Dutta 2009). *p21* is also a downstream gene of p53 and the expression of p21 is known to be regulated at the transcriptional level by both p53-dependent and p53-independent mechanisms (Benson, Mungamuri et al. 2014, Engeland 2022). On of such p53-independent mechanisms is mediated by binding of FOXQ1 to a segment of the p21 promotor to increase its expression (Kaneda, Arao et al. 2010, Engeland 2022).

FOXQ1 is also a known as transcriptional regulator of the TGF-β (Fan, Feng et al. 2014, Mitchell, Wu et al. 2022). TGF-β is a multifunctional cytokine that can induce EMT through activation of its signalling cascade (Miyazono 2009). Thereby, TGF-β itself induces the expression of several transcription regulators such as zinc finger E-box-binding homeobox 1 and 2 (ZEB1 &2), zinc-finger factors Snail (SNAI1), Twist related protein 1 (TWIST1). In contrast, knockdown of FOXQ1 blocked TGF-β induced EMT at both morphological and molecular level (Feuerborn, Srivastava et al. 2011, Zhang, Meng et al. 2011, Fan, Feng et al. 2014). Taken together, several studies have shown that numerous target genes of FOXQ1 are involved in tumour biogenesis, cell proliferation, invasion and EMT.

1.10. Transcriptional network of FOXQ1

Transcription protein FOXQ1 activates further TFs to initiate a cascade of regulatory events. Expression regulation of FOXQ1 induces a direct or indirect effect on the activity of the targeted gene (Liu, Wu et al. 2017). Similar to FoxQ1, members of the O subfamily of FOX are key molecular players in cancer pathogenesis. FOXO proteins acts downstream of PI3K/AKT, and ERK signalling pathway. Studies have revealed that FOXQ1 can interact with the DBD of FOXO's and thereby block their transcriptional activity (Matsumoto, Han et al. 2006, Tzivion, Dobson et al. 2011, Cui, Qiao et al. 2016). Furthermore, members of the FOXF subfamily function in cell cycle regulation, tumorigenesis and embryonic development. It has been stated that ectopic expression of FOXF2 inhibited the protein expression of cyclin D1, CDK4 and PCNA, thereby arresting the cell cycle from G1 phase to S phase transition and reduce cellular proliferation and tumorigenesis (Lo, Lee et al. 2016, Higashimori, Dong et al. 2018, He, Kang et al. 2020). Depletion of FOXF1 resulted in lethal effect in mice. It has been shown that FOXQ1 regulates Wnt/β-catenin signalling pathway and promotes osteogenic differentiation of mouse bone mesenchymal stem cells (Bolte, Flood et al. 2017, Xiang, Zheng et al. 2020). Interestingly, multiple studies showed putative FOXQ1 binding sites in the promotor regions of FOXF genes indicating a direct regulatory effect of the FOXF family by FOXQ1 (Lo, Lee et al. 2010, Kang, Yu et al. 2019, Xu, Liu et al. 2021). FOXQ1 is known as a direct transcriptional activator of microphthalmia-associated transcription factor (MITF) (Bagati, Bianchi-Smiraglia et al. 2018). MITF has a dominant function in melanoma progression and melanocyte differentiation. MITF regulates the expression of CDKN1A and plays a cytoprotective role in the response to DNA damage (Carreira, Goodall et al. 2005, Hartman and Czyz 2015). Hence, FOXQ1 induces differentiation in melanocytic cells via regulating the transcription of *MITF* gene (Hartman and Czyz 2015, Bagati, Bianchi-Smiraglia et al. 2018).

Twist Basic Helix-Loop-Helix transcription factor 1 (TWIST1) is a key mediator of metastasis that regulates the expression of various molecules involved in epithelial to mesenchymal transition (EMT). TWIST1 is expressed in T cells, dendritic cells, macrophages and natural killer cells and is important for lymphocytes maturation and function (Sosic, Richardson et al. 2003, Dobrian 2012). Studies have indicated that FOXQ1 directly regulates the transcriptional activity of TWIST1 to stimulate its expression resulting in increased cell invasion and migration in colorectal cancer (Abba, Patil et al. 2013).

Mouse double minute 2 (*MDM2*) is an oncogene overexpressed in different human cancers that regulates cell growth, survival, cell cycle, and invasion (Zhao, Yu et al. 2014). Most importantly, MDM2 is considered the most important negative regulator of the tumour suppressor p53, the retinoblastoma susceptibility gene product (Rb) and the growth suppressor p14 (Manfredi 2010). The results of previous studies have indicated that the transcription factor B cell lymphoma/leukaemia (BCL11A) regulates the expression of MDM2, while FOXQ1 upregulates the expression of BCL11A in acute myeloid leukaemia and colorectal cancer. Taking together, FOXQ1 through BCL11A and MDM2 regulates cell growth, cell cycle and proliferation (Mayo and Donner 2001, Bond, Hu et al. 2005, Zhang, Wang et al. 2016).

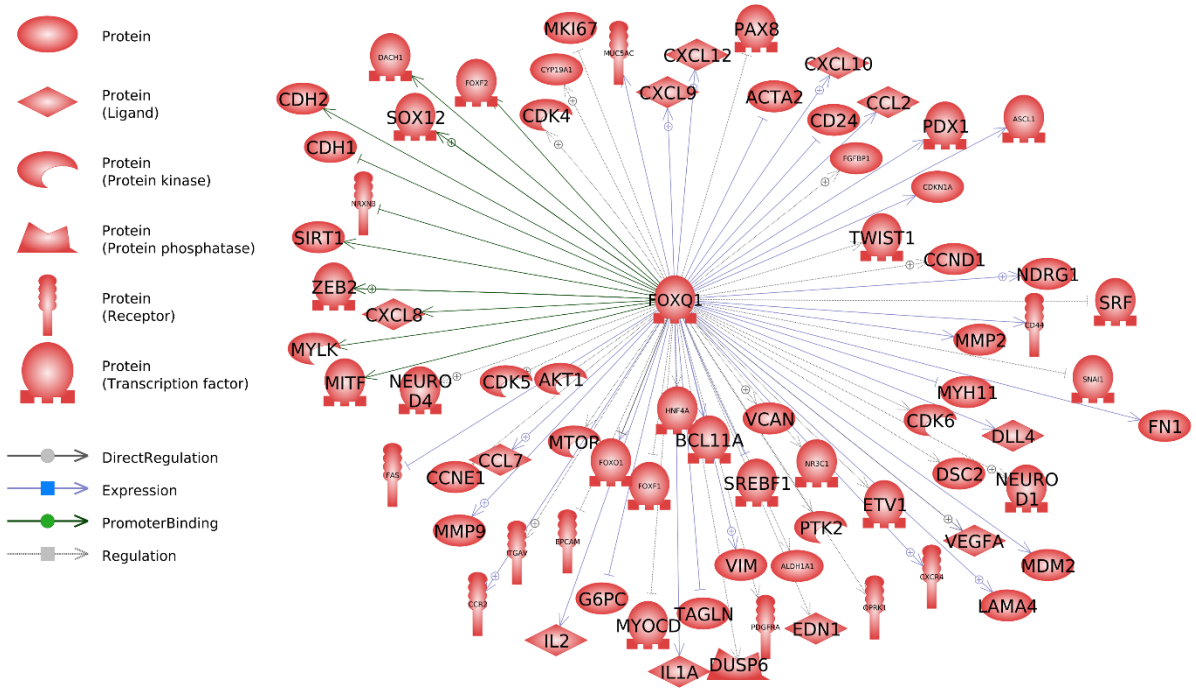


Figure 4: Downstream transcriptional targets of FOXQ1

FOXQ1 transcription factor induces the transcription of a large array of targeted genes. FOXQ1 directly and indirectly regulates the activation and expression of multiple transcription factors and proteins through post translational modification, including phosphorylation, also acetylation, methylation, and ubiquitination. CDH1, cadherin-1 or epithelial cadherin; CDH2, cadherin-2; DACH1, dachshund homolog 1; SOX12, SRY-Box transcription factor 12; FOXF2, Forkhead box F2; CDK4, cyclin-dependent kinase 4, MKI67, antigen KI-67; CXCL9, chemokine ligand 9; PAX8, paired-box gene 8; CXCL12, C-X-C motif chemokine ligand 12; CD24, signal transducer CD24; CCL2, chemokine ligand 2; FGFBP1, fibroblast growth factor-binding protein 1; ACSL1, Acyl-CoA synthetase long chain family member 1; CDKN1A, cyclin-dependent kinase inhibitor 1 ; *TWIST1*, twist family BHLH transcription factor 1; *CCND1*, cyclin D1; *NDRG1,* N-Myc downstream regulated 1; *SRF*, serum response factor, MMP2, matrix metalloproteinase-2; *MYH11*, myosin heavy chain 11; S*NAI1*, snail family transcriptional repressor 1; *CDK6*, cyclin dependent kinase 6; *FN1*, fibronectin 1; *DLL4*, delta like canonical notch ligand 4; *DSC2*, desmocollin 2; *NeuroD1,* neurogenic differentiation 1; *VCAN*, versican; *HNF4A*, hepatocyte nuclear factor 4 alpha; BLC11A, B-cell lymphoma/leukaemia 11A; *SREBF1*, sterol regulatory element binding transcription factor 1; *VIM*, vimentin; *PTK2*, protein tyrosine kinase 2; *VEGFA*, vascular endothelial growth factor A; *MDM2*, *MDM2* proto-

oncogene; FOXO1, Forkhead box protein O1; FOXF1, Forkhead box protein F1; *TAGLN*, transgelin; *MYOCD*, myocardin; *EDN1*, endothelin 1; IL1A, interleukin 1 alpha; *DUSP6*, dual specificity phosphatase 6; *MTOR*, mechanistic target of rapamycin kinase; G6PC, glucose-6-phosphatase catalytic-subunit-encoding *gene*; *IL2*, interleukin 2; *CCR2*, C-C motif chemokine receptor 2; *CCL7*, C-C motif chemokine ligand 7; *CCNE1*, cyclin E1; *MMP9*, matrix metallopeptidase 9; *AKT1*, *AKT* serine/threonine kinase 1; *CDK5*, cyclin dependent kinase 5; *MITF*, melanocyte inducing transcription factor; *CXCL8*, C-X-C motif chemokine ligand 8; *ZEB2*, zinc finger E-box binding homeobox 2; SIRT1, silent information regulator 1; *NRXN3*, neurexin 3-alpha. Figure generated by Dr. Dietrich Trümbach using pathway studio program (Elsevier).

Moreover, the Snail family transcription repressor 1 (SNAIL1) is another transcriptional target of FOXQ1. Studies have revealed that FOXQ1 regulates the expression of SNAIL at both the mRNA and protein level (Zhang, Liu et al. 2016). SNAIL represses Ras kinase inhibitor protein transcription in prostate cancer cells (Beach, Tang et al. 2008). Silencing SNAIL1 arrests cell cycle, reverses EMT and suppresses tumour cell proliferation (Cano, Perez-Moreno et al. 2000, Osorio, Farfan et al. 2016).

The Zinc finger E homeobox binding proteins family (ZEBs) is a transcription factor family that includes ZEB1 and ZEB2. ZEBs are best known for their function as transcriptional repressors driving epithelial to mesenchymal transition (Scott and Omilusik 2019). ZEB1 via binding to the promotor suppresses the expression of E-cadherin and correspondingly increases the expression of vimentin and N-cadherin (Wu, Zhong et al. 2020). It was found that FOXQ1 promotes HCC (hepatocellular carcinoma) metastasis and induces EMT by regulating the transcriptional activity of ZEBs through binding to their promotor (Vandewalle, Van Roy et al. 2009, Xia, Huang et al. 2014).

The member of the sirtuin family (SIRT1) functions as a transcription factor involved in several physiological process. SIRT1 is the most conserved mammalian nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase involved in a broad range of biological functions, including the control of gene expression, metabolism, inhibiting oxidative stress and neurodegeneration. Researchers have determined that SIRT1 overexpression may increase the risk of cancer in mammalian cells via inhibiting tumour suppressor p53 (Rahman and Islam 2011). Additionally, SIRT1 is controlling the acetylation of nuclear factor kappa B (NF-κB) and impart protection against chronic inflammation. FOXQ1 transcriptionally upregulates SIRT1 expression by binding to the *SIRT1* promotor region. In addition, FOXQ1 reduces the expression of the inflammatory cytokines interleukin-6 (IL-6) and IL-8 through modulating the SIRT1/NF-κB signalling pathway (Rahman and Islam 2011, Wang, Lv et al. 2017, Elibol and Kilic 2018). Recently is has been reported that sterol regulatory element-binding

proteins/transcription factors (SREBPs) regulate genes involved in lipid homeostasis and intracellular cholesterol levels (Brown and Goldstein 1997, Bertolio, Napoletano et al. 2019). Additionally, SREBPs overexpression increases cell proliferation in different cancers, whereas silencing of SREBPs exerts inhibitory effects on cell proliferation (Wang, Ling et al. 2020). An in vivo study has reported that FOXQ1 overexpression suppresses the expression of SREBP isoform SREBP-1c (Cui, Qiao et al. 2016).

The epidermal growth factor receptor (EGFR) is a transmembrane protein activated by extracellular EGF to signal downstream targets PI3K/AKT, MAPK/AKT in the cell (Wee and Wang 2017). Further, overexpression of FOXQ1 is closely associated with the activation of the EGFR signalling pathway (Zhang, Cao et al. 2022). FOXQ1 regulates the expression of EGF and thereby enhances activation of the EGFR signalling pathway. Abnormal activation of EGFR or its downstream effectors PI3K/AKT, MAPK/AKT by FOXQ1 can induce cell proliferation, invasion and apoptosis of tumour cells. On the contrary, knockdown of FOXQ1 suppressed the expression of EGFR and its downstream genes attenuated cell proliferation, migration and invasion (Sabbah, Hajjo et al. 2020, Luo, Wang et al. 2021)

To understand the regulatory mechanism underlying ferroptosis, it is worth to examine whether or not FOXQ1 is involved in the regulation of ferroptosis by comparing ferroptosis and the expression of ferroptosis-induced genes between control and FOXQ1 overexpressing cells. Therefore, the investigation and expansion of ferroptosis signalling pathways is the subject of this dissertation.

2. OBJECTIVE OF THE STUDY

Ferroptosis is an iron-dependent form of regulated necrotic cell death, driven by uncontrolled lipid peroxidation in the cellular membranes (Dixon, Lemberg et al. 2012). Targeting the regulatory network of ferroptosis emerges as a novel treatment option for previously untreatable diseases. Many transcriptional factors play crucial roles in shaping the ferroptosis sensitivity of cells through either transcription dependent or independent mechanisms (Dai, Chen et al. 2020). The aim of this study is to characterize the role of the TF FOXQ1 in ferroptosis and to potentially identify novel ferroptosis mediators. However, whether FOXQ1 and FOXQ1-regulated genes have a potential prognostic and predictive role in ferroptosis remains largely unknown. Therefore, a mouse cell line and a panel of human cancer cell lines overexpressing FOXQ1 should be generated to investigate the effect of FOXQ1-mediated on the sensitivity to ferroptosis. Furthermore, a knockout approach will be applied to explore the genotype-phenotype relationship of FOXQ1 to its targeted genes. In addition, a genome-wide crispr/cas9 screening should be applied in human cancer cell lines to recognize and characterize newly discovered ferroptosis regulators in more detail.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Equipment

Equipments	Company
Axioplan 2 Microscope	Carl Zeiss
Bio-Rad PCR	Bio-Rad C1000 Thermal Cycler, USA
qTOWER Real-time PCR/qPCR	Analytik Jena, Germany
Sonification device Q125	Qsonica
SpectraMax M5 Microplate Reader	Molecular Device GmbH, Biberach, Germany
LC-4000 Series UHPLC Chromatography System	JASCO, Germany
Nanolive's imaging-the CX-A	Nanolive SA, Switzerland
Water bath VWB 12	VWR, Radnor, USA
Vortex Genie 2	Scientific industries, Bohemia, New York, USA
Mixer Mill MM 400	Retsch GmBH, Haan, Germany
Multi-Tube Vortex Mixers VXMTDG	OHAUS Europe GmbH, Nänikon, Switzerland
Thermo Scientific™ Fresco™ 17 Microcentrifuge	Thermo Fisher Scientific, Waltham, USA
Fisherbrand™ GT4 Benchtop Centrifuge	Thermo Fisher Scientific, Waltham, USA
Fisherbrand™ GT4 Benchtop Centrifuge	Thermo Fisher Scientific, Waltham, USA
Thermo Scientific™ Fresco™ 17 Microcentrifuge	Thermo Fisher Scientific, Waltham, USA
Chemi Doc™ MP Imaging System	BioRad, Munich, Germany
Mini-PROTEAN® Tetra electrophoresis chamber	BioRad, Munich, Germany
Electrophoresis power supply	PEQLAB Biotechnologie GmbH, Erlangen, Deutschland
NanoDrop 1000 Spectrophotometer	PEQLAB Biotechnologie GmbH, Erlangen, Deutschland
Nikon Eclipse microscope	Nikon, Tokyo, Japan
Leica DFC3000G Microscope	Leica Microsystems GmbH, Wetzlar, Germany
Axioplan 2 Microscope	Carl Zeiss AG, Oberkochen, Germany
Olympus confocal microscope IX81	Olympus, Münster, Germany
LSM 880	Carl Zeiss AG, Oberkochen, Germany
Trans-Blot Turbo Transfer System™	BioRad, Munich, Germany
Thermo Mixer C	Eppendorf, Hamburg, Germany
Thermal Cylcer	G-Storm, Somerton, United Kingdom
Centrifuge Combi Spin FVl-2400N	Kisker Biotech GmbH & Co. KG, Steinfurt, Germany
Centrivap Cold Trap	Labconco Corporation, Kansas City, USA
RapidVap Vacuum Evaporation System	Labconco Corporation, Kansas City, USA
Leica CM3050 S Cryostat	Leica Biosystems Nussloch GmbH, Nussloch, Germany
Mr. Frosty™ freezing container	Thermo Fischer, Bonn, Germany
Roller 10 digital	IKA Labortechnik, Staufen, Germany
T 25 digital ULTRA-TURRAX® Dispergierer	IKA®-Werke GmbH & CO. KG, Staufen, Germany
2100 Bioanalyzer	Agilent
CB150 CO2 Incubator	Binder, Tuttlingen, Germany
CytoFLEX Flow Cytometer	Beckman Coulter, USA
Counting chamber	Paul Marienfeld

3.1.2. Disposables and Kits

Disposals and Kits	Company	Catalog no.
2 % Mini-PROTEAN® TGX StainFree™ Precast Gels	Bio-Rad, Munich, Germany	456-8043
µ-Dish 35 mm, low	ibidi GmbH, Gräfelfing, Germany	80136
Clarity Wetern ECL Substrate	Bio-Rad, Munich, Germany	1705061
Gibson Assembly® Cloning Kit	New England BioLabs	E5510S
Costar® reagent reservoirs	Sigma-Aldrich GmbH, Taufkirchen, Germany	4870
Roti®-Histokitt	Carl Roth GmbH & Co, Karlsruhe, Germany	6638.1
Pierce BCA Protein Assay	Thermo Fisher, Bonn, Germany	23225
PageRuler prestained protein ladder	Thermo Fisher, Bonn, Germany	26616
SuperSignal West Femto Maximum Sensitivity Kit	Life Technologies, Carlsbad, USA	34095
Tissue Tek® Compound Embedding Medium	Weckert Labortechnik, Kitzingen, Germany	4583
Cell culture dish 100 x 20 mm	Greiner Bio-One, Kremsmünster, Austria	664160
Cell Counter Slides for TC20™	Bio-Rad, Munich, Germany	1450011
Clarity ™ Western ECL Blotting Substrate	Bio-Rad, Munich, Germany	1705061
CryoTubes®	Thermo Fisher, Bonn, Germany	V7634-500EA
Lysing Matrix S	MP Biomedicals, Santa Ana, USA	6925100
Corning Tubes with Cap 14 ml	Thermo Fisher Scientific, Waltham, USA	9401352
Millex GP Filter 0,22µm	MilliporeSigma, Massachusetts, USA	SLGP033RS
Millex GP Filter 0,45µm	MilliporeSigma, Massachusetts, USA	SLHP033RS
Nunc 96 well plates	Thermo Fisher, Bonn, Germany	9407473
Nunc 60 x 15 mm plates	Thermo Fisher, Bonn, Germany	9476940
Nunc 6 well plates	Thermo Fisher, Bonn, Germany	9477145
Parafilm M®	Thermo Fisher, Bonn, Germany	12378039
PageRuler prestained protein ladder	Thermo Fisher, Bonn, Germany	26616
Plasmid Maxi Kit	Qiagen, Hilden, Germany	12163
Parafilm M®	Pechiney Plastic Packaging Company	PM996
QIAprep Spin Miniprep Kit	Qiagen, Hilden, Germany	27104
RNase-Free DNase Set	Qiagen, Hilden, Germany	79254
GeneRuler 1 Kb Plus DNA Ladder	Fermentas Life Science, Bonn, Germany	SM1331
Chromacol™ 10mL screw top round bottom vial	Thermo Fisher Scientific, Waltham, USA	71210135501
Fisherbrand™ Pasteur Pipets	Thermo Fisher Scientific, Waltham, USA	1154-6963
Trans-Blot Turbo Transfer Pack, PVDF	Bio-Rad, Munich, Germany	170-4156
Aqua-Poly/Mount	Polysciences, Inc., Warrington, USA	18606-20
Micro tube 0.5ml 9NC	Sarstedt AG & Co.KG, Nümbrecht, Germany	411.506.002
Cell Scraper with 2-position blade	Sarstedt AG & Co.KG, Nümbrecht, Germany	422-83.1832
Precellys Keramik-kit 1.4 mm 2 ml Tubes	VWR International , Radnor, USA	432-3751
12 % Mini-PROTEAN® TGX Stain-Free™ Precast Gels	Bio-Rad, Munich, Germany	456-8043
DNA Loading Dye 6x	Thermo Fisher, Bonn, Germany	R0611
ApopTag® Fluorescein In Situ Apoptosis Detection Kit	MilliporeSigma, Massachusetts, USA	S7110
Tissue Tek® Compound Embedding Medium	Weckert Labortechnik, Kitzingen, Germany	4583
Vectastain ABC HRP Kit	Vector Laboratories	PK-4000
Wizard® SV Gel and PCR Clean-Up System	Promega, Mannheim, Germany	A9282

3.1.3. Chemicals

Chemical	Company	Catalog no.
(1S,3R)-RSL3	Cayman Europe, Michigan, USA	19288
0.9 % Sodium Chloride injection solution	B. Braun Melsungen AG, Melsungen, Germany	-
10X PCR buffer	Thermo Fisher, Bonn, Germany	10342020
2-Mercaptoethanol 50 mM	Life Technologies, Carlsbad, USA	31350-010
2-Propanol	Merck KGaA, Darmstadt, Germany	1096342500
2-Propanol	Thermo Fisher Scientific, Waltham, USA	P/7508/17
4-Hydroxytamoxifen	(TAM) Sigma- Aldrich	H7904
3-Methyl-1-phenyl-2-pyrazoline-5-one (edaravone)	Sigma-Aldrich GmbH, Taufkirchen, Germany	M70800
4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma-Aldrich GmbH, Taufkirchen, Germany	D9542-10MG
AquaBluer ™ Cell Viability Assay Solution	MultiTarget Pharmaceuticals LLC, Salt Lake City, USA	6015
Acetic acid	Merck KGaA, Darmstadt, Germany	1000632500
Agarose	Invitrogen, Karlsruhe, Germany	15510-027
Ampicillin	Sigma-Aldrich GmbH, Taufkirchen, Germany	A9518
Arachidonic Acid	Sigma-Aldrich GmbH, Taufkirchen, Germany	A9673
Bambanker	VWR International , Radnor, USA	WAKO30214 681
Blasticidin S hydrochloride	Thermo Fisher, Bonn, Germany	A1113903
Bovine Serum Albumin, fatty acid free	Sigma-Aldrich GmbH, Taufkirchen, Germany	A8806-5G
BSA, fatty acid free	Sigma-Aldrich GmbH, Taufkirchen, Germany	A8806-5G
Butylated hydroxytoluene (BHT)	Sigma-Aldrich GmbH, Taufkirchen, Germany	W218405
Ceramide/Sphingoid Internal Standard Mixture II	Avanti Polar Lipids, Birmingham, USA	LM6005-1EA
Chloroform	Thermo Fisher Scientific, Waltham, USA	C/4966/17
Chloroquine diphosphate salt	Sigma-Aldrich GmbH, Taufkirchen, Germany	C6628
CryoStor® CS10	Stemcell Technologies, Vancouver, Canada	7930
cOmplete™ Protease Inhibitor Cocktail	Sigma-Aldrich GmbH, Taufkirchen, Germany	4693116001
Cumene hydroperoxide	Sigma-Aldrich GmbH, Taufkirchen, Germany	247502
D+Saccarose	Carl Roth GmbH & Co, Karlsruhe, Germany	46211
DAPT	Miltenyi Biotec B.V. & CO. KG, Bergisch Gladbach, Germany	130-110-489
Diethylenetriaminepentaacetic acid (DTPA)	Sigma-Aldrich GmbH, Taufkirchen, Germany	D6518405
Desthiobiotin elution buffer	IBA Lifesciences, Göttingen, Germany	2-1000-025
Dimethylsulfoxide (DMSO)	Agilent, Santa Clara, USA	600675
DMEM (1x)	Thermo Fisher, Bonn, Germany	21969035
DMEM/F-12-500 mL	Life Technologies, Carlsbad, USA	11320-074
DMH1	Tocris Bioscience, Bristol, UK	4126/10
DMSO	Sigma-Aldrich GmbH, Taufkirchen, Germany	D2650
DNA Agarose	BiozymBiozym Scientific GmbH, Hessisch Oldendorf, Germany	870055
DNA Loading Dye 6x	Sigma-Aldrich GmbH, Taufkirchen, Germany	R0611

Doxycycline hyclate	Sigma-Aldrich GmbH, Taufkirchen, Germany	D9891-1G
Dulbecco's Phosphate Buffered Saline (DPBS)	Thermo Fisher, Bonn, Germany	14190094
Erastin	Merck KGaA, Darmstadt, Germany	329600-5MG
Ethanol (EtOH)	Merck KGaA, Darmstadt, Germany	1009831000
Ethyleneglycol	Carl Roth GmbH & Co, Karlsruhe, Germany	9516.3
Ethylendiamintetraacetatic acid (EDTA)	Sigma-Aldrich GmbH, Taufkirchen, Germany	E9884
Fetal Bovine Serum (FBS)	Thermo Fisher, Bonn, Germany	10270106
Fetal Calf Serum (FCS)	Thermo Fisher, Bonn, Germany	10270
GeneRuler 1 kb Plus DNA Ladder-5 x 50 µg	Life Technologies, Carlsbad, USA	SM1331
Geneticin® Selective Antibiotic (G418 Sulfate) (50 mg/mL)	Thermo Fisher, Bonn, Germany	10131035
Glycerol	Sigma-Aldrich GmbH, Taufkirchen, Germany	G5516
Gentamicin sulfate	Sigma-Aldrich GmbH, Taufkirchen, Germany	G1914-250MG
Goat serum	Cell Signalling Technologies, Danvers, USA	5425
GlutaMAX Supplement-100 mL	Thermo Fisher, Bonn, Germany	35050038
Glycerin	Carl Roth GmbH & Co, Karlsruhe, Germany	3783.2
Hydrogen peroxide	Carl Roth GmbH & Co, Karlsruhe, Germany	8070.2
Human BDNF, research grade	Miltenyi Biotec B.V. & CO. KG, Bergisch Gladbach, Germany	130-093-811
Human CNTF, research grade	Miltenyi Biotec B.V. & CO. KG, Bergisch Gladbach, Germany	130-096-336
Human IGF-1, research grade	Miltenyi Biotec B.V. & CO. KG, Bergisch Gladbach, Germany	130-093-885
Insulin Solution, Human recombinant	Sigma-Aldrich GmbH, Taufkirchen, Germany	I9278-5ML
Ketamin 10 %	Veterinärarzneimittel	-
L-buthionine sulfoximine (BSO)	Sigma-Aldrich GmbH, Taufkirchen, Germany	B2515
L-Glutamine	Thermo Fisher, Bonn, Germany	25030081
Liproxstatin-1	Selleck Chemicals GmbH, Berlin, Germany	S7699
Liproxstatin-1	Sigma-Aldrich GmbH, Taufkirchen, Germany	SML1414
Linoleic acid	Sigma-Aldrich GmbH, Taufkirchen, Germany	L2376
Lonza™ AccuGENE™ Molecular Biology Water	Thermo Fisher Scientific, Waltham, USA	BE51200
Magnesium Chloride	Sigma-Aldrich GmbH, Taufkirchen, Germany	1374248-1G
Magnesium Chloride (50 mM)	Thermo Fisher, Bonn, Germany	10342020
Meloxicam Melosus 1.5mg/ml	Veterinärarzneimittel	-
Magnesium sulfate (MgSO4)	Sigma-Aldrich GmbH, Taufkirchen, Germany	M7774
MEM non-essential amino acid NEAA (100X)	Sigma-Aldrich GmbH, Taufkirchen, Germany	M7145-100ML
Methanol	Thermo Fisher Scientific, Waltham, USA	M/4056/17
Methanol	Sigma-Aldrich GmbH, Taufkirchen, Germany	322415
Methyl-Tert-Butyl Ether	Thermo Fisher Scientific, Waltham, USA	M/4496/17
Mygliol	ChemTik, Berlin, Germany	CTK5E4513
N2 SUPPLEMENT 5ML	Thermo Fisher, Bonn, Germany	17502048
Neurobasal Medium-500 mL	Life Technologies, Carlsbad, USA	21103-049
N-ethylmaleimide	Sigma-Aldrich GmbH, Taufkirchen, Germany	E3876
Oleic acid	Cayman Chemical, USA	90260
Protamine sulfate	Sigma-Aldrich GmbH, Taufkirchen, Germany	P3369-10G
Penicillin/Streptomycin	Thermo Fisher, Bonn, Germany	15140122

Proteinase K	Carl Roth GmbH & Co, Karlsruhe, Germany	7528.1
Paraffin wax	Polysciences, Warminster, USA	19652
Paraformaldehyde	Carl Roth GmbH & Co, Karlsruhe, Germany	0335.3
Puromycin dihydrochloride	Sigma-Aldrich GmbH, Taufkirchen, Germany	P9620-10ML
Paraformaldehyde (PFA)	Carl Roth GmbH & Co, Karlsruhe, Germany	0335.3
Page ruler Prestained, Protein Ladder	Thermo Fisher, Bonn, Germany	26616
Propylene glycol	Sigma-Aldrich GmbH, Taufkirchen, Germany	398039
Potassium chloride	Sigma-Aldrich GmbH, Taufkirchen, Germany	4504
Retinoic acid	Miltenyi Biotec B.V. & CO. KG, Bergisch Gladbach, Germany	130-117-339
Rapamycin	Sigma-Aldrich GmbH, Taufkirchen, Germany	r8781
Roti®-Phenol/Chloroform/Isoamylalkohol	Carl Roth GmbH & Co, Karlsruhe, Germany	A156.2
Skim Milk Powder	Carl Roth GmbH & Co, Karlsruhe, Germany	T145.2
SOC-Medium	Thermo Fisher, Bonn, Germany	15544034
Sodium acetate	Sigma-Aldrich GmbH, Taufkirchen, Germany	9888
Sodium Chloride (NaCl)	Sigma-Aldrich GmbH, Taufkirchen, Germany	W302600
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co, Karlsruhe, Germany	2326.2
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co, Karlsruhe, Germany	6771.1
SPLASH® LIPIDOMIX® Mass Spec Standard	Avanti Polar Lipids, Birmingham, USA	330707-1EA
Stem Flex medium	Thermo Fisher, Bonn, Germany	A3349401
Stem MACS Purmorphamine	Miltenyi Biotec B.V. & CO. KG, Bergisch Gladbach, Germany	130-104-465
Sodium pyrophosphate	Sigma-Aldrich GmbH, Taufkirchen, Germany	S6422
Stem Pro Accutase Cell Dissociation Reagent	Life Technologies, Carlsbad, USA	A1110501
Sybr® Safe DNA gel stain	Invitrogen, Karlsruhe, Germany	S33102
Tamoxifen	Sigma-Aldrich GmbH, Taufkirchen, Germany	T5648-1G
tertiary-butyl hydroperoxide	Sigma-Aldrich GmbH, Taufkirchen, Germany	C6628
Tris (Trizma-Base)	Carl Roth GmbH & Co, Karlsruhe, Germany	2449.2
Tris(2-carboxyethyl)phosphine hydrochloride	Sigma-Aldrich GmbH, Taufkirchen, Germany	C4706-2G
Trisodium citrate dihydrate	Sigma-Aldrich GmbH, Taufkirchen, Germany	S1804
Triton-X	Sigma-Aldrich GmbH, Taufkirchen, Germany	T9284-10ml
Trypsin 0,05% EDTA	Thermo Fisher, Bonn, Germany	25300054
Tween ® 20	Sigma-Aldrich GmbH, Taufkirchen, Germany	P9416
Tween20	Sigma-Aldrich GmbH, Taufkirchen, Germany	P9416
Valporic acid	Tocris Bioscience, Bristol, UK	2815
Water	Thermo Fisher Scientific, Waltham, USA	W/0106/17
X-tremeGene HP DNA Transfection Reagent	Roche Diagnostics, Mannheim, Germany	6366236001
Xylol Roticlear®	Carl Roth GmbH & Co, Karlsruhe, Germany	A538.1
Z-VAD-FMK	Enzo Life Sciences GmbH, Lörrach, Germany	ALX-260-020-M001
Y-27632 dihydrochloride; 10 mg	Tocris Bioscience, Bristol, UK	1254/10
γ-Linolenic Acid	Cayman Chemical, USA	90220

3.1.4. Enzymes

Enzymes	Company	Catalog no.
Complete™ Protease Inhibitor Cocktail	Roche Diagnostics, Mannheim, Germany	1697498
PhosSTOP™ Phosphatase inhibitor cocktail	Roche Diagnostics, Mannheim, Germany	4906837001
Proteinase K	Carl Roth GmbH & Co, Karlsruhe, Germany	7528.1
Horseradish Peroxidase (HRP)	Sigma-Aldrich GmbH, Taufkirchen, Germany	P8125
Restriction Endonucleases	New England Biolabs GmbH, Frankfurt, Germany	600677
RNase A	Thermo Fisher, Bonn, Germany	10777019
Herculase II Fusion DNA Polymerase	Agilent, Santa Clara, USA	various
Taq Polymerase	Thermo Fisher, Bonn, Germany	10342020

3.1.5. Antibodies

Primary antibodies	species	Company	Catalog no.
FOXQ1	rabbit	Abcam, Cambridge, UK	ab51340
FOXQ1	rabbit	Origene	TA345477
FLAG	rabbit	Cell Signaling	2368S
GPX4	rabbit	Abcam, Cambridge, UK	ab125066
XCT / SLC7A11	rabbit	Invitrogen	PA1-16893
ACSL4	mouse	Santa Cruz Biotechnologies, Heidelberg, Germany	sc-365230
GPX1	rabbit	Abcam, Cambridge, UK	ab22604
ACSL6	rabbit	ATLAS	HPA040470
ACSL5	rabbit	Origene	TA332397
CDK4	mouse	Cell Signaling	2906
CDK6	mouse	Cell Signaling	3136
Cyclin D1 (DCS6)	mouse	Cell Signaling	2926
TRXRD1 (TR1)	rabbit	Abfronitier	LF-PA0023
SEPP1	rabbit	Abcam, Cambridge, UK	ab193193
p21 Waf/Cip1 (12D1)	rabbit	Cell Signaling	2974
E-Cadherin (24E10)	rabbit	Cell Signaling	3195
Vimentin (D21H3)	rabbit	Cell Signaling	5741S
p-S6K	rabbit	Cell Signaling	92095
S6	rabbit	Cell Signaling	4858T
MDM2	rabbit	Cell Signaling	86934S
pMDM2	rabbit	Cell Signaling	3521S
VCP	mouse	Abcam, Cambridge, UK	ab11433
β-Actin	mouse	Sigma-Aldrich GmbH, Taufkirchen, Germany	A5441
β-Actin-peroxidase	mouse	Sigma-Aldrich GmbH, Taufkirchen, Germany	A3854
IBA1	rabbit	Genetex, Irvine, USA	GTX100042
GFAP	rabbit	Abcam, Cambridge, UK	ab7260

Secondary antibodies	species	Company	Catalog no.
goat anti-mouse IgG-HRP	goat	Santa Cruz Biotechnologies, Heidelberg, Germany	SC-2031
goat anti-rabbit IgG-HRP	goat	Santa Cruz Biotechnologies, Heidelberg, Germany	SC-2030

3.1.6. Cell lines

Pfa1: The TAM-inducible Gpx4 -/- MEF cell line was previously described by Seiler et al. 2008

Pfa1-p442-FOXQ1-OE: This cell line was generated by lentiviral infection of Pfa1 cells by stably overexpressed p442-FOXQ1.

Pfa1-pLV-EF1a-FOXQ1-OE: This cell line was generated by lentiviral infection of Pfa1 cells by stably overexpressed pLV-EF1a-FOXQ1.

Pfa1-Mock: This cell line was generated by lentiviral infection of Pfa1 cells by stably overexpressed pLV-EF1a-IRES vector.

A549, NCI-H1975, NCI-H1437, and NCI-H1573: Human lung cancer cell lines were obtained and cultured according to ATCC guidelines.

U-87, U-251, U-138, and U-373: Human brain cancer cell lines were obtained and cultured according to ATCC guidelines.

786-O: Human kidney cancer cell lines were obtained and cultured according to ATCC guidelines.

MDA-MB-468, MDA-MB-157, MDA-MB-436, MCF7, and BT-474: Human breast cancer cell lines were obtained and cultured according to ATCC guidelines.

All aforementioned human cell line (pLV-EF1a-FOXQ1-OE): All cell line was generated by lentiviral infection to stably overexpressed pLV-EF1a-FOXQ1-Flag.

All aforementioned human cell line (Mock): All cell line was generated by lentiviral infection to stably overexpressed pLV-EF1a-IRES empty vector.

HEK293T: Human embryonic kidney (HEK) 293T cells obtained from ATCC (ATCC® CRL-3216™) and used for lentiviral production.

3.1.7. Oligonucleotides

3.1.7.1. Oligos for cloning

Oligos	sequence
Ascl5_Gibson_for	ccggtcgaatcaagcttatcgataccgtcgacggatccttgccgccaccatgcttttattttaacttctt gt
Ascl5_Gibson_rev	attgctcggagggccccgggcggccgctacgtaaccggtcctactactcctcgatgctctcatag
Ascl3_Gibson_for	ccggtcgaatcaagcttatcgataccgtcgacggatccttgccgccaccatgaataaccacgtatctt caacac
Ascl3_Gibson_rev	attgctcggagggccccgggcggccgctacgtaaccggtcttattttcttccatacatccgctc
mActb-New_for	AAGAGCTATGAGCTGCCTGA
mActb_rev	GCCACAGGATTCCATACCCA
P442_mFoxq1_for	ccggtcgaatcaagcttatcgataccgtcgacggatccttgccgccaccatgaaattggaggtgttcg tcccacg
P442_mFoxq1_rev	aattgctcggagggccccgggcggccgctacgtaaccggtcttaaaccttatcgtcgtcatcctt
P442_hFOXQ1_for	ccggtcgaatcaagcttatcgataccgtcgacggatccttgccgccaccatgaagttggaggtgttcg t
P442_hFOXQ1_rev	aattgctcggagggccccgggcggccgctacgtaaccggtctcaggctaggagcgtctcc
pEN_TRE_hFoxq1_for	gacaccgggaccgatccagcctccgcggccccgaactagtccaccatgaagttggaggtgttcgt
pEN_TRE_hFoxq1_Myc_flag_rev	cctcattcaaacaattaccctgttatccctagtcgactagtttaaaccttatcgtcgtcat
p442_hFoxq1_PAM-Cod Opt_for	ccggtcgaatcaagcttatcgataccgtcgacggatccttgccgccaccATGAAGCTGGAAGT GTTTGT
p442_mFoxq1_PAM-Cod Opt_for	ccggtcgaatcaagcttatcgataccgtcgacggatccttgccgccaccATGAAACTTGAAGTT TTCGTA
p442_ß-Actin_c2s-PAM-Cod Opt_for	ccggtcgaatcaagcttatcgataccgtcgacggatccttgccgccaccATGGATGACGATAT CGCTGCT
pEN_TRE_mFoxq1Cod Opt_for	gacaccgggaccgatccagcctccgcggccccgaactagtccaccATGAAACTTGAAGTTTT CGTA

3.1.7.2. Oligos for sequencing

Oligos	sequence
pEN_TRE_seq_for	GTCGAGTTTACCACTCCCTA
pEN_TRE_seq_rev	TCAGAGATTTTGAGACACGGGC
pLV-EFa1_seq_for	GAGGGGTTTTATGCGATGGA
pLV-EFa1_seq_rev	ATATAGACAAACGCACACCGGC

3.1.7.3. Oligos for genotyping

Oligos	sequence
hFoxq1-geno-For	AGACGACTCCCTGGGCTCAGAT
hFoxq1-geno-rev	GCGAAGGAGCTGGAGAACTT
FOXQ1_hGeno-New_for	TGAAGTTGGAGGTGTTCGTC
FOXQ1_hGeno_rev	AGGATGCTGTCGATGGCGAA
mFoxQ1_Geno_for	AAAATGGGCAGCGATCTGGAG
mFoxQ1_Geno_rev	GCCAGATCCTCTTCTGAGATG

3.1.7.4. Oligos for CRISPR/Cas9 gene KO

Oligos	sequence
plentiCRISPRv2_G1_hFOXQ1_for	CACCGGCTTGGGCCGCCGCGTATA
plentiCRISPRv2_G1_hFOXQ1_rev	AAACTATACGCGGCGGCCCAAGCC
plentiCRISPRv2_G2_hFOXQ1_for	CACCGCGAGTACACCTTCGCCGAC
plentiCRISPRv2_G2_hFOXQ1_rev	AAACGTCGGCGAAGGTGTACTCG
FOXQ1_g3-for"	ACACCGgccgagtcgcggatggccaG
FOXQ1_g3-rev"	AAAACtggccatccgcgactcggcCG
plentiCRISPRv2_G1_mFoxQ1_for	CACCGCGAATACACCTTCGCCGAC
plentiCRISPRv2_G1_mFoxQ1_rev	AAACGTCGGCGAAGGTGTATTCGC
plentiCRISPRv2_G2_mFoxQ1_for	CACCGCTACGCTGCTGGCGTCGCGC
plentiCRISPRv2_G2_mFoxQ1_rev	AAACGCGCGACGCCAGCAGCGTAGC
PTEN_1-gRNA-exo#1_rev	ACACCGttaaaaccggcccgggtcccG
PTEN_1-gRNA-exo#1_for	AAAACgggacccgggccggttttaaCG
PTEN_2-gRNA-exo#5_rev	ACACCggtgggttatggtcttcaaaG
PTEN_2-gRNA-exo#5_for	AAAACtttgaagaccataacccaccG

3.1.7.5. Oligos for qPCR

Oligos	sequence
qPCR_EGFR-For	GAAACGTCTGCCGCAAATTC
qPCR_EGFR-rev	GGTTCACATCCATCTGGTACG
qPCR_KRAS-For	AGGACTTAGCAAGAAGTTATGGAA
qPCR_KRAS-rev	TCGAACTAATGTATAGAAGGCATCA
qPCR_RAF1-For	GAGCACTGTAGCACCAAAGTA
qPCR_RAF1-rev	TACGACGCATAGTCAAAGAAGG
qPCR_MAP2K1-For	CTCCATGGCCAACTCCTTC
qPCR_MAP2K1-rev	TCTCAGCCGCATCTCCT
qPCR_MAPK1-For	TTCTAACAGGCCCATCTTTCC
qPCR_MAPK1-rev	TTCAGGTCTTCTTGTGATGGG
qPCR_PIK3CA-For	CTCGACTTTGCCTTTCCATTT
qPCR_PIK3CA-rev	GAGTGTCTGTGTAATCAAACAAGT
qPCR_AKT1-For	CCTCTGCTTTGTCATGGAGTA
qPCR_AKT1-rev	CACAATCTCAGCGCCATAGA
qPCR_MTOR-For	GGCATCCAGCAGGATATCAA
qPCR_MTOR-rev	GTCCTTCTTTAGCTGTGGAATCT
qPCR_hFoxq1-For	TCAACGACTGCTTCGTCAAG
qPCR_hFoxq1-rev	GAAGGTGTACTCGCTGTTGG
qPCR_hFoxq1-Cod Opt-For	CTCACCTGTATTGTCCACTGAG
qPCR_hFoxq1--Cod Opt-rev	GAGTTTCAACAGGGTAGGGAAG
qPCR_Gpx4-For	CGATACGCTGAGTGTGGTTT
qPCR_Gpx4-rev	CGGCGAACTCTTTGATCTCTT
qPCR_mFoxq1-For	TCTCGCTCAACGACTGTTTC
qPCR_mFoxq1-rev	AAGGTGTATTCGCTGTTGGG
qPCR_h-GAPDH-For	CCAAGGTCATCCATGACAACT
qPCR_h-GAPDH-rev	ATCACGCCACAGTTTCCC
qPCR_h-actb(ß-Actin)-For	GGCCAGGTCATCACCATT
qPCR_h-actb(ß-Actin)-rev	GATGTCCACGTCACACTTCA
qPCR_h-Acsl4-For	CGACCTAAGGGAGTGATGATG

qPCR_h-Acsl4-rev	CAGAGAGTGTAAGCGGAGAAG
qPCR_h-AIFM2-For	ACCGGCATCAAGATCAACAG
qPCR_h-AIFM2-rev	GTCGGCACAGTCACCAAT
qPCR_h-SLC7A11-For	GTGGCCTACTTTACGACCATTA
qPCR_h-SLC7A11-rev	CAGGAGAGGGCAACAAAGAT
qPCR_h-p21-For	CTGCCCAAGCTCTACCTTC
qPCR_h-p21-rev	CATGGTCTTCCTCTGCTGTC
qPCR_mFoxq1-Cod Opt-For	TGCTTCGTGAAGGTCTTGAG
qPCR_mFoxq1-Cod Opt-rev	CGTCTCCTAAACACTCCATCAG
qPCR_hFoxq1#2-For	TGAAGTTGGAGGTGTTCGTC
qPCR_hFoxq1#2-rev	ATCTGAGCCCAGGGAGT
qPCR_KRAS#2-For	GTAGGCAAGAGTGCCTTGAT
qPCR_KRAS#2-rev	CTCATGTACTGGTCCCTCATTG
qPCR_h-RPS6KB1-For	GTAACAGGAGCAAATACTGGGA
qPCR_h-RPS6KB1-rev	CCACGATGAAGGGATGCTTTA
qPCR_h-EIF4EBP1-For	CACGCTCTTCAGCACCA
qPCR_h-EIF4EBP1-rev	TTTGGTCACAGGTGAGTTCC
qPCR_h-p53-For	GTTTCCGTCTGGGCTTCTT
qPCR_h-p53-rev	GCAGGTCTTGGCCAGTT
qPCR_m-MTOR-For	CACTGCTGAATATGTGGAGTTTG
qPCR_m-MTOR-rev	AACTTGCTGGAAGAAGAAGGT
qPCR_m-RPS6KB1-For	GTGAACAGAGGGCCAGAAA
qPCR_m-RPS6KB1-rev	CTTCCCAGTATTTGCTCCTGT
qPCR_m-CDKN1A (p21)-For	AAGTGTGCCGTTGTCTCTTC
qPCR_m-CDKN1A (p21)-rev	AGTCAAAGTTCCACCGTTCTC
qPCR_m- p53-For	AACTTACCAGGGCAACTATGG
qPCR_m-p53-rev	AGCTGGCAGAATAGCTTATTGA

3.1.8. Bacteria

DH5α E.coli: F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ (lacZYA-argF) U169, hsdR17 (rK-mK+), λ–

3.1.9. Cloning vectors

3.1.9.1. Cloning vector 442-PL1-IRES-PURO

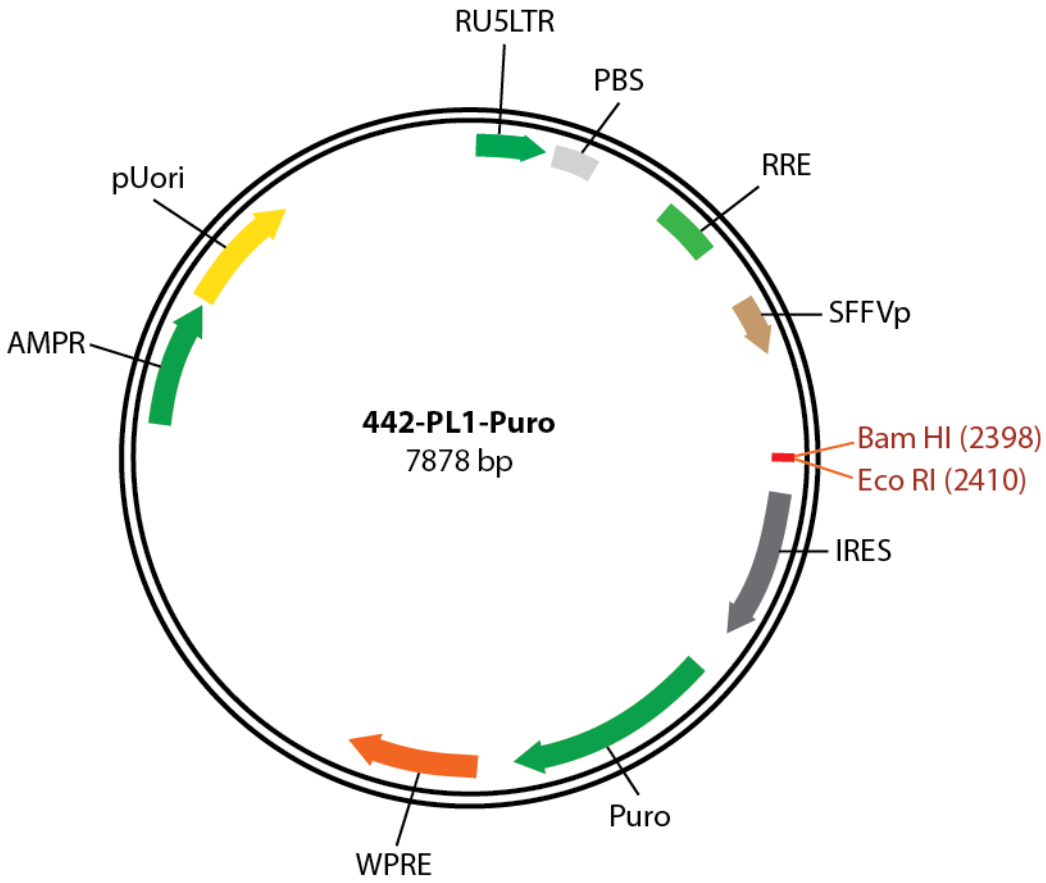


Figure 5: Map of the transfer vector 442-PL1 IRES puro.

442-PL1 transfer vector was generated by modifications of the human immunodeficiency virus (HIV) lentiviral vector (kind gift from Dr. Timm Schröder, ETH Zürich). The displayed transfer vector consists of a modified and reduced viral genome, lacking the required proteins for infection and integration of the virus. Restriction site used for cloning are indicated by BamHI and EcoRI. The expression target cDNA is regulated by the SFFV promotor after successful transduction. Internal ribosomal entry site (IRES), Rous sarcoma virus Promotor (RSV), RU5 LTR (RU5), rev-responsive element (RRE), polypurine tract (PPT), spleen foci forming virus (SFFV), puromycin-acetyltransferase (Puro) (puromycin resistance), post-transcriptional regulatory element (PRE), self-inactivating 3' LTR (SIN), β-lactamase (Amp), origin of replication (pUC ori).

3.1.9.2. Cloning vector pLV-EF1a_Puro

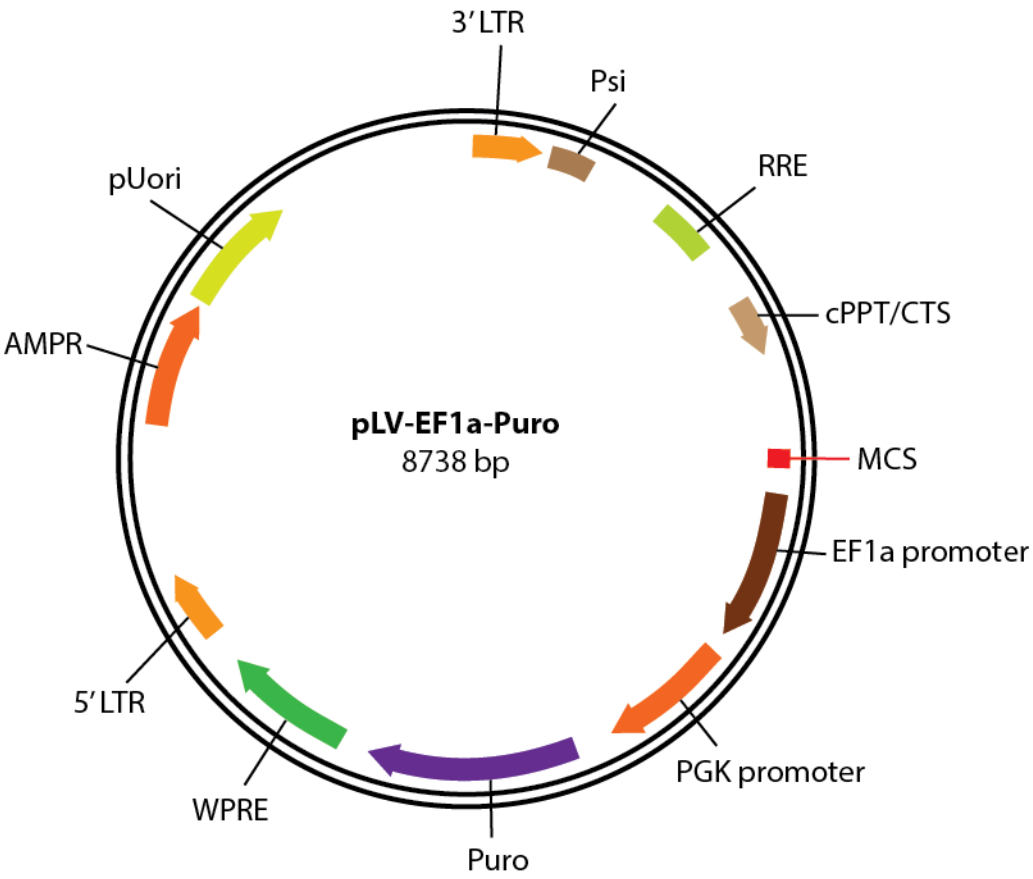


Figure 6: Map of the gene expression vector pLV-EF1a-puro.

The displayed transfer vector is sgRNA. Lentiviral vector expresses gRNA with the human U6 promoter and PURO with the EF1 promoter to allow for PURO selection of transduced cells, pLV-EF1a-puro (Addgene #85132). Restriction sites used for cloning are indicated by XhoI, BamHI and EcoRI. Abbreviations: self-inactivating 3´LTR (SIN), retroviral packaging element (Psi pack), rev responsive element (RRE), central poly-purine tract (cPPT), multiple cloning site (MCS), elongation factor-1α promoter (EF1a), restriction sites used for cloning (BamHI/EcoRI), phosphoglycerate kinase promoter (PGK promoter), puromycin selection marker (PuroR), woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), promoter for ampicillin resistance *(AMPR),* 5' terminal repeat (LTR), origin of replication (pUC ori).

3.1.9.3. Cloning vector lentiCRISPR v2

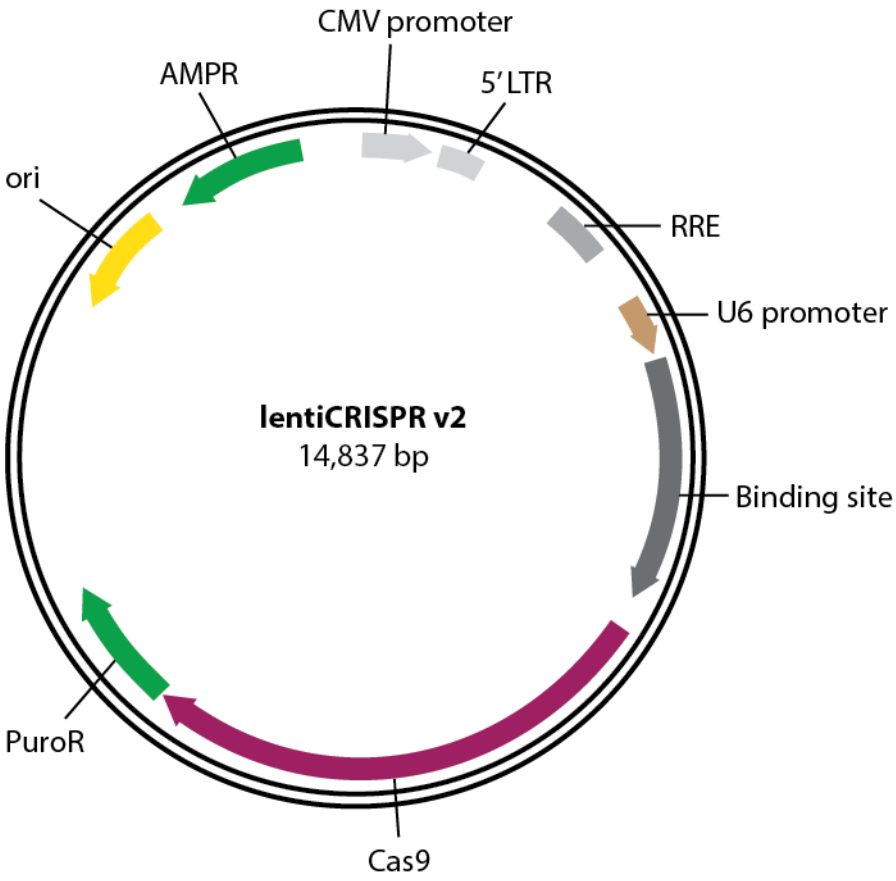


Figure 7: Map of the sgRNA expression vector lentiCRISPR v2

The displayed vector is lentiviral expression vector for Streptococcus pyogenes Cas9 and sgRNA in the improved one vector system (lentiCRISPR v2, Addgene #52961). Abbreviations: Cytomegalovirus (CMV), rev responsive element (RRE), origin of replication (ori), terminal repeat (5' LTR), puromycin selection marker (PuroR), restriction sites used for cloning (BamHI/EcoRI).

3.1.10. Software and online tools

Software	Supplier
Adobe Illustrator	
Biorender	BioRender, Toronto, Canada
FinchTV 1.4	Geospiza, Inc., free software
GraphPad Prism 8.0	GraphPad Software Inc., USA
Image Lab	Bio-Rad, Munich, Germany
multalin.toulouse.inra.fr	INRAE Institute
Vector NTI	Thermo Fisher, Bonn, Germany
ZEN	Carl Zeiss AG, Oberkochen, Germany

3.2. Methods

3.2.1. Molecular biology standard methods

3.2.1.1. Plasmid DNA amplification and purification

For plasmid amplification, bacteria transformed with plasmid DNA were incubated overnight 16-20 h at 37°C on LB plates containing the corresponding antibiotic selection. Afterwards, a single bacterium was selected from agar plates. Overnight bacterial cultures were set up for mini-preparation (2 mL) or maxi-preparation (200 mL) and incubated overnight at 37 °C under constant shaking. After overnight incubation plasmid DNA was isolated and purified using either a plasmid mini or maxi Kit (QIAGEN) according to manufacturer's instructions. DNA concentration and quality was determined using a 1000 Spectrophotometer (NanoDrop).

3.2.1.2. Ligation of DNA fragments by Gibson cloning

The Gibson assembly method allows the ligation of two or more blunt-ended fragments without the need for restriction enzyme digestion. Instead, the fragments contain a least 40 bp overlapping sequence on both 5' and 3' ends that allows seamless joining of adjacent fragments. The vector DNA was linearized by digestion with DNA specific targeting restriction enzymes. To achieve maximal sequence fidelity, Herculase II Fusion polymerase or *Thermo* Scientific *Taq* DNA polymerase was used for the PCR reaction in a 50 µL or 30 µL reaction mix in the presence of 4 % DMSO, 0.2 mM dNTP, 0.5 µM of the individual reverse and forward primer, 2 µL of DNA template and 5 x reaction mix. The vector DNA and the PCR product were separated by agarose gel electrophoresis, and both DNAs were isolated from agarose gel by using the Wizard® SV Gel and PCR clean up system (Promega). The concentration and quality of the DNA fragments were measured by using 1000 NanoDrop Spectrometer. For the ligation reaction, 10 µl of the Gibson Assembly® Master Mix (NEB) and 100 ng of vector was used together with a 2-fold molar excess of the desired insert in a total volume of 20 µl. The reaction mix was incubated in a thermocycler at 50°C for 15 min and 5 µl of the ligation reaction was used for bacteria transformation.

3.2.1.3. Ligation of DNA fragments by T4-DNA-ligase

T4 DNA Ligase catalyses the formation of phosphodiester bonds between 5' phosphate and 3' hydroxyl termini to join the DNA fragments. Measured amounts of DNA fragments were used in a molar ratio of 1:3 to 1:5. Following the manufacturers' protocol, usually 50-100 ng of backbone DNA was mixed with the corresponding amount of insert DNA, T4-DNA-ligase and 5x ligase buffer in total volume to 10 µl adjusted with nuclease free H_2O. The ligation reaction was incubated for 10 or 60 min at room temperature or alternatively for 16 h at 4 °C and then proceeded with bacteria transformation.

3.2.1.4. Amplification of DNA fragments for cloning

In the PCR technique, DNA is amplified in vitro by a series of polymerisation cycles. The PCR reaction mix was set up in 50 µl final volume containing the template DNA (~100 ng), 0.5 µM of both forward and reverse primer, 200 µM dNTPs, 100 nM of DNA template and 8 % DMSO and 1 unit of Herculase II Fusion polymerase or Taq DNA polymerase. The following program was set up on the 96 well PCR machine.

PCR Master Mix using Taq DNA polymerase	
Forward primer (10 µM)	1.3 µL
Reverse primer (10 µM)	1.3 µL
10X PCR Buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl)	2.5 µL
MgCl2 (50 mM)	0.75 µL
Deoxyribonucleotide triphosphate (dNTP)	0.5 µL
Taq DNA Polymerase (5U/µl)	0.3 µL
dH2O	16.35 µL
Sample DNA	2 µL
Total volume	25 µL

Table 1: Standard protocol for PCR Master Mix preparation using Taq DNA polymerase

PCR Master Mix using *Herculase II Fusion* DNA polymerase	
Forward primer (10 µM)	2.6 µL
Reverse primer (10 µM)	2.6 µL
5× Herculase II Reaction Buffer	10 µL
Dimethyl sulfoxide (DMSO)	2 µL
Deoxyribonucleotide triphosphate (dNTP)	1.25 µL
Herculase II Fusion DNA polymerase	0.5 µL
dH2O	28.5 µL
Sample DNA	3 µL
Total volume	50 µL

Table 2: Standard protocol for PCR Master Mix preparation using *Herculase II Fusion* DNA polymerase.

Standard program for polymerase chain reaction (PCR)			
No of cycles	Steps	Temperature	Time
1x	Denaturation	95 °C	5 min
35x	Denaturation	95 °C	30-60 sec
	Annealing	58 - 75°C	30-60 sec (primer recommended)
	Elongation	72 °C	Taq 1 min/0.5 kb, Herc 1 min/1 kb
1x	Extension	72 °C	5 min
1x	cooling	4 °C	until required

Table 3: Standard protocol for DNA amplification by PCR reaction

3.2.1.5. Restriction digestion

Restriction enzymes obtained from New England Biolabs GmbH and as per manufacturer´s protocol 50 µL of reaction mix were applied to digest vector DNA. The linearised DNA was separated using 0.8-1 % agarose gel and isolated using Wizard® SV Gel and PCR Clean Up System (Promega). The concentration of DNA was measured by a NanoDrop 1000 Spectrophotometer.

3.2.1.6. Agarose gel electrophoresis

Agarose gel electrophoresis is a routine method used for DNA separation according to its size. Depending on size DNA fragments were separated in 0.8 %, 1 % or 2 % agarose gel, made of agarose in 1 x TAE buffer containing SYBR® Safe DNA stains in a ratio of 1:10,000. The gel running chamber contains 1 x TAE buffer allowed to solidify. Before loading the DNA samples in the gel pockets, they were mixed with 6x DNA loading dye (Thermo Fisher), and voltage 100-120 V was applied to enable the separation of the fragments. For visualisation of the DNA separated fragments, the ChemiDoc™ UV transilluminator (BioRad) was used.

TAE buffer (10 ×): 200 mM Tris, 100 mM sodium acetate, 6 mM EDTA (pH 7.5)

3.2.1.7. DNA fragments extraction from agarose gel

The specific required DNA fragments separated in the agarose gel fragments were isolated using a scalpel. Following the manufacturer´s protocol the isolated DNA was purified by applying Wizard® SV Gel and PCR Clean Up System (Promega). The DNA content was measured by a 1000 Spectrophotometer (NanoDrop).

3.2.1.8. PCR product column purification

For column purification, DNA/PCR products were purified using either the QIAquick PCR Purification Kit (28104 QIAGEN) following the manufacturer's protocol. The DNA content was measured by a 1000 Spectrophotometer (NanoDrop).

3.2.2. Gene Cloning

3.2.2.1. Cloning genes of interest into plasmid vector

Cloning into (pLV-EF1a-IRES, and 442-PL1-IRES)-PURO/NEO/Blast viral expressing vector:

The third-generation lentiviral vector 4 *pLV-EF1a-IRES-Blast* (Plasmid #85133) Addgene and 442-PL1-IRES-puro (a kind gift from Prof. Dr. Timm Schröder, ETH Zurich) were used to express both human and mouse FOXQ1 flag tagged codon, optimised using Gibson cloning (see 3.2.3).

Therefore, *pLV-EF1α-IRES-blast* plasmid was first linearised with BamHI/EcoRI, while 442- PL1-IRES-puro plasmid was first digested with XbaI at 37°C for 1 h, then BstbI was added, and the temperature was raised to 65°C for another hour to inactivate the restriction endonuclease. PCR was performed to generate *pLV-EF1α-IRES-Blast* _human FOXQ1 flag tagged codon optimised and 442-PL1-IRES-puro_ mouse_FOXQ1 for the insertion via Gibson cloning. The gene string was amplified with designed primers to add ~40 bp homology to each end of the digested vector.

(Cloning FOXQ1 into pSlik-Neo)

For a doxycycline-inducible expression of human FOXQ1 the plasmid pSLIK-Neo (Plasmid #25735) and pSLIK-Zeo (Plasmid #25736) Addgene were used via Gibson cloning.

(Cloning of lentiviral sgRNA plasmids (pLenti-CRISPRv2-Blast)

For the generation of cell lines with a stable expression of sgRNAs, the plentiviralV2 (pLenti_CRISPR_v2Blast (Plasmid# 98293)/Puro (Plasmid# 98290)) was used to clone the guide sequence (~20-21 bp) that represents the CRISPR target into the vector. The lentiviral vector was linearised with restriction enzymes and used to generate stable sgRNA expressing cells. The digested vector was separated on a 1% agarose gel, extracted from the gel using Wizard® SV Gel and PCR clean up system (Promega) and DNA concentration was determined using the spectrophotometer (NanoDrop). Online platform (http://crispor.tefor.net/) was used to design and optimise CRISPR guides. Protocol from (3.2.4) was followed to clone the guides into the linearised vector. Two oligos per guide (forward and reverse) were ordered (Invitrogen) consisting of the guide sequence (20 bp) and overhangs matching the digested backbone at the restriction site.

Forward oligo: CACC(G)N20GT

Reverse oligo: TAAAACN20(complementary to forward oligo) (C)

The ordered oligos were dissolved in dH2O at a final concentration of 100 µM. To gain small fragments of double stranded oligos that can be ligated with the digested vector, 1 µM of each (forward and reverse) oligo stock was added to 10 µl of 1 × TE buffer and heated to 95°C for 5 min. Oligos were allowed to anneal by cooling down to room temperature before using them

in a ligation reaction. For the ligation reaction 50-100 ng linearised vector (pLenti_CRISPR_v2Blast/Puro) was mixed with 4 µl oligos in 10 µl reactions incubated at RT 10 min or at 4°C overnight. Electro-competent DH5α cells were transformed, and the sequence of single colonies was validated via Sanger sequencing (GATC Biotech) was used for virus production.

List of guides (gRNA) used for cloning.

Names of the oligoes	Sequences
plentiCRISPRv2_G1_mFoxQ1_for	CACCGCGAATACACCTTCGCCGAC
plentiCRISPRv2_G1_mFoxQ1_rev	AAACGTCGGCGAAGGTGTATTCGC
plentiCRISPRv2_G2_mFoxQ1_for	CACCGCTACGCTGCTGGCGTCGCGC
plentiCRISPRv2_G2_mFoxQ1_rev	AAACGCGCGACGCCAGCAGCGTAGC
plentiCRISPRv2_G1_hFOXQ1_for	CACCGGCTTGGGCCGCCGCGTATA
plentiCRISPRv2_G1_hFOXQ1_rev	AAACTATACGCGGCGGCCCAAGCC
plentiCRISPRv2_G2_hFOXQ1_for	CACCGCGAGTACACCTTCGCCGAC
plentiCRISPRv2_G2_hFOXQ1_rev	AAACGTCGGCGAAGGTGTACTCG
plentiCRISPRv2_G3_hFOXQ1_for	ACACCGGCCGAGTCGCGGATGGCCAG
plentiCRISPRv2_G3_hFOXQ1_rev	AAAACTGGCCATCCGCGACTCGGCCG
plentiCRISPRv2_PTEN_1-gRNA-exo#1_for	AAAACGGGACCCGGGCCGGTTTTAACG
plentiCRISPRv2_PTEN_1-gRNA-exo#1_rev	ACACCGTTAAAACCGGCCCGGGTCCCG
plentiCRISPRv2_PTEN_2-gRNA-exo#5_for	AAAACTTTGAAGACCATAACCCACCG
plentiCRISPRv2_PTEN_2-gRNA-exo#5_rev	ACACCGGTGGGTTATGGTCTTCAAAG
plentiCRISPRv2_PPARD_1-gRNA-exo#3_for	AAAACAGAGCTCAATGGGGGACCACG
plentiCRISPRv2_PPARD_1-gRNA-exo#3_rev	ACACCGTGGTCCCCCATTGAGCTCTG
plentiCRISPRv2_PPARD_2-gRNA-exo#6_for	AAAACTTTTGGTCGGATGCCGGAGGCG
plentiCRISPRv2_PPARD_2-gRNA-exo#6_rev	ACACCGCCTCCGGCATCCGACCAAAAG

Table 4: List of the CRISPR_gRNAs used for cloning.

3.2.3. General methods for gene transformation

3.2.3.1. Transformation of chemically competent bacteria

Chemical competent E. coli strain DH5α was used for bacterial transformation. Bacteria stored at -80°C and were thawed on ice for 15 min before use. 50 µl of thawed cells were transferred to a fresh 1.5 mL Eppendorf tube and were mixed with 2 µl plasmid DNA. After maxing gently further incubated on ice for 10 min. Heat shock (42°C) was applied for 2 min to enhance the uptake of the plasmid, and then bacterial cell suspension was immediately placed on ice. After adding 500 µL of S.O.C. medium, the bacteria were allowed to regenerate for 60 min at 37°C. Next, bacterial cells were centrifugated (210 × g, 1 min), supernatant was removed, and cell

pellet was resuspended in the residual medium. Bacteria were then plated directly on LB agar plates containing antibiotic selection markers (ampicillin (100 µg/mL)).

LB Agar: 20 mM MgSO4, 10 mM KCl, 1 % (w/v) Trypton, 0.5 % (w/v) bacto yeast extract, 0.5 % (w/v) NaCl, 1.2 % bacto agar

3.2.3.2. Lipofection

Lipofection also known as liposome transfection is a lipid-mediated DNA transfection procedure which results in the transfer of genetic material into a cell. Cells were seeded on 6-well plate and were allowed to 45-50 % confluency prior to transfection. 200 µL of serum-free DMEM medium was mixed with 2 µg plasmid DNA and 6 µL (1:3 ratio (DNA: reagent)) X-tremeGENE HP DNA Transfection Reagent (Roche) of room temperature was added. The transfection complex was incubated for 15-30 min at room temperature and applied dropwise to the cells after medium change. After 48 h incubation specific selection was added to the medium. Expression of the transfected DNA could be observed 48-72 h after transfection via immunoblot analysis.

3.2.3.3. Lentiviral transduction

Viral transduction for MEFs was performed using third generation lentiviral vectors (lentiCas9-Blast/Puro (Addgene #52962), pLV-EF1a-IRES-Blat/Puro (Addgene #85133/85132), and 442-PL1-IRES-puro (a kind gift from Dr. Timm Schröder, ETH Zürich)) together with the third generation packaging system containing pEcoEnv-IRES-puro (ecotropic envelope and glycoprotein), pMDLg_pRRE (structural protein Gag and enzyme cluster Pol) and pRSV_Rev (post-transcriptional regulation protein Rev). While for human cell line, third generation lentiviral vectors together with the third-generation packaging system containing pLenti-ORF expression construct pMD2.G (Addgene 12259) and packaging plasmids psPAX2 (Addgene 12260) were used under the biosafety condition of S2 lab. Viral particles can only infect rodent cells due to their ecotropic envelope proteins, to this end, HEK293T cells were used to produce replication-incompetent viruses. HEK293T cells were seeded in 6 or 10 well plate allow to reach 70% confluency after overnight incubation. Cells were co-transfected with the transfer

vector and the vectors from the packaging system in a fixed molar ratio by lipofection (3.3.2). 72 h after transfection supernatant containing viral particles was collected from the HEK293T cells and a 0.45 µm low protein binding syringe filter was used for sterile filtration. Aliquots of infectious supernatant were stored at -80°C. For lentiviral transduction an aliquot of infectious supernatant was thawed, mixed 1:1 with Standard DMEM, supplemented with (8 µg/mL) protamine sulfate to enhance viral transduction and added to the pre seeded cells. Antibiotic selection was started 48 h after viral transduction.

3.2.3.4. Doxycycline-inducible expression of FOXQ1

Transfection of doxycycline-dependent pSlik vector containing the FOXQ1-Flag into human cells was conducted via lipofection using the X-tremeGene HP DNA Transfection Reagent. About 50,000 cells/well were cultured on 6 well dish and infected with 1:1 of doxycycline inducible plasmid cloned with FOXQ1 (pSlik_FOXQ1-Flag-OE). 48 h after infection cells were selected with neomycin for at least 1 week. Doxycycline-inducible pSlik_FOXQ1-Flag expression was verified by Western blot using antibody against flag after cells were treated for 24 h with different concentration of doxycycline. In order to determine the level of FOXQ1 expression necessary for ferroptosis execution, 6-well plates were seeded treated overnight with different concentrations of doxycycline and ferroptosis was induced by increasing concentration of RSL3. After 24 h incubation, the cell viability was assessed using the AquaBluer assay method.

3.2.4. General methods of cell cultures

3.2.4.1. Standard methods of cell culture

MEfs (Pfa1) and various human cancer cell lines (lung, Kidney, brain and breast cancer cell lines) were cultured in standard DMEM medium. Cell culture incubators were operated under at 37°C with 5 % CO_2 humid conditions without oxygen level. Cultures were maintained around 70-80% confluency by periodically splitting them onto fresh cell culture plates. For cells splitting procedure, cells were washed with DPBS to remove residual culture medium and trypsinized using one volume of Trypsin-EDTA (0.05%) in incubator at 37°C for 3-5 min or once

cells appear detached. Two volumes (2 ml) of pre-warmed growth medium to inactivate trypsinization and a fraction of the detached cells was transferred to a fresh cell culture plates already containing standard DMEM medium.

Standard DMEM: DMEM, 10 % FCS, 1 % glutamine, 50 U/ml penicillin G, 50 µg/mL streptomycin

3.2.4.2. Cells counting using haemocytometer

In order to determine the cells number, cells were washed twice with PBS, harvested by trypsinisation and resuspended with Standard DMEM. The cell suspension was centrifuged at 400 g for 5 min and the cell pellet was resuspended in 5 ml Standard DMEM. 10 µl of the resuspended cells was used to determine cell number using the haemocytometer.

Total cells/ml = (Total cells counted) / (Number of squares counted) x (Dilution factor x 10,000 cells/ml).

3.2.4.3. Cell viability assay

To analyse the sensitivity of WT and FOXQ1-OE in both mouse and human cells, a dose-dependent cytotoxicity of RSL3 induced ferroptosis was assessed using the AquaBluer assay method. To his end, cells were seeded in a 96-well plate (2000 cells/well) and incubated overnight at 37°C with 5 % CO_2. The next day, cells were treated with increasing concentrations of different compounds including (1S, 3R)-RSL3, erastin and BSO for 24 h. The compound reflects the activity of the mitochondrial electron transport chain. Oxidized AquaBluer® enters the cells and gets reduced by electrons such as NADPH and FADH2 derived from active mitochondria. Active mitochondria reduce AquaBluer resulting in a colour change of the medium from blue to pink and the reduced compound has an altered fluorescent emission on a different wavelength. For cell death inhibition, cells were pre-incubated for 2 h with different cell death inhibitors (liproxstatin-1, and Z-VAD-FMK) before cell death was elicited using the aforementioned compounds. To determine cell viability, AquaBluer® was added to the cells 24 h after treatment (1:100) and further incubated for 6 h at 37°C.

Fluorescence of the reagent was measured at 562 nm using the SpectraMax microplate reader (Molecular Device GmbH).

3.2.4.4. Cells cryopreservation and thawing

Cryopreservation is used to store cells in liquid nitrogen for long-time storage. For cryopreservation cells were trypsinised when they reached approximately 80% confluency. Cells were centrifuged and the cell pellet was resuspended in Standard DMEM containing 10% DMSO and transferred to cryo vials which were stored overnight in freezing container 'Mr. frosty' at -80°C before they were transferred to liquid nitrogen; or by using cell freezing medium (COS banker) where cells can be directly transferred into cryo vials and put in liquid nitrogen. For thawing cells the cryo-vials were placed in a water bath at 37°C and soon after being defrosted the cell suspension was immediately transferred into 6 well plate containing 2 ml Standard DMEM.

3.2.5. General methods related proteins

3.2.5.1. Protein purification from cells and tissue

In order to extract proteins for immunoblot analysis, cells were washed twice with ice-cold PBS and LCW lysis buffer in the presence of proteinase and phosphatase inhibitors (Roche) added and incubated on ice for 30 min. Cell lysate was collected using scrapers and transferred to Eppendorfs. Samples were incubated on ice for 15 min before removing the cell debris by centrifugation (14,000 x g, 15 min, 4°C). Supernatant was transferred to fresh Eppendorfs which were stored at -80°C, or 6 × loading buffer was added to 20 µg of the protein samples stored at -20°C.

LCW Lysis Buffer: 0.5 % TritonX-100, 0.5 % sodium deoxycholate salt, 150 mM NaCl, 20 mM TRIS, 10 mM EDTA, 30 mM Na-Pyrophosphate, pH 7.5.

3.2.5.2. Protein quantifications

Protein lysates were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific) following the manufacturer`s instructions. Absorbance was measured using SpectraMax microplate reader (Molecular Device GmbH).

3.2.5.3. Protein separation

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate proteins. 3 µL of Sample Loading Dye (6x) was added to 25 µg of each protein sample and complemented with MilliQ H2O to a final volume of 20 µL. Samples were heated to 95°C for 5 min and proteins were separated on precast 12% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (BioRad). The western blot chamber was filled with 1 x running buffer and the protein METHODS - 50 - samples along with protein ladder (Prestained Protein Page Ruler) were allowed to migrate at 80 V for 2-3 hours depending on the size of proteins to be separated. Gels were separated from their plastic scaffold and proteins were transferred to a PVDF (polyvinylidene difluoride) membrane for subsequent Western blot analysis.

Running buffer (10 x): 1% SDS, 250 mM Tris, 2.5 M glycine

3.2.5.4. Western blot analysis

For immunoblot analysis the SDS gel was transferred to a ready to use Trans-Blot® Turbo™ PVDF membranes (Bio-Rad) and protein transfer was conducted using the standard program (30 min, 25 V, 1 A) or m (7 min, 25 V, 2.5 A) in the semi-dry Trans-Blot® Turbo™ Transfer System (Bio-Rad). After that, the blotting cassette was disassembled, and the membranes were placed into a 50 mL falcon and washed with TBS-T before blocking was started. The membrane was then incubated under constant shaking at room temperature for 1 h in blocking buffer (5% non-fat milk) dissolved in Tris-Buffered Saline supplemented with 0.1% Tween-20 (TBS-T). Next, the membranes were then incubated with primary antibody diluted according to manufacturer`s protocol in the corresponding blocking solution for an overnight incubation at 4°C on a shaker. Three washes were carried out with TBS-T for 5 minutes at room temperature. The membrane was then incubated with secondary antibody diluted according

to manufacturer`s protocol at room temperature for 1 hour on a shaker. Finally, the membrane was washed again 3 times with TBS-T for 5 minutes. To visualise, the membrane was placed in a transparent plastic wrap and Clarity™ Western ECL Blotting Substrate (Bio-Rad) was applied to the membrane for 1 min. The excess reagent was removed, and the blot was placed into the ChemiDoc™ imaging system (Bio-Rad) for imaging. To redevelop with different antibody, antibodies were stripped from the PVDF membrane with 0.4 M NaOH for 7 min. After all, protein bands were quantified using the ImageLab software (Bio-Rad)

TBS (10 x): 1.5 M NaCl, 250 mM Tris, adjust pH to 7.4

TBS-T: 100 mL TBS (10 x), 900 mL dH2O, 1 mL Tween (0.1%)

Blocking solution: 5 w/v % BSA or skim milk diluted in TBS-T

NaOH: 20 g NaOH, volume adjusted to 1000 mL (0.5 M)

3.2.6. Methods related to RNA

3.2.6.1. Total RNA isolation

Following the manufacturer´s protocol, RNeasy Mini Kit (Qiagen) was used to isolate total RNA form the cells. 1 x 106 cells were plated on a 10 cm cell culture plate. On the following day, the medium was removed, and the cells were washed 2 times with ice-cold DPBS (Gibco) and harvested by adding 350 µL RLT Buffer. The isolated RNA concentrations and quality was determined by a NanoDrop spectrometer and stored at -80°C for further application.

3.2.6.2. cDNA synthesis

According to manufacturer`s instructions, 2 µg of the isolated RNA was used as template for cDNA synthesis utilizing the Reverse Transcription System Kit (Promga) according to manufacturer`s instructions. The isolated cDNA concentration and quality were determined by a NanoDrop spectrometer, and cDNA was stored at -20°C for longer use or at 4°C for short time.

3.2.6.3. Quantitative real time PCR

In order to determine gene expression (mRNA) level of OXQ1 in MEFs and different human cancer cell lines, quantitative real-time (qRT) PCR was performed using the TaqMan® gene expression assay following the manufacturer`s protocol (Applied Biosystems). 100 ng of first strand cDNA was used in qPCR reactions and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control. Quantitative real-time PCR was conducted in triplicates at the qTOWER 3 real-time PCR (Analytik Jena).

3.2.6.4. RNAseq samples preparation

RNeasy Mini Kit (Qiagen) was used to isolate total RNA from both mouse and human (Mock and FOXQ1-OE) cell lines following the manufacturer´s protocol (see 3.2.5.1). 2-3 µg of the isolated RNA of each cell line was used as template for cDNA synthesis (see 3.2.5.1). From 0.2 -1 (ng/ul) in volume of 45 ul was prepared for each sample. The isolated cDNA concentrations and quality were determined by a 1000 spectrometer (NanoDrop) and the prepared samples were sent for sequencing to the Core Facility Genome Sequencing, Helmholtz Centre Munich.

3.2.7. CRISPR/Cas9 technology

3.2.7.1. CRISPR/Cas9 genome wide screening

In order to uncover potential candidate genes that mediate RSL3 induced ferroptosis in human breast cancer cells (BT474), a CRISPR-mediated genome-wide knockout screen was conducted. BT474 cells were seeded at low density (300,000 cells) onto 3 x 15 cm dishes and were infected with these lentiviral particles containing a sgRNA library (Brunello, 73179 addgene), which comprises about 76441 sgRNAs targeting human genome at an MOI of 0.3 to achieve genome-wide mutagenesis. Two days after infection, cells were selected with increasing concentrations of (1S, 3R)-RSL3 (100-250, 500 nM) for 7 days. Genomic DNA was extracted from selected and unselected cells pools. DNA was harvested from the RSL3 250 nM treated and untreated cell pool and was amplified by PCR using primers embedded with two different barcodes enabling the preparation suitable for next-generation sequencing (NGS). The PCR product from both treated and untreated were pooled equimolarly and were sent to

the PrimBio research institute for NGS analysis. The NGS results were provided as a separate FASTQ files for each barcode. The necessary sgRNA guide information was extracted from single reads of the FASTQ files using the software ENCoRE (Trumbach, Pfeiffer et al. 2017). Counting the number of each sgRNA sequenced per sample determined the sgRNA distributions of the RSL3 treated and untreated cells. Results were sorted into excel sheet to display sgRNAs top hits of the selected cell pool.

3.2.7.2. CRISPR/Cas9 mediated depletion of FOXQ1

Using the online tool http://crispr.mit.edu single sgRNA guides were designed to target single exon gene FOXQ1. Guides were cloned using annealed oligonucleotides with specific overhangs complementary to the plentiCRISPRv2-Blast vector (see 3.2.1.9). Pfa1 and many human cancers cell lines were seeded onto a 6-well plate (200,000) and were infected on the same day with the desired sgRNA expressing plasmid plentiCRISPRv2-FOXQ1-KO (mouse, human) using the X-tremeGENE HP agent according to the manufacturer's recommendations (Roche). After 48 h cells were treated with specific antibiotic selections and analysed for several days.

3.2.8. Cell cycle analysis

3.2.8.1. Flow cytometry analysis

To determine cell cycle analysis, flow cytometry analysis was performed on cells infected with gRNA-not-targeting compared to the cells infected with gRNA-FOXQ1. To do this, equal number WT (Pfa1, and human) were seeded and infected with a non-targeting gRNA, FOXQ1-gRNA (FOXQ1-KO) and without gRNA transfection (Etoposide). After 48 h of transfection cells were further incubated with corresponding antibiotic selections (blasticidin (10 µg/ml) for 24 h. On the day 3 of transfection, cells without gRNA were incubated with etoposide (3 µg/ml) for 6 h prior to FACS analysis. In total 72 h of incubations cells were trypsinised, fixed with 70% chill ethanol and analysed on a flow cytometer CytoFLEX (Beckman Coulter CytoFLEX™) using the 488 nm laser for excitation and detection filters 610/20 nm band-pass for PI.

3.2.8.2. Cell fixation

Cell fixation was carried out prior to flow cytometer. Seeded cells were trypsinised, $1x10^6$ cells transferred in a 5 mL tube and centrifuged at 300 g for 3 minutes. Supernatant was removed and cells were washed 2X with 500 µl PBS to remove residual medium. PBS was removed and cells were re-suspended in 1-3ml cold 70% ethanol drop by drop to the cell pellet while vortexing for 30 sec. Cells were then incubated at 4°C for 30 min or overnight. Next day, cells were centrifuged at 300 g for 3 minutes, aspirate the supernatant and washed 2X with PBS. After that, cells were treated with ribonuclease (50-100 µl of a 100 µg/ml stock of RNase) to ensure only DNA, not RNA, is stained. The pellet was re-suspended in 0.5 mL of PI staining solution (from 50 µg/ml stock solution) and incubated at 37°C for 30 min, avoiding light. The solution was filtered through FACS tube and analysed by CytoFLEX (Beckman Coulter CytoFLEX™).

3.2.8.3. Live cell imaging by Nanolive

In order to observe morphological changes that characterize ferroptosis in cells, a non-invasive long-term live cell imaging technology (Nanolive, 3D Cell Explorer, Lausanne, Switzerland) was used. To his end, human cancer cells (Mock, FOXQ1-OE) were seeded at low density on µ-Dish 35 mm low (80136, i-bidi) and allowed to grow overnight. Next day, cells were treated with 100 nM RSl3 to induce ferroptosis. Using 3D Cell Explorer and Eve software v1.8.2 (Nanolive), live-cell imaging was performed for next 8 h. During this time, images were obtained at 10 min intervals, the cells were maintained at 37 °C, and 5% CO_2 by using a temperature controlled incubation chamber.

3.3. Statistical analysis and data presentation

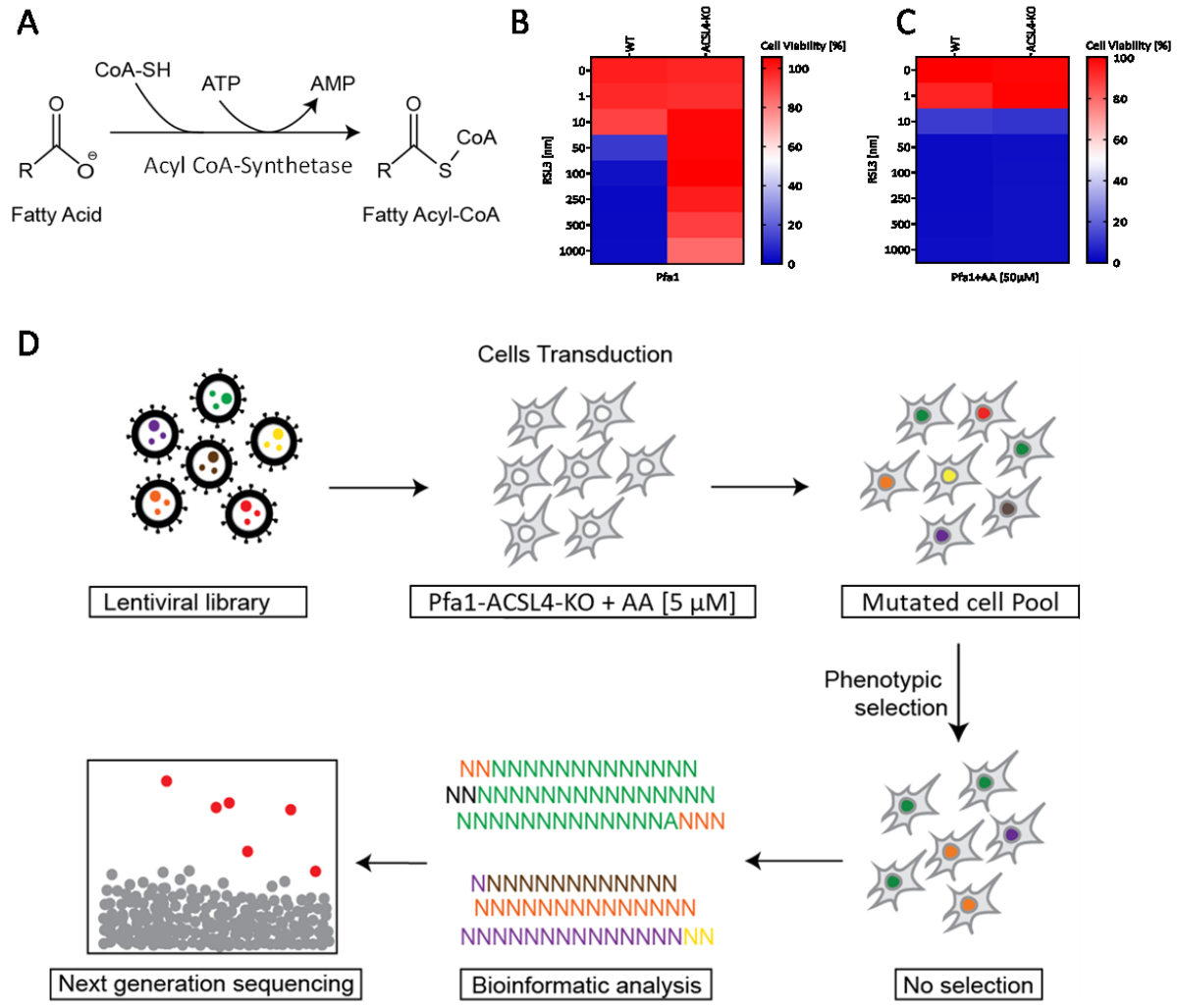
Statistical analysis was conducted using GraphPad Prism 5.0 software. Data are presented as mean ± s.d. unless stated otherwise

4. RESULTS

4.1. Genome-wide CRISPR screen uncovers ferroptosis regulators in Acsl4 KO cells.

Acyl-CoA synthetase long-chain family member 4 (ACSL4) is an essential ferroptotic gene that determines ferroptosis sensitivity via modulating the cellular phosphor-lipid composition (Doll, Proneth et al. 2017, Chen, Yang et al. 2021). ACSL4 is an important enzyme for PUFA metabolism with a substrate preference for arachidonic acid (AA) [5 µM] and promote RSL3 induced ferroptosis (Doll, Proneth et al. 2017, Kuwata and Hara 2019). Fatty acids are converted to fatty acyl-coenzyme A (CoA) by a two-step reaction. The reaction is catalysed by acyl-CoA synthetase and is an energy dependent reaction that requires one molecule of CoA and one molecule of adenosine triphosphate (ATP) (Li, Melton et al. 2007). In this process, two high energy bonds of ATP are consumed, and AMP and pyrophosphate are produced alongside with a fatty acyl-CoA (Fig. 8A) (Li, Klett et al. 2010, Kuwata, Nakatani et al. 2019).

The TAM-inducible Gpx4$^{-/-}$ cells (Pfa1) Pfa1-WT and Pfa1 Acsl4-KO cells were seeded on to a 96-well plate with or without AA [50 µM] supplementation as ACSL4 exhibits substrate preference for AA (Feuerborn, Srivastava et al. 2011, Sen, Kan et al. 2020). On the next day, cells were treated with increasing concentration of RSL3. As previously shown (Doll, Proneth et al. 2017), Pfa1 Acsl4-KO cells seeded with the supplementation of AA were highly sensitive to RSL3-induced ferroptosis (Fig. 8B). Supplementation with AA rendered Pfa1 Acsl4-KO cells susceptible to RSL3 induced ferroptosis (Fig. 8C). This suggested the presence of compensatory pro-ferroptotic mechanisms even in the absence of ACSL4 to sensitise the cells to ferroptosis (Feuerborn, Srivastava et al. 2011, Friedmann Angeli, Schneider et al. 2014, Doll, Proneth et al. 2017). CRISPR/Cas9 is an efficient and versatile genome-editing technology that has enabled researchers to identify networks of genetic regulation based on genome-wide phenotypic screens (Li, Yang et al. 2020). Thereby, CRISPR/Cas9 knockout libraries can target thousands of genes simultaneously in a single experiment they are assumed to introduce mutations in all targeted genes. To this end, a CRISPR-mediated genome-wide knockout screen was conducted to reveal potential candidate genes that mediate RSL3 induced ferroptosis in Pfa1-Acsl4-KO cells supplemented with AA. The lentiCRISPRv2 plasmid was used to deliver sgRNAs and Cas9 together and induce loss of function in the targeted genes on a genomic scale (Koike-Yusa, Li et al. 2014, Wang, Wei et al. 2014, Doench, Fusi et al. 2016).



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Gene Symbol	Description	STARS Score	p-value
Acsl3	**Acyl-CoA Synthetase Long Chain Family Member3**	**8.40941742**	**0**
Foxq1	**Forkedhead Box 1**	**5.45581702**	**0.000008**
Nup210	**Nucleoporin 210**	**4.39368442**	**0.00011192**
Obox1	**Oocyte Specific Ho-ebox1**	**4.15606515**	**0.00024418**
Zfp940	**Zing finger protein 940**	**4.07862168**	**0.00028566**
Sval3	**Seminal vesicle antigen-like 3**	**4.02402478**	**0.00031981**
Olfr1410	**Olfactory receptor 1410**	**3.91138074**	**0.00044831**
Cyp7b1	**Cytochrome P450 Family 7 Subfamily B Member 1**	**3.83365276**	**0.00051469**
Ogdh	**Oxoglutarate Dehydrogenase**	**3.81567182**	**0.00058249**
Slc25a36	**Solute Carrier Family 25 Member 36**	**3.79890788**	**0.00059957**

Figure 8: **Genome-wide CRISPR screen to uncover ferroptosis regulators beyond Acsl4**

(A) Enzymatic reaction for the formation of acyl-CoA from a fatty acid, catalysed by Acyl-CoA synthetase. In this irreversible reaction, long chain fatty acids are linked to coenzyme A (CoA) at the expense of ATP. **(B-C)** RSL3-induced ferroptotic cell death in Pfa1 (WT) and Pfa1 ACSL4-KO cell lines. Acsl4 KO cells are resistant to ferroptosis compared to WT cells (**B**) but can be sensitized by supplementation of arachidonic acid [5 µM] (**C**). RSL3 dose-dependent cell viability was assessed 24 h after treatment using AquaBluer. Data shown represent the mean ± s.d. of n = 3 wells of a 96-well plate, one representative of three independent experiments. **(D)** A schematic outline of the workflow of the performed genome wide CRISPR/Cas9 knockout screen. Pfa1 Acsl4-KO cells were

selected with 100 nM RSL3 in the presence of arachidonic acid [5 µM] after transduction with a lentiviral CRISPR library. **(E)** List of top 10 genes with enriched sgRNAs after selection. The content of sgRNA guides in the NGS data set was extracted using the ENCoRE tool and analyzed for enrichments using the STARS gene-ranking algorithm for genetic perturbation screens (Broad Institute). Data kindly provided by Dr. Sebastian Doll.

Pseudo-typed (Moloney Murine Leukemia Virus) lentiviral particles of the CRISPR/CAS9 library (Brie, 73632 Addgene) were produced in HEK293T cells and Pfa1 ACSL4-KO cells with addition of AA were transduced at an MOI of 0.3 to minimize superinfection of cells (Doench, Fusi et al. 2016) (Fig. 8D). This library contained about 70,000 sgRNAs, covering each gene with around four different guides. The resulting mutant cell pool was expanded under antibiotic pressure and divided into two equally sized sub pools. While one sub pool was selected with 100 nM RSL3 in the presence of 50 µM AA, the other pool was not subjected to selection (control). After 48 h of phenotypic selection, the genomic DNA was extracted from the surviving cell pools, amplified with specific primers and equimolar amount of DNA was sent for NGS. NGS reads provided as FASTQ files were filtered to identify and quantified the containing sgRNAs using the ENCoRE tool (Trumbach, Pfeiffer et al. 2017). The distribution of sgRNAs from the selected sample was compared to the distribution obtained from the unselected control samples to identify potentially accumulated distinct sgRNAs. Statistical analysis using bioinformatics tool ENCoRE developed by Dr. Dietrich Trümbach (Trumbach, Pfeiffer et al. 2017), identified an enrichment of sgRNAs targeting the genes ACSL 3 and FOXQ1 with a corrected p-value (false discovery rate, FDR) (Fig. 8E). ACSL 3 is the closest homologue of ACSL 4 with potential functional compensation at the level of AA, while FOXQ1 was found as promising candidate for further investigations that has not been associated with ferroptosis before.

4.2. Validation of FOXQ1 as a novel regulator of ferroptosis in MEFs

FOXQ1 is a member of the forkhead transcription factor family, previously known as hepatocyte nuclear factor 3 forkhead homolog 1 (HFH1) (Sun, Cheng et al. 2013). The FOX family exhibits functional diversity in key biological processes including oxidative stress and cell proliferation (Golson and Kaestner 2016). FOXQ1, is a single exon gene and characterized by a highly conserved winged-helix DNA-binding domain. Importantly, FOXQ1 is the only member of the FOX family identified in our CRISPR/Cas9 screening.

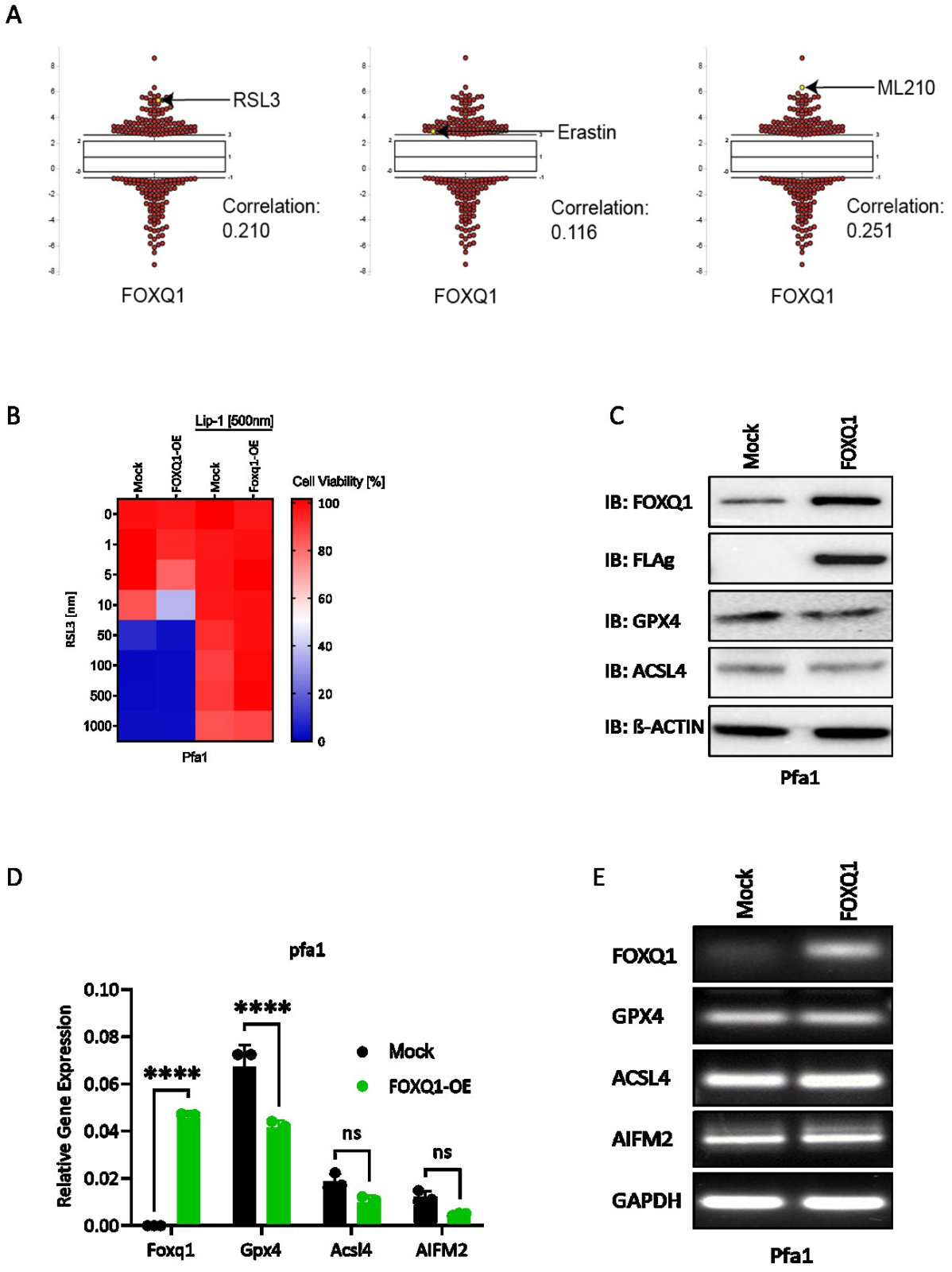

Figure 9: Generation and analysis of FOXQ1 overexpressing Pfa1 cell line.

(A) Cancer Therapeutics Response Portal (CTRP) www.broadinstitute.org/ctrp revealing a correlation between the expression level of genes and ferroptosis inducing compounds. CTRP identified FOXQ1 expression correlated with cellular resistance to ferroptosis mediator RSL3 (correlation: 0.210), erastin (correlation: 0.116), and ML210 (correlation: 0.251). **(B)** RSL3 dose dependent cell viability assay with or without Lip-1, analysed 12 h after

treatment p<0.001 (two-way ANOVA). **(C)** Immunoblot analysis demonstrating the successful generation of FOXQ1-Flag expressing Pfa1 cells. The expression level of FOXQ1, GPX4 and ACSL4 normalized to β-ACTIN and was analysed in the FOXQ1 and Mock overexpressing Pfa1 cells. **(D)** Relative mRNA expression levels were assessed by qPCR (n = 3). Relative gene expression was normalised to the reference gene GAPDH. Codon optimized FOXQ1-Flag was highly expressed in Pfa1 cells. While the relative expression of GPX4, ACSL4 and AIFM2 was slightly downregulated in FOXQ1-OE cells compared to the control (Mock) cells. **(E)** Electrophoresis of qPCR-amplified products on 2% agarose gel, stained with ethidium bromide and comparison of relative expression level of ß-actin. Data shown represents the mean ± s.d. of n = 3 wells of a 96-well plate from an experiment performed independently three times.

Studies have shown that FOXQ1 is an important transcription factor regulating the expression and activity of many genes and participate in cancer progression (Kaneda, Arao et al. 2010, Brown and Webb 2018). Based on this multi-functional role of FOXQ1 it was worth further exploring its role in the context of ferroptosis. Analysis of data from the Cancer Therapeutics Response Portal (CTRP) revealed that the expression of FOXQ1 correlated with cellular resistance to the ferroptosis-inducing compounds RSL3, erastin, and ML210 (Fig. 9A) (Basu, Bodycombe et al. 2013, Viswanathan, Ryan et al. 2017). Though the CRTP analysis were contrary of what we found in our CRISPR screen. FOXQ1 was identified the top hit in the selected cell pool, while its high expression correlates with resistance to ferroptosis-inducing compounds. But still the identified correlation of FOXQ1 with ferroptosis was found to be very interesting.

To determine the role of FOXQ1 in ferroptosis regulation, I overexpressed and knocked out FOXQ1 in Pfa1 WT cells (2.1). Lentiviral overexpression of FOXQ1 did not induce any phenotypic effect on the cells. No changes in the cell morphology, nor significant changes in cell proliferation were observed. To induce ferroptosis, cells were incubated with increasing concentration of RSL3 with or without lip-1 (liproxstatin-1 is a potent inhibitor of ferroptosis) and cell viability was assessed after 24 h of incubation. Viability assay revealed that FOXQ1 overexpression sensitised Pfa1 cells to ferroptosis induced by RSL3 (Fig. 9B). FOXQ1 overexpression was verified by western blot analysis using specific monoclonal antibodies against FOXQ1 and FLAG as the cloned FOXQ1 was labelled with a FLAG tag. A significant increase in FOXQ1 protein was detected in overexpressing cells compared to Mock (Fig. 9C). Additionally, I measured the protein expression of the known players of the ferroptosis pathway Gpx4, Acsl4 (Dixon, Lemberg et al. 2012, Doll, Proneth et al. 2017). ß-actin was used as a protein loading control. Further, I isolated the total RNA from cells overexpressing FOXQ1 and an empty vector (Mock). Reverse transcription was used to synthesise cDNA and quantify the expression of FOXQ1-FLAG via qPCR analysis (Fig. 9D). After completion of the qPCR, the

samples were run on a 2% agarose gel (Fig. 9E). Relative gene expression normalised to GAPDH revealed the transcriptional downregulation of GPX4, ACSL4 and AIFM2 mRNA. To this end, FOXQ1 overexpression increased Pfa1 cells sensitivity to ferroptosis and provided a first proof that FOXQ1 expression can induce changes in a cell's ferroptosis sensitivity which is to be explored in different cell lines.

4.3. FOXQ1 overexpression in lung cancer cells increased sensitivity to ferroptosis

Cells' survival and proliferation can be influenced by many factors. Recent studies have demonstrated that the level of FOXQ1 expression affects cellular proliferation via direct or indirect regulation of the Wnt signalling pathways (Christensen, Bentz et al. 2013, Fan, Zhang et al. 2014). In line with this, the suppression of FOXQ1 inhibited cell division and led to cell death, presumably by apoptosis (Zhang, Ma et al. 2015). Although, the effect of FOXQ1 on cell survival and cell cycle regulation has been mentioned in many studies, the detailed mechanism is not completely understood. To confirm the functional role of FOXQ1 in cell survival, I analysed the protein expression of FOXQ1 and GPX4 in a series of human cancer cell lines using a monoclonal antibody against FOXQ1 and GPX4. Endogenous protein expression of FOXQ1 was observed in most cell lines by western blot analysis (Figure 11A). High levels of protein expression were detected in A549, 786-O and U-87 cell lines. While the protein expression of FOXQ1 was low in U-373, U-251, MDA-MD-436 and MDA-MB-157 cell lines (Fig. 10A).

To further test our hypothesis, a panel of human cancer cell lines (WT) from different organs (lung, kidney, brain and breast) were used to analyse the protein expression of FOXQ1 and GPX4 normalised to β-ACTIN. To analyse the sensitivity to ferroptosis the cell lines were treated with increasing concentrations of the ferroptosis inducers RSL3 and erastin with or without the ferroptosis inhibitor Lip-1 (500nM). 24 h after treatment, the cell viability was assessed using AquaBluer assay (Fig. 10B, C). Cancer cell ferroptosis has been linked to the expression and activity of certain proteins (GPX4, XCT and ACSL4) (Dixon, Lemberg et al. 2012, Doll, Proneth et al. 2017, Sato, Kusumi et al. 2018).

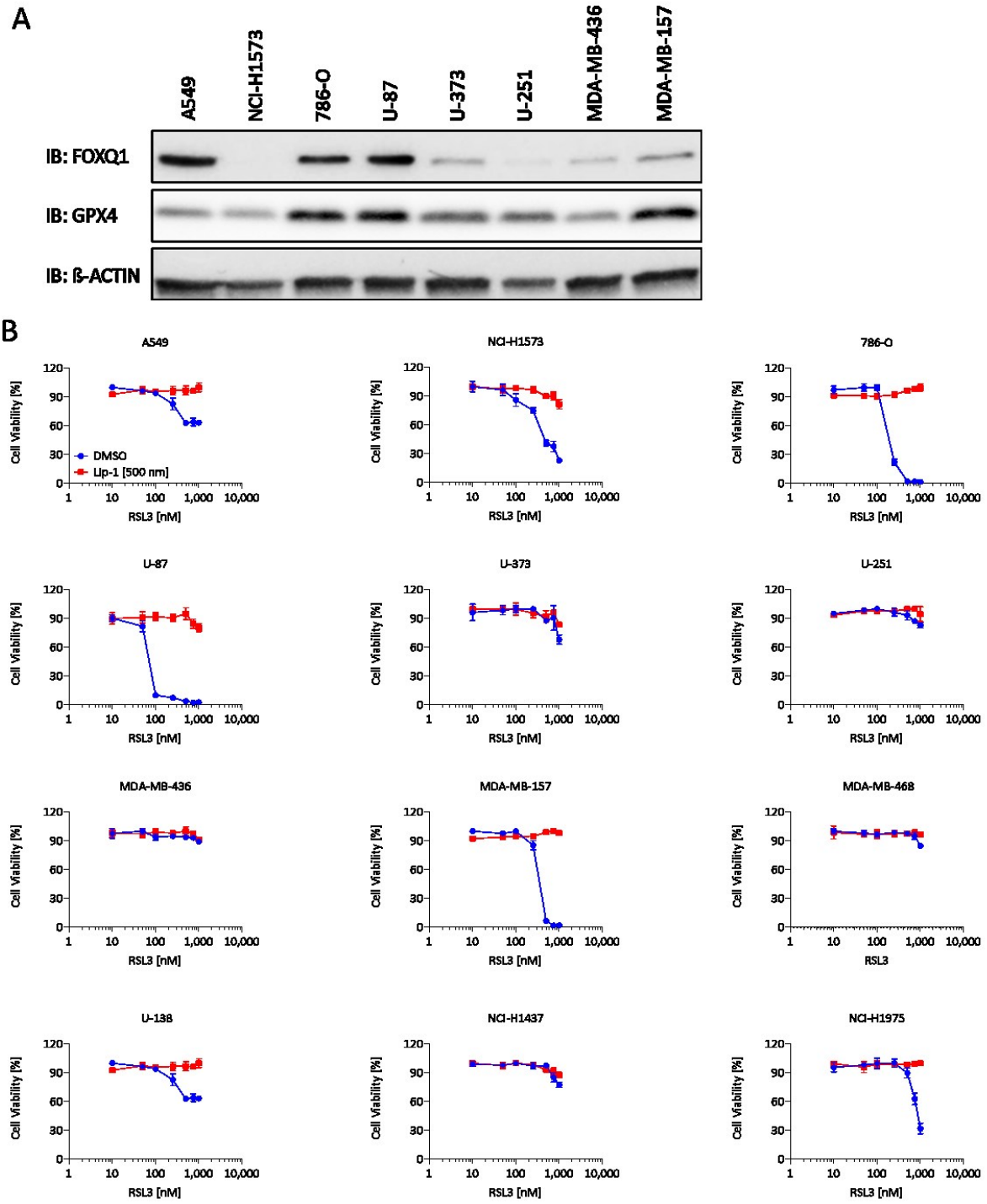

Figure 10a: Endogenous FOXQ1 expression analysis in a panel of cancer cell lines.

(A) Immunoblot analysis of different cancer cell lines using FOXQ1 monoclonal antibody. Western blot analysis revealed FOXQ1 expression to be cell type dependent. **(B)** Different cell lines were cultured at a density of 2500 cells per well (96 well plate) and grown for 24 h. Dose-dependent toxicity of RSL3 in a panel of human cancer cell lines from different origins (lung, brain, breast, and kidney) treated with or without Lip-1 [500 nm]. Data shown represent the mean ± s.d. p< 0.001 (two-way ANOVA), of n = 3 wells of a 96-well plate, from three independent experiments. Cell viability was assessed 24 h after RSL3 treatment using Aqua bluer method.

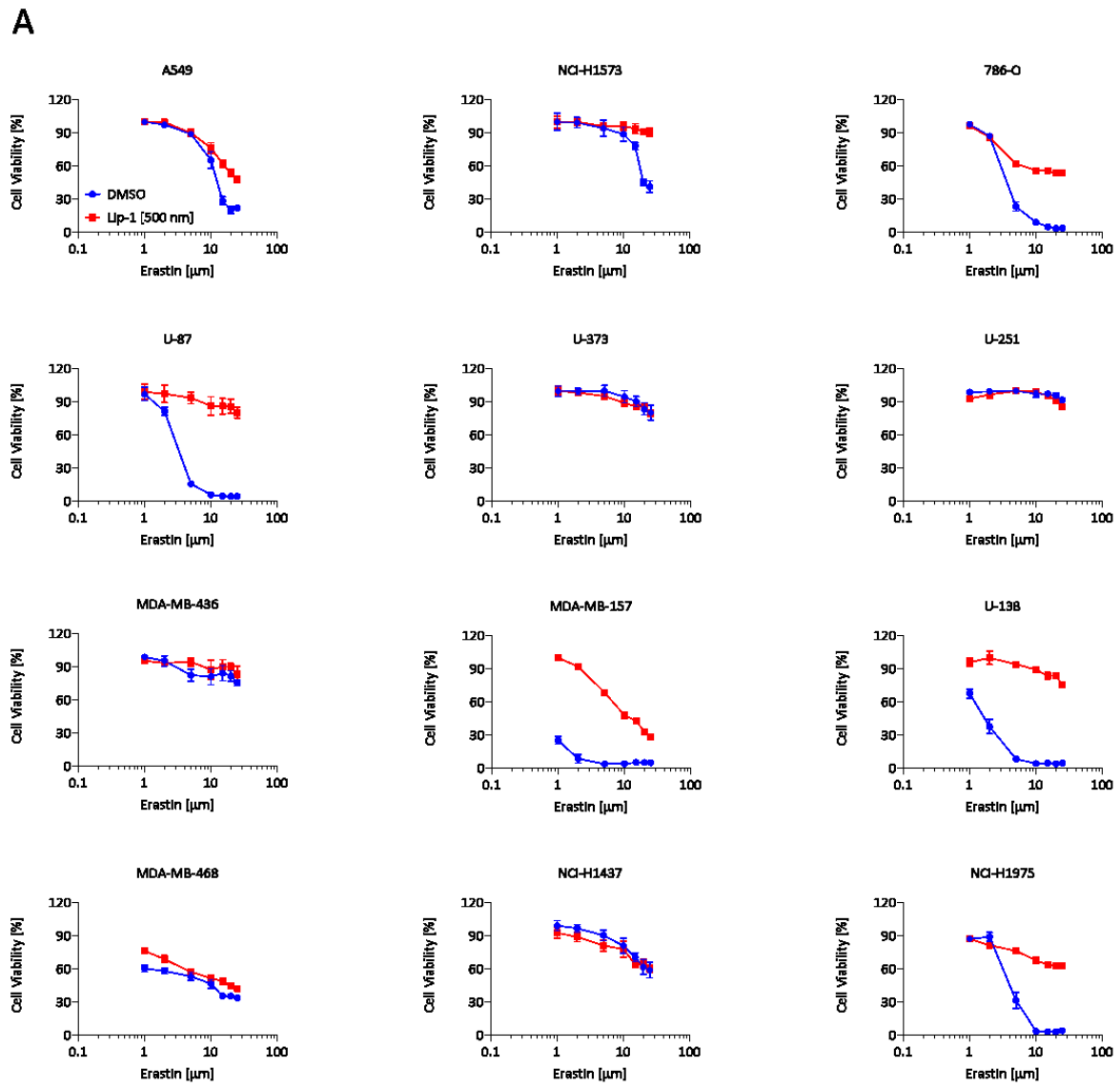

Figure 10b: Panel of human cancer cell lines treated with erastin.

(A) Different cell lines were cultured at a density of 2500 cells per well (96 well plate) and grown for 24 h. Dose-dependent toxicity of erastin in a panel of human cancer cell lines from different origins (lung, brain, breast, and kidney) treated with or without Lip-1 [500 nm]. Data shown represent the mean ± s.d. p< 0.001 (two-way ANOVA), of n = 3 wells of a 96-well plate, from three independent experiments. Cell viability was assessed 24 h after RSL3 treatment using Aqua bluer method.

In order to investigate if and how these known regulators of ferroptosis are influenced by FOXQ1, I overexpressed a C terminal flag-tagged version of human FOXQ1 in a panel of human cancer cell lines (A549, NCI-H1975, and NCI-H1437) (Fig. 11). To this end, FOXQ1 was codon optimised and cloned into a lentiviral transfer plasmid (pLV-EF1a-IRES-Puro, Addgene #85132). A second generation lentiviral packaging system (pMD2.G, the G envelop plasmid and psPAX2, the packaging plasmid) was used to produce vesicular stomatitis virus G (VSV-G) pseudotype lentiviral particles in HEK293T cells, and the lung cancer cell lines A549, NCI-

H1975, and NCI-H1437 were subsequently infected in a biosafety level 2 environment. The forced expression of FOXQ1-flag was verified by immunoblot analysis using a FLAG antibody (Figure 11A). Notably, different expression levels were achieved among the different cell lines. I also performed immunoblot analysis of genes whose expression have been previously linked to ferroptosis resistance.

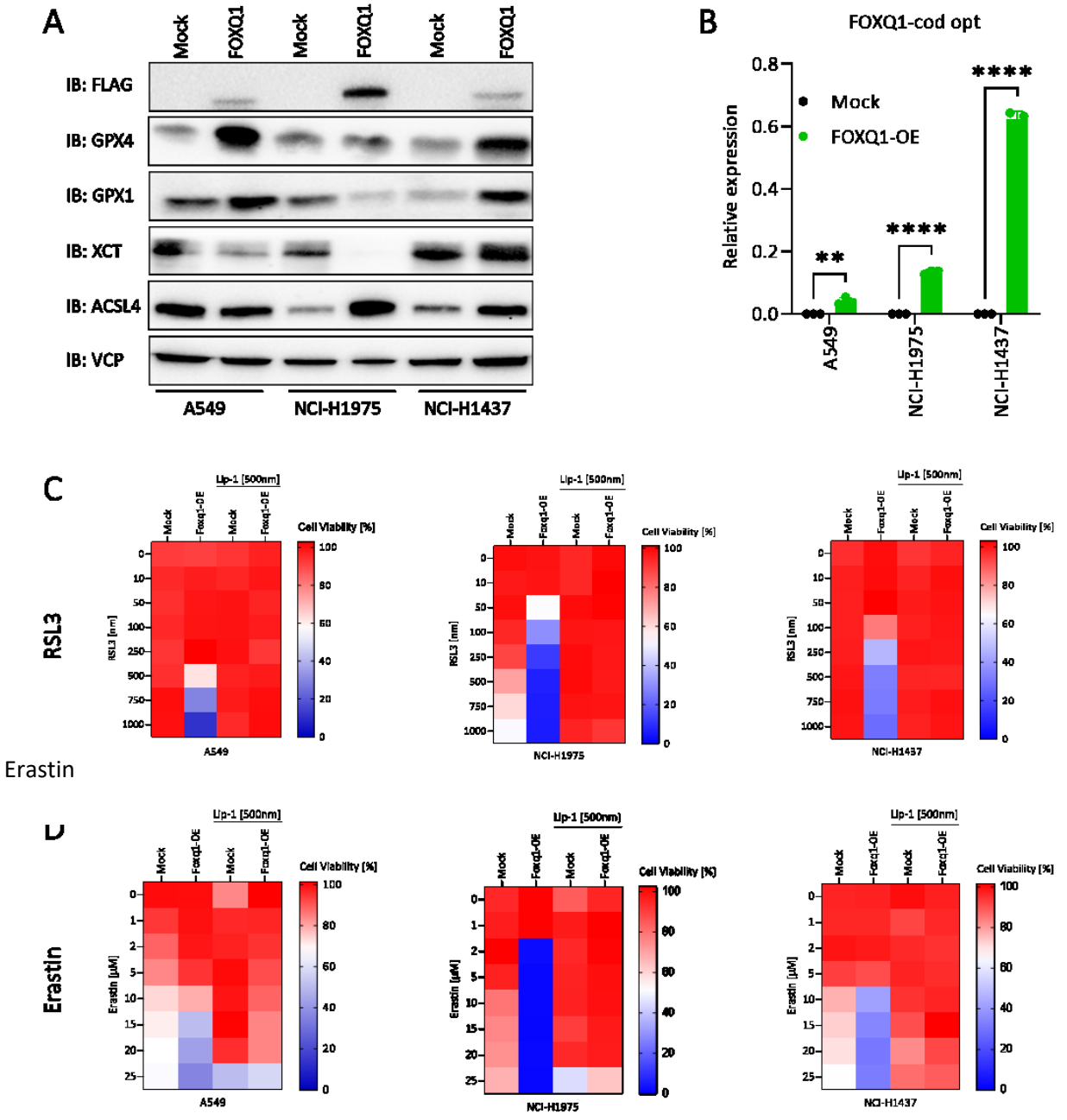

Figure 11: FOXQ1 overexpression regulates ferroptosis sensitivity in different lung cancer cell lines.

(A) Immunoblot analysis of Flag antibody demonstrating the successful generation of FOXQ1-flag expressing A549, NCI-H1975 and NCI-H1437 cell lines (n = 3). Protein expression analysis of GPX4, GPX1, XCT and ACSL4 in both control and FOXQ1-OE cells using specific antibodies against each gene. **(B)** Relative gene expression levels analysed by qPCR and normalised to the reference gene GAPDH (n = 3). **(C-D)** Dose dependent toxicity of the

ferroptosis inducing agents (RSL3 and erastin). Panel of lung cancer cells overexpressing FOXQ1 were treated with increasing concentrations of the GPX4 inhibitor RSL3 and system xCT inhibitor erastin with or without the ferroptosis inhibitor Lip-1. Cell viability was measured using the Aqua Bluer method. Data shown represent the mean ±s.d. of n=3 wells of a 96 well plate, $p<0.001$ (two-way ANOVA) from three independent experiments.

Interestingly, protein expression of the key ferroptosis regulators (GPX4 and XCT) and the pro-ferroptotic gene (ACSL4) was strongly influenced by the overexpression of FOXQ1 (Figure 11A). Furthermore, the overexpression of FOXQ1 in A549, NCI-H1975, and NCI-H1437 was conformed and quantified by quantitative PCR (qPCR) with specifically designed qPCR primers for codon optimised FOXQ1 (Fig. 11B). In accordance with previous observations of Pfa1-FOXQ1-OE, where FOXQ1 overexpression in Pfa1 cells increased sensitivity to induced ferroptosis, I intended to induce ferroptosis in human lung cancer cell lines overexpressing FOXQ1 and compare it to control (Mock). Equal numbers of A549, NCI-H1975 and NCI-H1437 cells infected either with control or FOXQ1-OE lentiviral particles were seeded on to a 96-well plate in triplicates. On the next day cells were treated with an increasing concentration of RSL3 and erastin in the absence or presence of lip-1. The cell viability was assessed after 24 h after treatment using the Aqua Bluer assay. Cells overexpressing FOXQ1 were found be sensitised to both RSL3 and erastin compared to Mock expressing cells. In particular, I observed that the impact of the ferroptosis induced by RSL3 and Erastin was different on each cell line (Fig. 11C-D). The induced ferroptotic cell death could be inhibited byLip-1 treatment in both Mock and FOXQ1-OE cells.

4.4. FOXQ1 mediated resistance to ferroptosis is cell context dependent

Studies have revealed that many human cancers cell lines exhibit strong dependency on system xc^- to facilitate cystine inflow for glutathione synthesis (Zhang, Trachootham et al. 2012, Liu, Duong et al. 2017, Jyotsana, Ta et al. 2022). Additionally, it has been demonstrated that GPX4 plays a crucial role in ferroptosis inhibition by utilising glutathione to detoxify lipid peroxidation (Dixon, Lemberg et al. 2012, Li, Long et al. 2022, Ma, Du et al. 2022). Moreover, ACSL4, an enzyme involved in the activation of polyunsaturated fatty acids, regulates ferroptosis and proliferation in cancer cells (Doll, Proneth et al. 2017, Cheng, Fan et al. 2020). Together, cysteine-glutamate antiporter system xc^-, GPX4 and ACSL4 are the primary components regulating ferroptosis (Dixon, Lemberg et al. 2012, Doll, Proneth et al. 2017, Sato, Kusumi et al. 2018). From previous analyses (Fig. 12A) I assumed that FOXQ1 might have the

functional role of FOXQ1 in terms of regulating genes transcription (Fig. 12A). Therefore, I sought to generate more FOXQ1-flag overexpressing cancer cell lines originating from different tissues to explore the function of FOXQ1 in different transcriptional contexts.

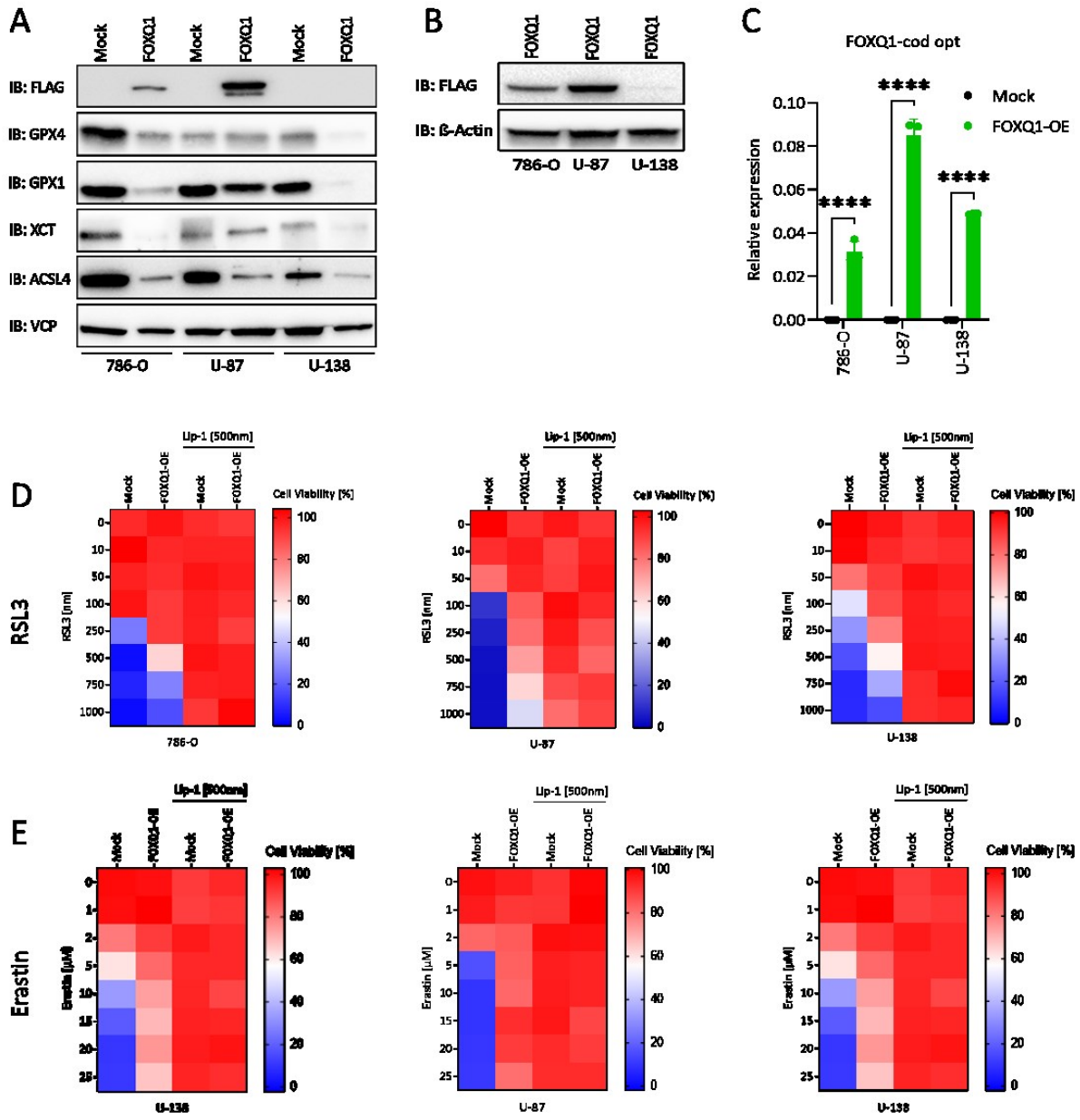

Figure 12: FOXQ1 overexpression increase resistance to ferroptosis in 786-O, U-87 and U-138 cells

(A) Immunoblot analysis of VCP, FOXQ1-flag, GPX4, GPX1, XCT and ACSL4 in 786-O, U-87 and U-138 cells with (FOXQ1 OE) and without (Mock) overexpression of FOXQ1-flag using specific antibodies. **(B)** The WB was repeated with only the FOXQ1 OE cell lines to confirm FOXQ1-flag overexpression **(C)** qPCR analysis of the relative expression of codon optimised FOXQ1 expression in 786-O, U-87 and U-138 cell lines with and without FOXQ1 overexpression normalised to the reference gene GAPDH (n = 3 technical replicates). **(D-E)** Cells overexpressing FOXQ1 were treated with increasing concentrations of RSL3 and erastin with or without the ferroptosis inhibitor Lip-1. Cell viability was measured 24 h after treatment using the Aqua Bluer method. Data shown represent the mean ±s.d. of n=3 wells of a 96 well plate, $p<0.001$ (two-way ANOVA) from three independent experiments.

Overexpression of FOXQ1-flag in the clear cell carcinoma cell line 786-O (kidney), and the glioblastoma cell lines U-87 and U-138 (brain) was performed as previously described for the lung cancer cell lines. Afterwards, a morphological change and slow cell proliferation rate was observed in U-87-FOXQ1-OE cells, half in comparing to the control one. However, the reduced cell growth rate was not observed in other cell lines overexpressing FOXQ1. To further verify the expression of FOXQ1, whole cell protein and RNA was extracted from the generated cell lines and analysed (Fig. 12A, B). Following the protocol of previous experiments, immunoblot analysis and qPCR quantitative analysis revealed that the exogenous FOXQ1 protein and mRNA expression strongly differed between cell lines. The obtained results from western blots highlighted this difference in the expression level of FOXQ1-OE in between the cell lines analysed. For further characterisation of the cell lines, I also detected known key players of ferroptosis (Fig. 12A). This immunoblots analysis revealed a largely different response of the key ferroptotic players as compared to the previously observed pattern from lung cancer cell lines.

The expression of xCT, GPX4 and ACSL4 were found to be significantly decreased in FOXQ1 overexpressing cells though the effect of decreasing the expression of GPX4 and xCT was lower in U-87 cell line compared to 786-O and U-138 cell lines. The observation was highly unexpected since FOXQ1 overexpression was previously observed to increase the protein expression of GPX4 and ACSL4 in the lung cancer cell line (Fig. 11A). The housekeeping gene VCP was used as a loading control to normalise the protein expression analysis. From the obtained data, one could state that the difference in protein expression in between the Mock and FOXQ1-OE cell lines was not caused by the amount of protein loaded. Our hypothesis is, that FOXQ1 overexpression had a negative regulatory effect on the protein expression of xCT, GPX4 and ACSL4 in the cellular context of kidney and glioma cells in contrast to lung cell lines, where a positive regulation was observed (Fig. 12A). To investigate how the overexpression of FOXQ1 influenced the sensitivity to ferroptosis, control (Mock) and FOXQ1 overexpressing cells were treated with different concentrations of both RSL3 and erastin with and without Lip-1 (Fig. 12C-D). In accordance with the protein analysis, FOXQ1 overexpression did not sensitise cells to ferroptosis, instead an increased resistance was observed in 786-O, U-87 and U-138 cell lines. This result indicates that FOXQ1 overexpression can have different effects on the ferroptosis sensitivity, depending on the cellular context. This finding prompted me to

further investigate the role of FOXQ1 in even more human cancer cell lines (U-251, MCF7, MDA157, and MB-MDA546).

To address this, I successfully generated human breast cancer cell lines (MDA-MB-436, MCF7 and MDA157) and glioblastoma cell lines (U-251, U-373) stably overexpressing FOXQ1 (Fig. 13).

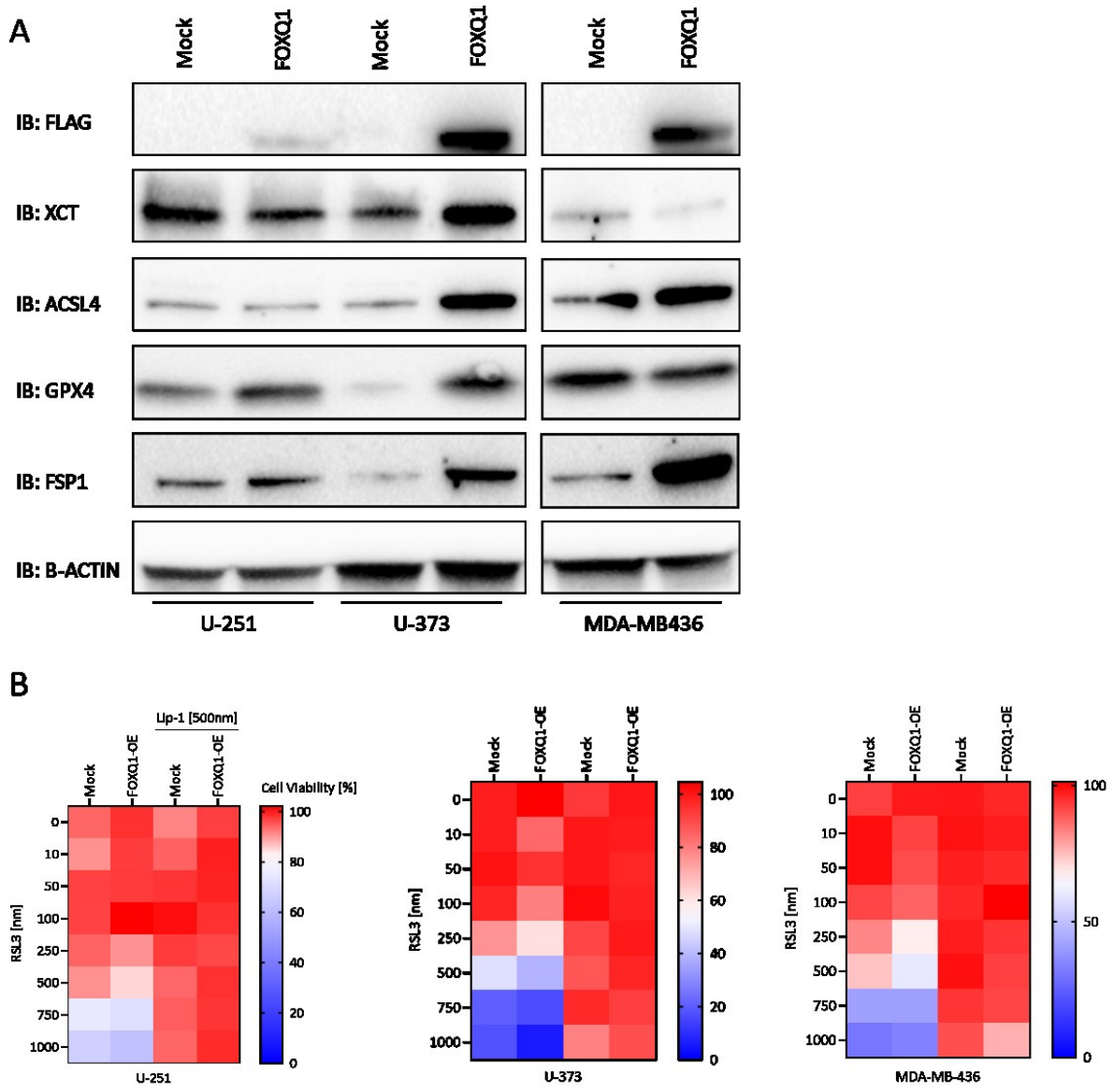

Figure 13: FOXQ1 overexpression does not mediate ferroptosis effect.

(A) Immunoblot analysis of the glioblastoma (U-251, U-373) and breast cancer (MDA-MB-436) cell lines expressing empty vector (Mock) and flag tagged FOXQ1. **(B)** All the three cell lines were treated with increasing concentration of RSL3 for 24 h. Cell viability was examined 24 h after treatment using Aqua Bluer method. Data shown represent the mean ±s.d. of n=3 wells of a 96 well plate, $p<0.001$ (two-way ANOVA) from three independent experiments.

Meanwhile, at the time of antibiotic selection and medium replacement, I noticed that some breast cancer cell lines (MCF7 and MDA157) failed to survive selection beyond five days after transduction withFOXQ1. In the other cell lines (U-251, U-373 and MDA-MB-436), FOXQ1 overexpression was conformed via immunoblot analysis using antibody against flag (Fag. 14A). From immunoblot analysis it can be concluded that the protein expression of GPX4, xCT and ACSL4 in MDA-MB-436-FOXQ1-OE and U-251-FOXQ1-OE cells is not regulated compared to control. However, an upregulated protein expression of GPX4, xCT and ACSL4 was detected in U-373-FOXQ1-OE cells compared to control. To further investigate how the overexpression of FOXQ1 influenced the sensitivity to ferroptosis, control and FOXQ1 overexpressing cells were treated with different concentrations of RSL3 with and without Lip-1 (Fig. 13B). Equal numbers of cells (Mock and FOXQ1-OE) were cultured and treated with increasing concentration of RSL3. Cell viability was examined 24 h after RSL3 treatment but the induced ferroptosis in between the control and FOXQ1 overexpressed cells was not as effective like in other cell lines (Fig. 11, 13). The small difference between the control and overexpressed groups might be due to slight variations in cell proliferation. This observation strongly suggests that FOXQ1 mediated ferroptosis effects and regulation of different ferroptotic genes is cell context dependent.

4.5. Doxycycline inducible expression of FOXQ1

Based on the knowledge gathered from my previous experiments, the next goal was to generate an optimal doxycycline (dox)-inducible Tet-on system for FOXQ1 overexpression. Several studies have revealed involvement of FOXQ1 in regulating many signalling pathways and cell cycle genes (Christensen, Bentz et al. 2013, Peng, Luo et al. 2015). CDK interacting protein-1 (p21) is a downstream gene of p53 and FOXQ1 has a direct effect on the expression of p21 (Kaneda, Arao et al. 2010, Zhang, Yang et al. 2015). Further studies have shown that p53 dependent cell cycle associated genes are controlled by p53–DREAM pathway. Irregularity in this pathway promotes chromosomal instability which might lead to aneuploidy (Engeland 2018). Cell function determinately follows the expression of most genes but this effect is very sensitive when the expression of subset genes is increased. Gene regulations to maintain specific cell states are controlled by thousands of transcription factors (Lee and Young 2013). Though the knowledge to understand the underlying mechanisms of how expression levels of

certain genes impact the cell proliferation is limited. However, in human cells many mechanisms contribute to regulation of gene expression in a specific manner.

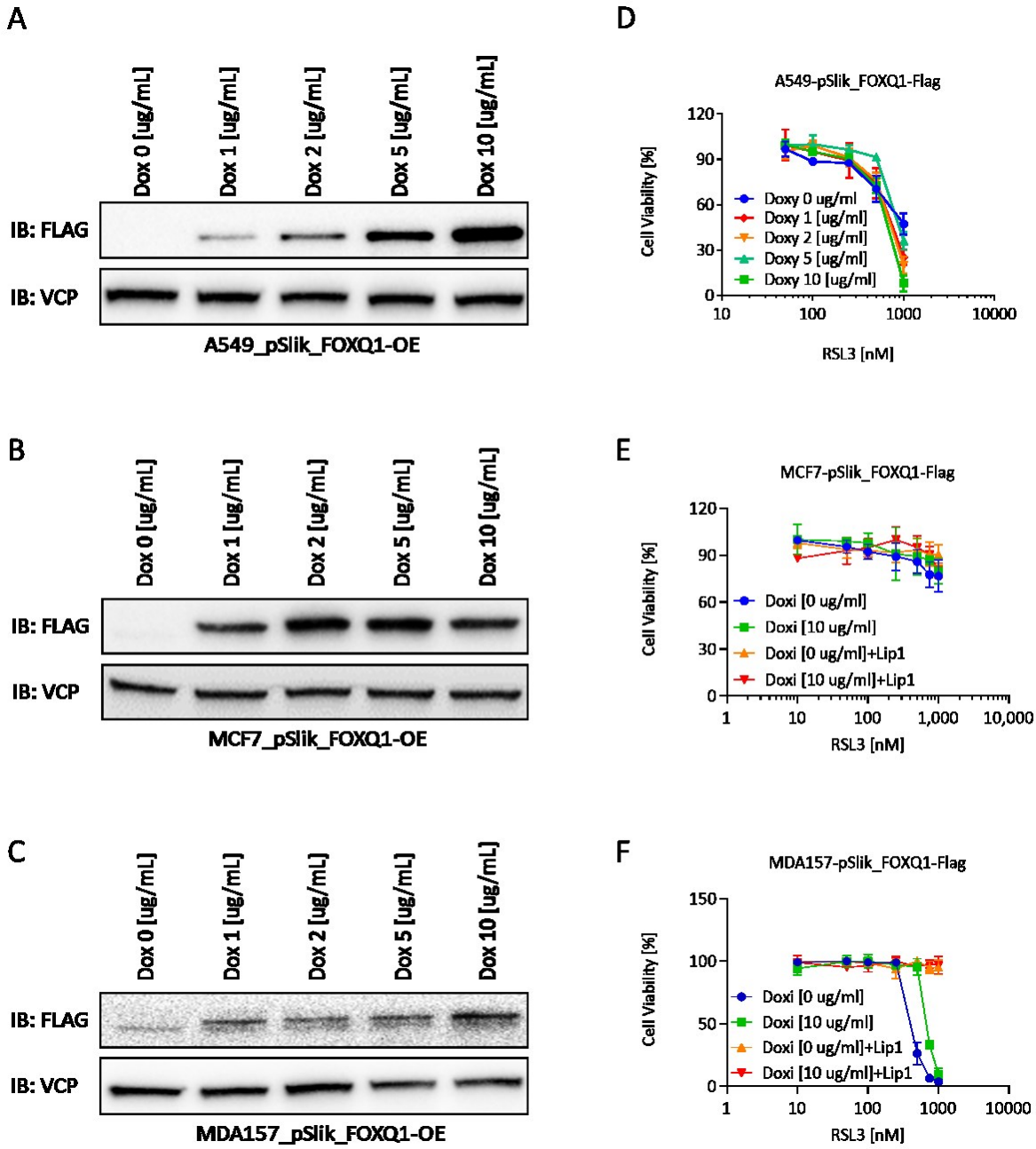

Figure 14: Doxycycline dependent FOXQ1 overexpression.

(A-C Immunoblot analysis demonstrating the doxycycline inducible expression of FOXQ1-Flag in A549, MCF7 and MDA157 cell using a tet-on inducible vector (pSlik). FOXQ1-flag detected with flag antibody and VCP used as a housekeeping gene to indicate general protein loading. **(D)** The cells were incubated without and with different concentration of doxycycline for 24 h. After further incubation for 24 h with different concentrations of RSL3, cell viability assay was performed using the Aqua Bluer. Data shown represent the mean ±s.d. of n=3 wells of a 96 well plate, $p<0.001$ (two-way ANOVA). **(E-F)** Both MCF7_pSlik-FOXQ1-OE and MDA157_pSlik-FOXQ1-OE cell lines were incubated with or without doxycycline for 24 h and using the Aqua Bluer method, cell viability assay was performed after further incubation for 24 h with increasing concentration of RSL3 and with or without Lip-1. Data shown represents the mean ±s.d. of n=3 wells of a 96 well plate, $p<0.001$ (two-way ANOVA).

Sometimes overexpression of certain proteins requires the regulation of multiple genes that cause cellular abnormalities which may be detrimental to proper biological functions. To overcome the irregularity in the expression of certain proteins it is important to estimate the protein expression limit (Emilsson, Thorleifsson et al. 2008). My previous experiments demonstrated, that the two cancer cell lines MCF7 and MDA157 could not tolerate the stable overexpression of FOXQ1 due to cycle arrest or slow cell growth rate. For this reason, a doxycycline inducible expression system (pSlik, Tet-on) was used to allow precise and efficient spatiotemporal control expression of FOXQ1 in these cell lines. In this system, the expression of the gene of interest (pSlik_FOXQ1-OE) was depended on the availability of tetracycline (Tc) or tetracycline-derivatives like doxycycline in the cell culture medium (Das, Tenenbaum et al. 2016). To this end, A549 cells were transfected with doxycycline inducible plasmid cloned with FOXQ1 (pSlik_FOXQ1-Flag-OE). As from previous experiments, A549 stably overexpressing FOXQ1 were found be sensitised to RSL3 compared to Mock expressing cells (Fig. 11C). Further, MCF7 and MDA-157 cells were stably transfected with doxycycline inducible plasmid cloned with FOXQ1 (pSlik_FOXQ1-FLAG-OE). To consider the optimised induction by doxycycline, I examined the expression pattern of FOXQ1 upon treatment with different concentrations of doxycycline in A549, MCF7 and MDA-157 cell lines (Fig. 14). Western blot analysis showed a doxycycline concentration dependent gradual expression of the pSlik_FOXQ1-FLAG-OE detected by anti-flag antibody (Fig. 14A-C). The doxycycline induced FOXQ1 expression reached its maximum with 10 µM doxycycline 24 h after treatment. At the concentration of 20 µM doxycycline I observed toxic effects.

To further support these findings, cell death assays were performed to analyse the FOXQ1 expression dependent increased sensitivity of the cells to ferroptosis (Fig. 14B-C). To this end, A549, MCF7 and MDA157 (overexpression pSIiK_FOXQ1-FLAG) cell lines were seeded in medium supplemented with and without different concentration (0-10 µM) of doxycycline 24 h. Subsequently, cells were incubated with increasing concentration of RSL3 for 24 h. Cell viability assays were used to assess the cells sensitivity to RSL3 induced ferroptosis (Fig. 14D-E). The effect of the induced ferroptosis in the cells expressed with doxycycline dependent FOXQ1 (pSIiK_FOXQ1-FLAG) was not as strong as in the cells which stably overexpressed FOXQ1 (A549-FOXQ1-OE). On the other hand, MCF7 and MDA157 cell lines tolerated the doxycycline dependent controlled expression of FOXQ1 identified via western blot analysis using anti-flag antibody (Fig. 14C, E). Cell viability experiments showed that the

overexpression of doxycycline dependent FOXQ1-Flag in MCF7 and MDA157 cells slightly suppressed RSL3 induced ferroptosis (Fig. 14D, F).

4.6. FOXQ1 knockout is lethal

Many publications revealed the impact of FOXQ1 on other transcription factors and regulating the functional role of several genes via regulating their transcription (Christensen, Bentz et al. 2013, Peng, Luo et al. 2015). Together with my findings of cell type based contrary effects of FOXQ1 mediating ferroptosis differently, it further urged to challenge the specificity of FOXQ1 in ferroptosis and cell cycle regulation. TFs and their targeted genes have important functions in biological processes, including cell cycle regulation, oxidative stress, and many others. TFs can activate or repress expression of important genes. Gene knockout is a potent and irreversible mean to generate a complete loss of gene function (Hall, Limaye et al. 2009, Wang, Wang et al. 2019). Knocking-out a particular TF is one of the most important approaches to explore genotype-phenotype relationships of a distinct TF and its targeted genes in a tissue/cell type specific manner. Hence, to uncover the critical role of FOXQ1 I used CRISPR-Cas9 technology to nullify FOXQ1 expression in both mouse and human cell lines.

To this end, an online tool (*http://www.crisprscan.org/*) was used to design sgRNA-guides targeting the endogenous *FOXQ1* gene and minimal predicted off target sites. Two individual sgRNAs were selected to target the single exon *FOXQ1* gene and cloned into the lentiviral expression plasmid lentiCRISPRv2-Blast (pLentiCRISPRv2) carrying a blasticidin resistance cassette (Sanjana, Shalem et al. 2014). This plasmid expressed both hSpCas9 and one of the sgRNAs (Fig. 15A) (Nageshwaran, Chavez et al. 2018). In a first approach Pfa1 and Pfa1-ACSL4-KO cells were seeded with and without arachidonic acid supplementation at a low density and were transfected with plasmids expressing both sgRNAs targeting *FOXQ1*. As a control, cells were infected with pLentiCRISPRv2-Blast plasmids containing non-targeting guide RNAs (control). After 48 h of transfections, a halt in cell growth was observed in both Pfa1 and Pfa1-ACSL4-KO cells transfected with *FOXQ1* gRNAs in compared to the cells transfected with non-targeting gRNA (Fig. 15B).

Subsequently, the corresponding antibiotic selections (blasticidin (10 µg/ml)/puromycin (2 µg/ml)) were supplied to the medium of transfected cells. For about 72 h post-transfection a significant decrease in cell population was observed in *FOXQ1* knocking out cells. In contrast,

cells transfected with non-targeting gRNA were growing normally. The cell medium with selection supplements was replaced with normal cell medium.

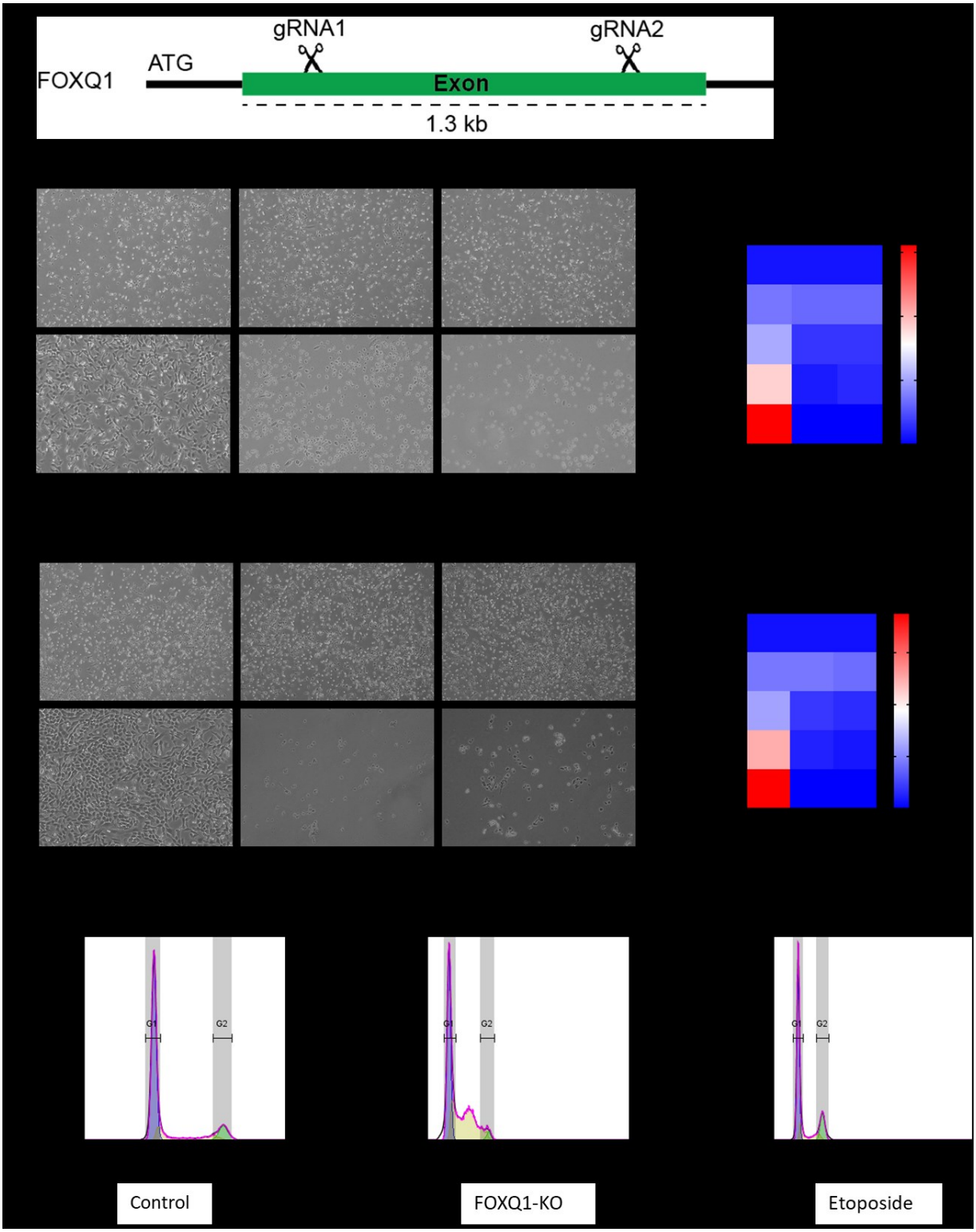

Figure 15: CRISPR/Cas9 knockout FOXQ1 is lethal.

(A) Schematic description of the guide RNAs targeting *FOXQ1*. Two gRNAs cloned into lentiCRISPR/Cas9 vector with blasticidin/puromycin resistance to knockout the single exon gene *FOXQ1* in Pfa1–WT and Acsl4 KO cells. **(B, C)** Microscopic images of Pfa1 and Pfa1-ACSL4-KO cells. Equal number of cells were seeded and transfected

with not-targeting gRNA (control), two gRNAs to target *FOXQ1* with and with arachidonic acid (AA) (5 µM). 48 h after transfection antibiotic selection on cell was initiated for further 48 h. Cell viability was lost in both cell lines transfected with *FOXQ1*-KO gRNAs compare to non-targeting gRNA. **(D, E)** Cell counting assay (n=3) to monitor cell proliferation. Cells were cultured at a density of 30000 cells per well (24 well plate), transfected with gRNAs with or with AA (5 µM) and incubated for 48 h. After 48 h cells were trypsinised and counted using a counting chamber (start of proliferation experiment) and the rest of the cells were treated with specific selection. Medium was replaced and cells were counted each day until day 6 following the same method. On day 6 the number of live cells from the *FOXQ1*-KO completely lost. **(F)** Cell cycle analysis assay from DNA content estimation by flow cytometry using the cell permeable Propidium Iodide (PI) florescent dye. FACS analyses showed *FOXQ1*-KO cells was associated with an increased number of cells in S phase (yellow portion) and a corresponding decrease in the cell numbers in G2 (green portion) phase comparing to the control and etoposide treated cells.

Unfortunately, even with replacing medium cells transfected with *FOXQ1* gRNAs did not recover its normal growth rate. More strikingly, *FOXQ1*-KO cells (Pfa1 and *Pfa1-ACSL4*-KO) with and without the supplements of AA could not be passaged further. In other words, FOXQ1 depletion arrested the cell cycle and induced cell death on both Pfa1 and Pfa1-ACSL4-KO cells (Fig. 15B). To scrutinize the lethal effect of FOXQ1 on cell growth, I performed a cell proliferation assay (Fig. 15C). On day 1, equal number of cells were seeded and transfected with *FOXQ1* gRNAs and non-targeting gRNA. 48 h after transfection cells were trypsinised and counted using a counting chamber. And on the same day medium was changed and corresponding antibiotic selections (blasticidin (10 µg/ml)/puromycin (2 µg/ml)) were added to the remaining wells. Afterwards, cells were counted each day, until the viability of the cells transfected with *FOXQ1*-gRNA were completely lost (Fig. 15C).

Since the targeted knockout of *FOXQ1* halted cell proliferation leading to reduced numbers of cultured cells I was curious to investigate the cell cycle regulations impacted by *FOXQ1* knocking out. To this end, flow cytometry was used to measure the cellular DNA content in different phase of cell cycle using the cell permeable propidium iodide (PI) staining dye to measure to measure ethanol or methanol fixed cell cycle (Poulin, Matthews et al. 1994). PI is a membrane impermeant dye binds to double stranded DNA by intercalating between base pairs. The fluorescence intensity correlates with the amount of DNA contained by the cells in that particular cell cycle (Kim and Sederstrom 2015). To perform the experiment, equal numbers of Pfa1-WT cells were cultured and transfected with a non-targeting gRNA (control), *FOXQ1*-gRNA (*FOXQ1*-KO) or were treated with the cell cycle inhibitor etoposide (Litwiniec, Gackowska et al. 2013). Etoposide induces G_2/M cell cycle arrest and cell death via DNA

damage (Litwiniec, Gackowska et al. 2013). 48 h after transfection, control cells and *FOXQ1*-KO cells were treated with the corresponding antibiotic selections (blasticidin (10 µg/ml).

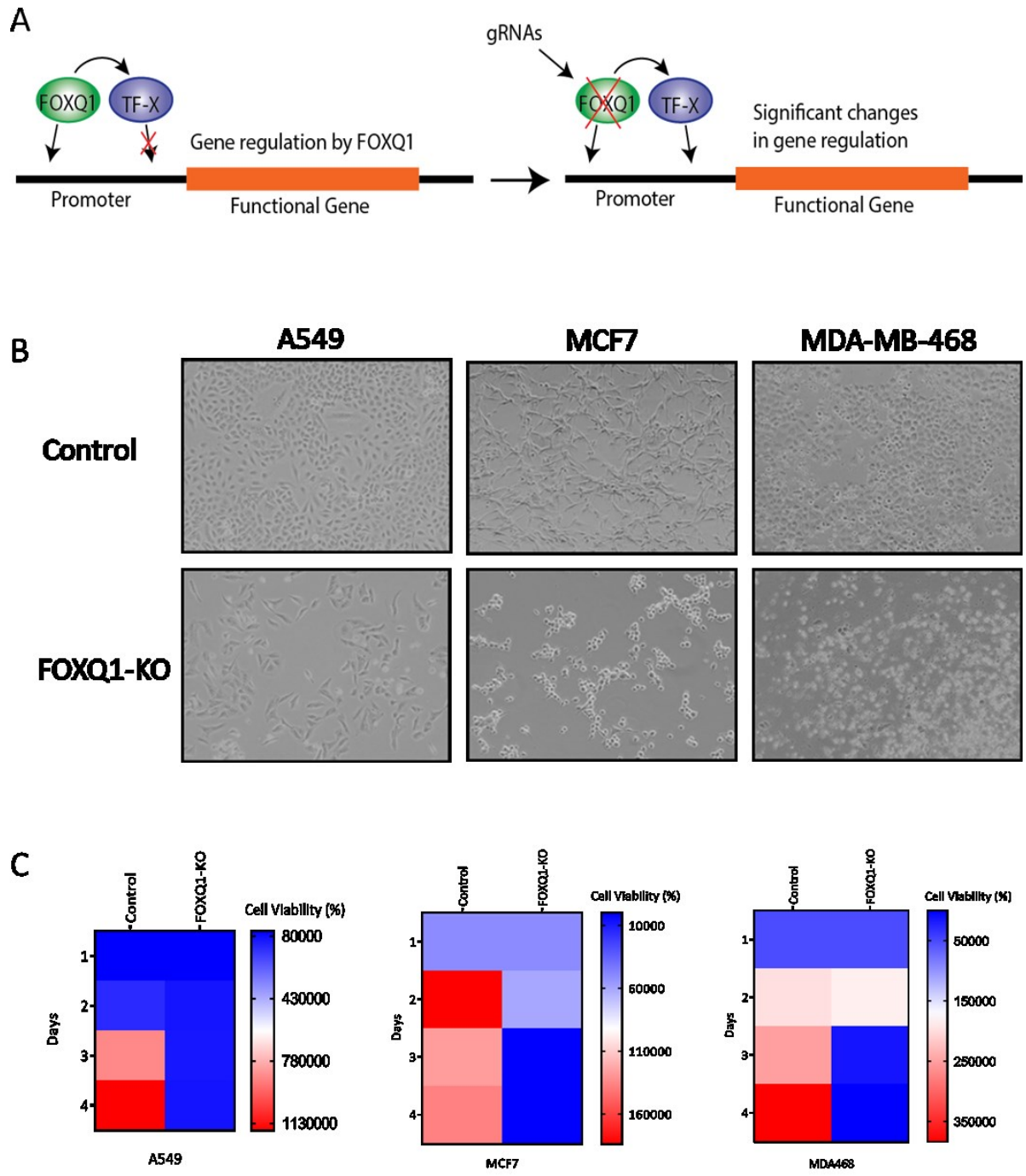

Figure 16: CRISPR/Cas9 knocking out FOXQ1 has lethal affect in cancer cells.

(A) Schematic representation of the hypothesis. My hypothesis is that directly or indirectly FOXQ1 regulates another TFs (TFX) or downstream genes. Knocking out *FOXQ1* unmask the function role of the TFX, which in turn binds to the promoter of the functional gene and induce significant changes in the gene regulation. **(B)** Microscopic images of A549, MCF7 and MDA468 cells. Equal number of cells were seeded and transfected with not-targeting gRNA, two gRNAs to target single exon *FOXQ1*. 48 h after transfection cell were incubate for further 48 h with specific selection. Morphological observation reveal *FOXQ1*-KO cells in stress condition and cell number

was reduced with passage of time. **(C)** Cell counting assay to monitor cell growth. Cells were cultured at a density of 50000 cells per well (18 well plate), transfected with gRNA. After 48 h of transfection medium was replaced and cells were counted (day 3) and the rest of the cells were treated with specific selection. Medium replacement and cells counting continued until the living cells lost.

Subsequently, in total 72 h after transfections cells were trypsinised and analysed on a flow cytometer (CytoFLEX (Beckman Coulter CytoFLEX™)) using the 488 nm laser for excitation and detection filters 610/20 nm band-pass for PI. As positive control for cell cycle inhibition, cells were incubated with etoposide (3 µg/ml) 6 h prior to fluorescence-activated cell sorting (FACS) analysis. Computer analysis of DNA content determined the discrimination of the cells in particular phases of cell cycle. As expected, in FACS analyses *FOXQ1*-KO cells showed an increased fraction of cells in S phase and a corresponding decrease in the cell numbers in G2 phase as compared to the non-targeting and etoposide treated cells. In particular, FACS data analysis revealed that *FOXQ1*-KO induces a cell cycle arrest in S phase (Fig. 15D).

Several studies have elucidated the functional role of FOXQ1 in cell cycle regulation and cell proliferation (Zhang, Li et al. 2015, Li, Zhang et al. 2016, Wang, Lv et al. 2017). Others have shown that the increased expression of certain genes regulates cellular growth (Emilsson, Thorleifsson et al. 2008). Based on obtained data from my experiments on the functional role of FOXQ1 regulating cell cycle progression in Pfa1 cells (Fig 16), and the observation of Dia et al., I hypothesized that FOXQ1 as a transcription factor might regulate the function of another TF (eg. transcription factor X, hypothetical name TFX), possibly a protein-protein interaction partner (Dai, Dai et al. 2009, Voordeckers, Pougach et al. 2015). Upon knocking out *FOXQ1*, TFX might get released and bind to the promoter of the desired gene leading to the significant changes in the gene expression (Fig. 16A). To test whether *FOXQ1*-KO would lead to cell death and cell cycle arrest in different cellular contexts, an online tool (*http://www.crisprscan.org/*) was used to design sgRNA-guides with minimal predicted off target sites and targeting the endogenous *FOXQ1* gene. Two individual sgRNAs were selected and cloned into the lentiviral expression plasmid lentiCRISPRv2-Blast carrying a blasticidin resistance cassettes (Sanjana, Shalem et al. 2014). This plasmid expressed both hSpCas9 and one of the sgRNAs (Nageshwaran, Chavez et al. 2018).

Number of human cell lines were infected with sgRNAs targeting *FOXQ1* under biosafety level S2 conditions (Fig. 16B). LentiCRISPRv2 cloned with gRANs targeting specifically human *FOXQ1* introduced into multiple cell lines. Single exon gene (*FOXQ1*) was targeted with two different

gRNAs containing blasticidin and puromycin resistance. While pLentiCRISPRv2 cloned with a non-targeting gRNA was used as control.

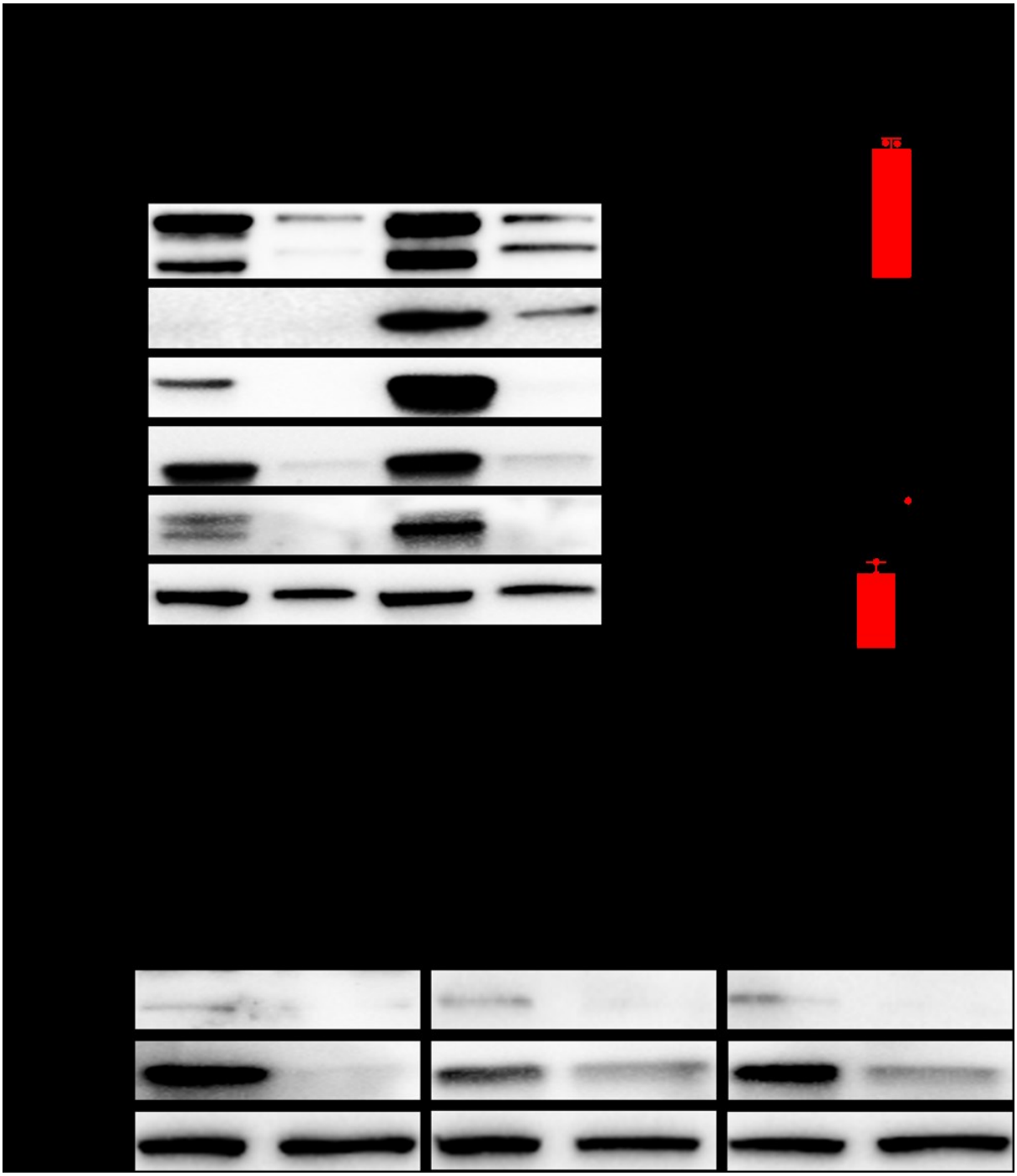

Figure 17: FOXQ1 depletion downregulate cyclin-dependent kinases.

(A) Immunoblot analysis of the A549-WT and flag tagged A549-FOXQ1-OE. Whole cells extracted were analysed by immunoblot using antibodies against FOXQ1, Flag, CDK4, CDK6, Cyclin D and the reference gene VCP. **(B)** qPCR analysis from both A549-WT cells (upper) and A549-FOXQ1-OE cells (lower) infected with non-targeting-sgRNA (control) and sgRNA targeting endogenous FOXQ1 (*FOXQ1*-KO). Data normalised to the reference gene *GAPDH* (n = 3). Data analysed show the relative expression of FOXQ1 downregulated in both the A549-WT and A549-FOXQ1-OE cell lines. **(C)** Immunoblot analysis of A549, MCF7 and MDA-MB436 cell lines with and without *FOXQ1*

KO. Cell lysates were isolated after 24 h of antibiotic selection before all the infected died and proteins expression was analysed by immunoblotting using antibodies against FOXQ1, CDK4 and the housekeeping gene VCP.

After 48 h of infection the antibiotics for selection were supplied to the medium. Afterwards, cells were observed for morphological changes and cell counting assays were performed.

The morphological phenotype observed by inverted microscope and limited proliferation rate revealed that *FOXQ1*-KO cells experienced stress under antibiotic selection and finally all cells treated with guides targeting FOXQ1 died after multiple rounds of medium replacement. More strikingly, the cell counting assay uncovered that the cell death rate was different in between the A549, MCF7 and MDA-MB-428 cell lines, presumably based on cell type or cell population doubling time of the particular cell lines (Fig. 16C).

To further investigate how FOXQ1 depletion induced cell death inA549 cells, A549 WT and A549 FOXQ1 OE cells were either transduced with sgRNAs targeting the endogenous *FOXQ1* or non-targeting sgRNAs. 48 h after transfection, cells were incubated with specific antibiotic selection for 24 h. The transfected cells were then trypsinised and collected for protein extraction before the infected cell died completely. Cell lysates were analysed via immunoblotting using antibodies against FOXQ1 (endogenous), FOXQ1-Flag, CDK4, CDK6 and VCP (Fig. 17A). Western blot analysis indicated remarkable and rapid degradation of endogenous FOXQ1 (lower band) (Fig. 17A) in both A549-WT and A549-FOXQ1-OE transfected with FOXQ1 gRNA. Furthermore, an increased expression of FOXQ1 was detected in the A549-FOXQ1-OE (Non-Targeting) compare to A549-WT (Non Targeting) (Fig. 17A). Additionally, some unspecific binding of the FOXQ1 antibody (top band) (Fig. 17A) was observed at higher molecular weights. Next, I aimed to determine whether FOXQ1 deficiency altered the expression of genes known to regulate cell cycle progression. Cyclin-dependent kinases CDKs and cyclins are the main regulatory components of cell cycle control (Malumbres 2014). Cyclin-dependent kinases 4 and 6 (CDK4/6) by the association with cyclin D directly regulate cell cycle progression from G1 to S phase. Direct modulation or downregulation in CDKs results in cell cycle arrest (Ding, Cao et al. 2020). Therefore, the expression of CDK 4/6 and cyclin D was analysed in both A549-WT and A549-FOXQ1-OE cell with and without endogenous FOXQ1 KO (Fig 18A). Western blot analysis confirmed a depletion of the CDK4/6 and cyclin D in cell with endogenous *FOXQ1*-KO. Additionally, total RNA was extracted 12 h after transfecting cells with the CRISPR CAS9 system (Fig. 17B). Furthermore, qPCR analysis was performed on A549-WT and A549-FOXQ1-OE to verify downregulation of endogenous FOXQ1 targeted with

FOXQ1-gRNAs. The qPCR data was normalized to the reference gene *GAPDH*. The results revealed that the relative gene expression of endogenous FOXQ1 was downregulated in cells with guides targeting *FOXQ1* compared to cells receiving non-targeting guides both in A549-WT and A549-FOXQ1-OE cells. Similar western blot experiments were conducted in A549, MCF7 and MDA-MB-468 cells confirming the result that FOXQ1 depletion downregulated CDK4 (Fig. 17C).

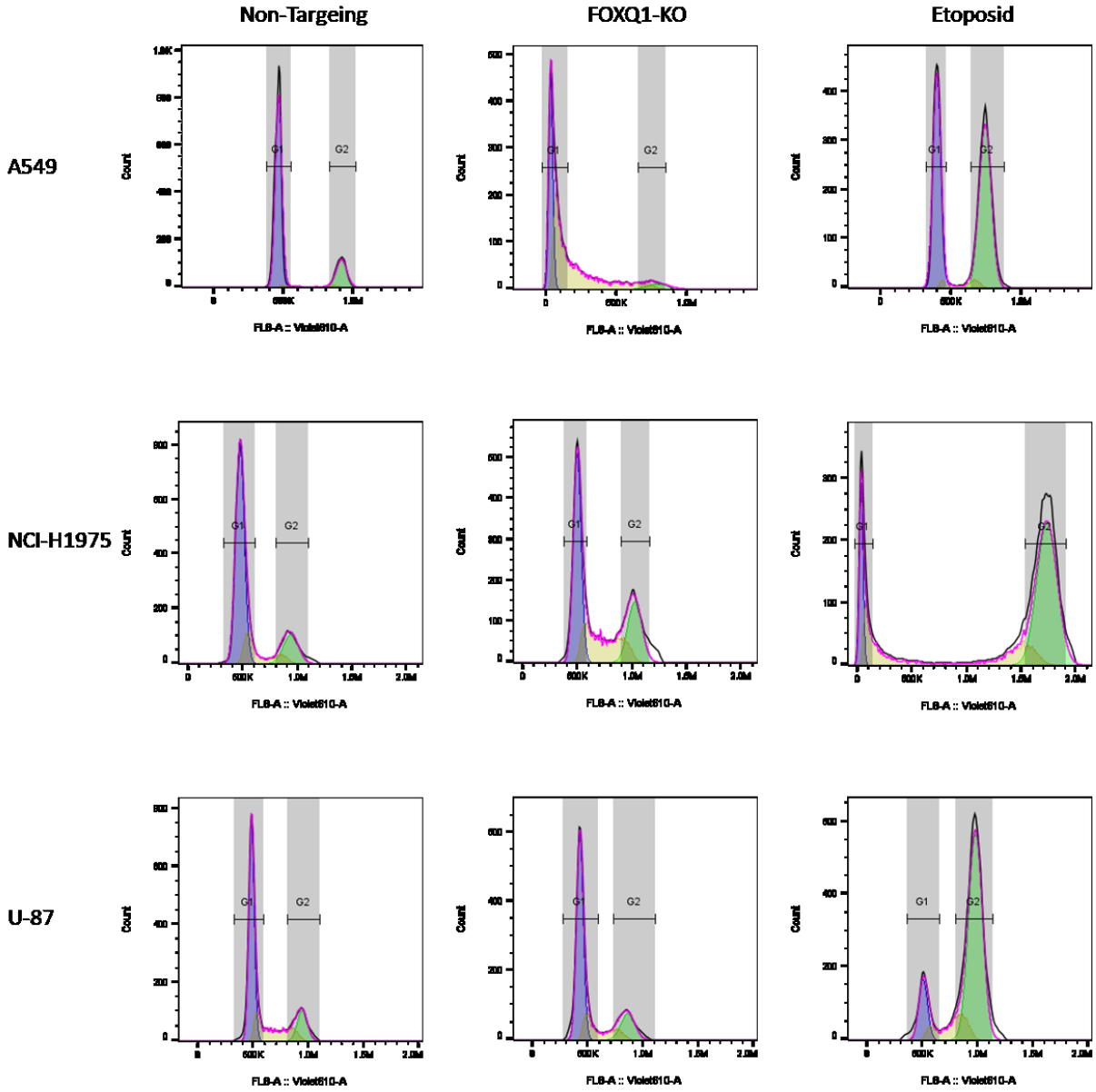

Figure 18: FOXQ1 depletion is arresting the cell cycle in cancer cells.

Cell cycle analysis using flow cytometry after fixation of cells and propidium iodide (PI) staining. Flow cytometry analysis of A549, NCI-H1975 and U-87 cells transfected with control (non-targeting), *FOXQ1*-KO (gRNA-*FOXQ1*) and etoposide treated are presented. Samples were fixed and analysed 48 h after transfection. The measured DNA content revealed the cell distribution within the major phases of the cell cycle. Increased DNA content at S phase (yellow) or G2 phase (green) was analysed compared to the haploid DNA status of G1 phase (blue).

To further authenticate the functional role of FOXQ1 in regulating cell cycle and proliferation, cell cycle was performed using flow cytometry analysis (Fig. 18). Human cancer cells (A549, NCI-H1975 and U-87) were transfected with sgRNAs or treated with etoposide (cell cycle inhibitor inducing G2/M arrest) following the protocol of previous experiments. Cells were fixed and stained with propidium iodide (PI) to visualize the DNA content of each cell in a FACS analyser. The result showed an increased DNA content in S phase in the *FOXQ1*-KO cells comparing to the control ones indicating a cell cycle arrest in between G1 and S phase. Treatment of cells with Etoposide arrested cell cycle in G2 phase indicated by an increased number of cells with high DNA content. Taking together, these results demonstrated that FOXQ1 depletion is lethal. FOXQ1 plays crucial role in cell cycle progression, while knocking out directly or indirectly trigger cell cycle arrest and induce cell death.

4.7. Total RNA sequencing analysis of FOXQ1 overexpressing cancer cell lines

Recently, a number of studies have revealed alterations in FOXQ1 and related genes that participate in cancer metastasis (Liu, Wu et al. 2017, Mitchell, Wu et al. 2022). FOXQ1 modulated biological effects via regulating several signalling pathways and the activities of other TFs (Abba, Patil et al. 2013, Peng, Huang et al. 2014). In addition, FOXQ1 was shown to regulate genes involved in biological pathways of RNA processing and protein metabolism (Liu, Wu et al. 2017, Pizzolato, Moparthi et al. 2022). Data from my previous experiments revealed a unique function of FOXQ1, as FOXQ1 overexpression directly or indirectly mediated the ferroptosis sensitivity in A549, NCI-H1975 and NCI-H1437 cell lines. FOXQ1 depletion was lethal and resulted in altering the expression of certain proteins (CDK4/6 and cyclin D) (Fig 18). Therefore, to examine the alteration in the expression of genes and post translation processing of proteins, RNA processing mechanisms are considered to be most important events to analyse (Manning and Cooper 2017, Szeto, Tran et al. 2021). Total RNA sequencing (RNA-seq) based transcriptomic studies have revealed many cellular abnormalities in cancer cells, including various misregulated biological processes, mitochondrial dysfunction and oxidative stress responses (Boo and Kim 2020, Destefanis, Avsar et al. 2021) regulating the global gene expression in different genetic backgrounds (Fig. 19).

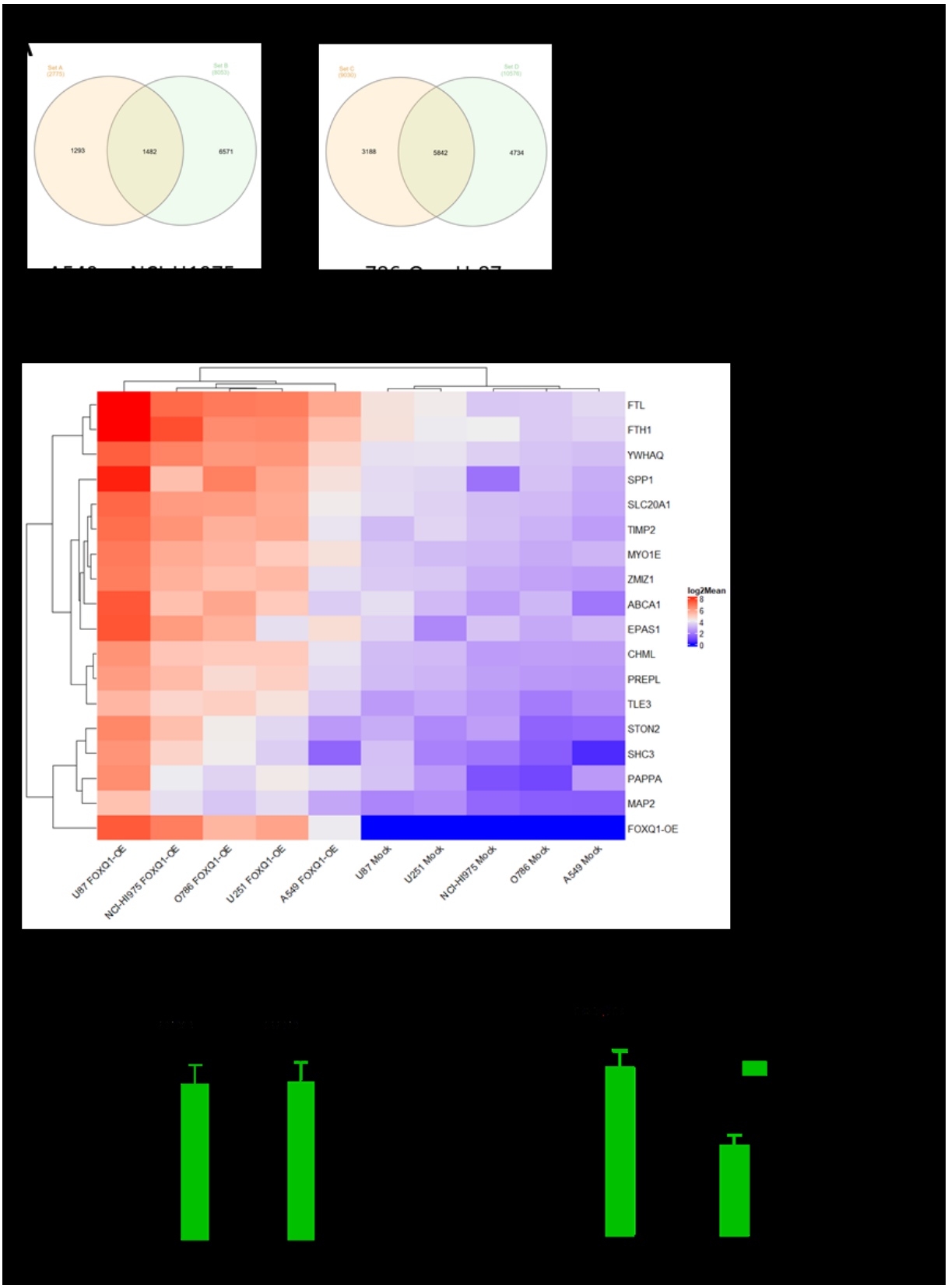

Figure 19: Multiple genes crucial for cell biological function are significantly regulated by FOXQ1

(A) Venn diagram represents overlap of significantly DEGs with an adjusted p-value < 0.05 between human cell lines "http://www.interactivenn.net''. FOXQ1 overexpression regulated 1482 DEGs mutually in both A549 and NCI-H1975 cells. 5842 DEGs are shared both 786-O and U-87 cells by FOXQ1 overexpression. **(B)** The top 18 significantly upregulated genes in FOXQ1-OE cells compare to mock be illustrated in the heatmap. Data were

normalised by mean centering and divided by the standard deviation. Mean values are represented by white squares (1:1, no change), red indicate up- and blue indicate down-regulation compared to the mean value, respectively. **(C)** Bar chart illustrating the RNA-seq of two selected differentially upregulated genes *FTL/FTH1* from both 786-O and U-87 (Mock vs FOXQ1-OE) cells. Black bar graphs represent relative mRNA expression changes of the upregulated genes derived from transcriptomic analysis of mock cells and green bar graphs represent relative mRNA expression changes of the upregulated genes from FOXQ1-OE cells.

To accomplish this, total RNAs were isolated from control (Mock) and FOXQ1-OE cell lines (A549, NCI-H1975, 786-O, U-87 and U-251). 2-3 µg of the isolated RNA of each cell line was used as template for cDNA synthesis by reverse transcriptase. The cDNA concentrations was determined by a UV/VIS spectrometer (NanoDrop) and samples were sent for next generation sequencing to the Core Facility Genome Sequencing, Helmholtz Centre Munich. A FASTQ file containing the separate sequence reads was provided. With the help of Dr. Dietrich Trümbach the sequences were analysed, aligned and quantified to determine the amount of each transcript present in the samples. RNA-seq data using different cell lines was compared on the basis of FOXQ1-OE mediating ferroptosis in these cell lines. A549, NCI-H1975 cell lines increased sensitivity to RSL3 and erastin-induced ferroptosis with overexpression of FOXQ1 (Fig. 11), while, 786-O, U-87 cell lines become resistant to RSL3 and erastin-induced ferroptosis with overexpression of FOXQ1 (Fig. 12). Intensive data analysis was performed to compare the RNA expression profile of control and FOXQ1 expressing cell lines A549, NCI-H1975 (left) and 786-O, U-87 (right) using a Venn diagram (Fig. 19A).

Partial overlap of significantly differentially expressed genes (DEGs) is displayed in the Venn diagram (Fig. 19), with an adjusted p-value < 0.05 between human cell lines analysed, a total of 1293 DEGs to be regulated in Set A (A549-Mock vs A549-FOXQ1-OE) and 8053 DEGs were found to be regulated in Set B (NCI-H1975-Mock vs NCI-H1975-FOXQ1-OE) (Fig. 19A-left), while, a total of 1482 DEGs were found to be mutually regulated in in both Set A and Set B. Subsequently, a total of 9030 DEGs were found to be regulated in Set C (786-O-Mock vs 786-O-FOXQ1-OE) and 10576 DEGs were found to be regulated in Set D (U-87-Mock vs U-87-FOXQ1-OE), while a total of 5842 DEGs were found to be mutually regulated in in both Set C and Set B (Fig. 19A-right). In FOXQ1-OE cells left (NCI-H1975-Mock vs NCI-H1975-FOXQ1-OE) and right (U-87-Mock vs U-87-FOXQ1-OE) the pathways linked to DEGs were significantly enriched in the EGFR signalling pathway, cell cycle regulation pathway, protein and lipids metabolism and cytokine signalling (Fig. 0B). Biological processes linked to DEGs were significantly enriched in cell metabolic processes, cell cycle arrest, cell proliferation and cellular response to stress and others (Fig. 19C).

Furthermore, using enrichment analysis by GeneRanker of 300 DEGs from the overlap of all sets of human cell lines were further subcategorized into top upregulated and downregulated DEGs. The identified genes were illustrated in the heatmap to precisely visualise hierarchical clustering of 18 upregulated DEGs in human cell lines overexpressing FOXQ1 (Fig. 19B). The top 18 significantly differentially upregulated genes were FTL, FTH1, YWHAQ, SPP1, SLC20A1, TIMP2, MYO1E, ZMIZ1, ABCA1, EPAS1, CHML, PREPL, TLE3, STON2, SHC3, PAPPA, MAP2, and FOXQ1 (overexpressed). All the significantly differentially upregulated genes have multi-functional roles in cells. These genes are involved in several biological process including cellular metabolism, iron homeostasis, signal transduction, protein and lipid metabolism, tyrosine kinase signalling pathway, induction of oxygen regulated genes, translocation of specific phospholipids and associated diseases (Morawski, Schulz et al. 2011, Villanueva, Waki et al. 2011, Chen, Yin et al. 2019, Koumakis, Millet-Botti et al. 2019, Wang, Hao et al. 2019, Li, Fan et al. 2020). FOXQ1 differentially upregulated genes were listed, among which Ferritin Heavy Chain 1 (FTH1) and Ferritin Light Chain (FTL) upregulation was more then 10-fold higher in FOXQ1-OE cells compare to control (Fig. 19C, D). FTH1/FTL, key subunits forming ubiquitous intracellular ferritin that plays an important role in the proliferation, iron metabolism and maintenance of the cellular iron balance in cells (Zhou, Zhao et al. 2018, Di Sanzo, Quaresima et al. 2020). FTH possesses enzymatic activity and oxidize ferrous (Fe^{2+}) iron into ferric (Fe^{3+}) iron, while FTL confers stability to the ferritin complex (Alkhateeb and Connor 2013). Ferritin complex regulates cell proliferation in an iron-dependent manner, while, dysregulation of intracellular iron content is toxic and leads to oxidative cell death (Oshiro, Morioka et al. 2011). Recently is has been reported that increased expression of FTL and FTH1 suppresses ferroptosis (Mbah and Lyssiotis 2022). Others have reported that increased FTH1 expression induces metabolism and oxidative cell death (Karim, Bajbouj et al. 2022). While from our study FOXQ1 mediated ferroptosis in a contrary manner in the different cell lines. Hypothetically, FOXQ1 directly or indirectly regulated FTH1/FTL expression and mediated ferroptosis cell-context dependent. This finding may go some way to explain how FOXQ1 depletion regulated cell cycle and cellular proliferation, though the underlying mechanism remains to be investigated.

4.8. FOXQ1 regulated ferroptosis associated genes

To shed light on total regulated (upregulated and downregulated) key genes and to better understand how mRNA and protein expression are altered via FOXQ1 overexpression I intended to analyse post-translation expression of different ferroptosis-associated genes (Fig. 20). Total protein expression analysis was performed in number of cell lines of both control and FOXQ1-OE. To do this, same protein amount was loaded for the Western blot analysis and the detected protein expression was directly compared between the control and FOXQ1-OE cell lines (Fig. 20A). The expression of a number of ferroptosis-associated genes was detected by specific antibody against each gene in all samples, yet to varying proportions. *VCP*, a housekeeping gene was used as a loading control to normalise the protein expression analysis. When comparing the protein expression level between control and FOXQ1-OE cells thioredoxin reductase 1 (TR1), selenoprotein P (SEPP1), Apoptosis inducing factor mitochondria associated 2 (AIFM2), CDK-interacting protein 1 (p21), E-cadherin (E-Cad), Vimentin (VIM), and S6 ribosomal protein (pS6) were differentially regulated (Fig. 20A). Protein expression of TR1, SEPP1, pS6 was slightly downregulated (except from NCI-H1975). While protein expression of p21 was downregulated in FOXQ1-OE cells (except A549), E-Cad was not detected in 786-O, U-87 and U-138 cell line. VIM was weakly detected in lung cells, while downregulated in the FOXQ1-OE (786-O, U-87 and U-138) cells (Fig. 20A).

Additionally, ferroptosis-associated genes expression were further characterised using volcano plot analysis of the RNA-Seq data (Fig. 20B-D). Volcano plots generated using individual data sets of each specific cell line revealed the top regulated ferroptosis genes. In total 60661 variables were analysed with p adjusted values (padj) ≤ 0.05, $\log_2 FC \geq 1.5$ and $\log_{10}$ p-values for each individual time point. The well-known ferroptosis associated genes in red labelled with names GPX4, SLC7A11 (xCT), AIFm2, TNXRD1, TP53, CDKN1A (p21), GCLM, vimentin (VIM) E-cadherin (CDH1) were highly regulated in all four cell lines (A549, NCI-H1975, 786-O and U-87). Of note, FOXQ1 mRNA and protein levels were not always consistent, indicating that FOXQ1 expression might also be subject to post-translational regulation.

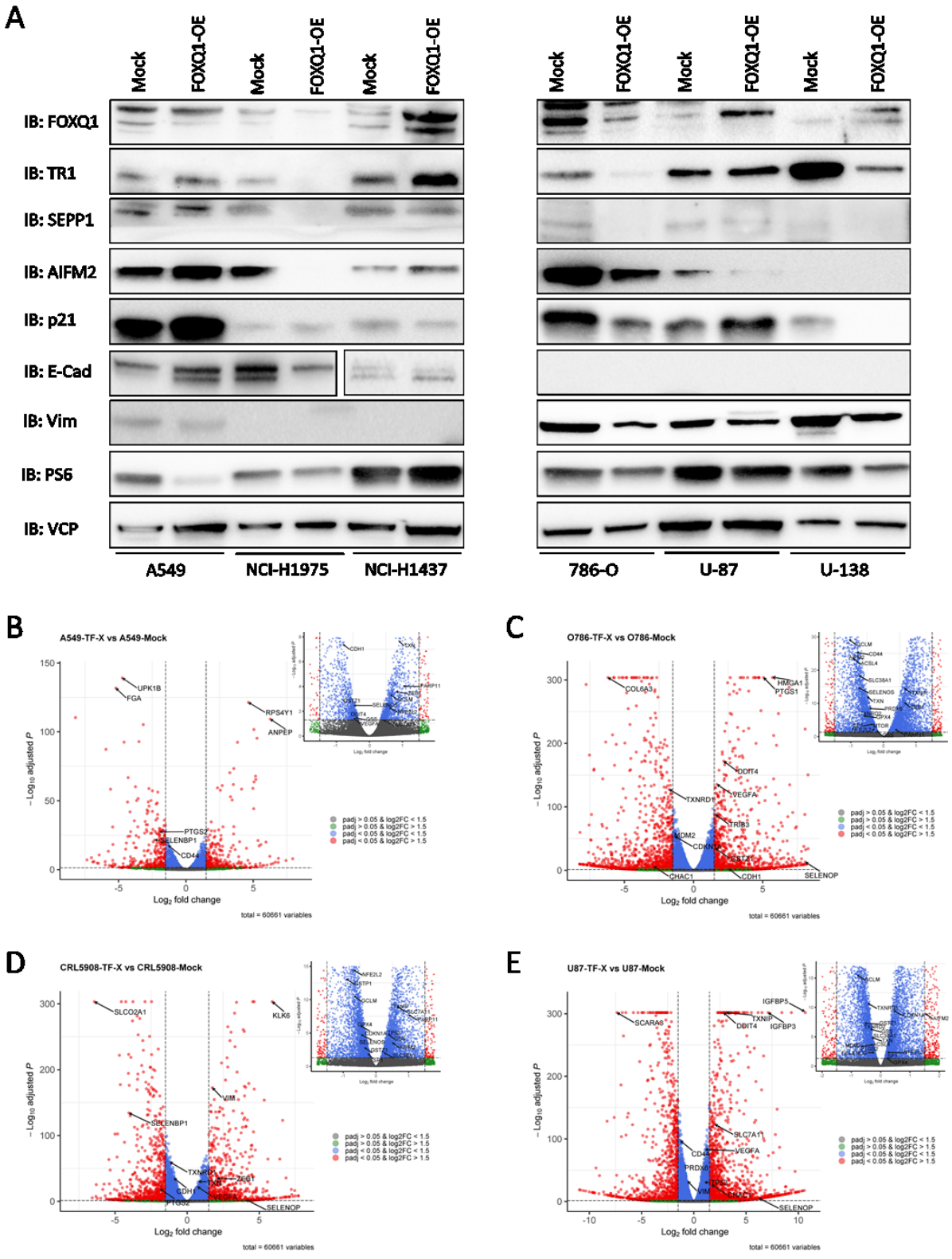

Figure 20: FOXQ1 overexpression regulates ferroptosis genes expression.

(A) Protein expression analysis in a panel of cancer cells expressing empty plasmid (Mock) and FOXQ1. The Western blot analysis with specific antibodies against FOXQ1, TR1, SEPP1, AIFM2, p21, E-Cad (only lung cancer cells), VIM, and pS6 revealed regulation via overexpression of FOXQ1 in cells. VCP expression is widely used for normalisation purposes in Western blotting **(B-E)** Volcano plots of DEGs of cells overexpressing FOXQ1. Each

point represents the average value of one transcript in three replicate experiments. The expression difference is considered significant for a log 2-fold change of 1 (outer light grey broken vertical lines) and for a P-adjusted value of 0.05 [log (FDR) of 1.5, dark broken horizontal line]. Points are coloured according to their average expression in all data sets. Names and outlined points represent virulence factors. FDR, false-discovery rate.

RNA-Seq analysis was focused to compare relative genes expression in between Mock vs FOXQ1-OE cells and in between the cell lines associated with FOXQ1 mediated ferroptosis. To this end, enrichment pathway analysis and Gene Ontology (GO) of differentially expressed genes (DEGs) based on cellular networks and biological processes were performed by comparing with databases (Fig. 21). The cut-off criteria, p-value ≤ 0.05, and $\log_2$FC ≥ 1.5 (FC, fold change) were used respectively in identifying top 10 pathway-based networks and important biological functions of a specific gene. In FOXQ1-OE cells left (NCI-H1975-Mock vs NCI-H1975-FOXQ1-OE) and right (U-87-Mock vs U-87-FOXQ1-OE) the pathways linked to DEGs were significantly enriched in EGFR signalling pathway, cell cycle regulation pathway, protein and lipids metabolism and cytokine signalling (Fig. 21B). Biological processes linked to DEGs were significantly enriched in cell metabolic processes, cell cycle arrest, cell proliferation and cellular response to stress and others (Fig. 21C). Collectively, the inclusive enrichment analysis indicated distinct transcriptional regulations associated with FOXQ1 overexpression. Cell proliferation and the epidermal growth factor receptor (EGFR) signalling pathway were highly regulated among the top 10 pathways (Fig 22C). The EGFR tyrosine kinase signalling pathway is one of the most important pathways regulating cellular growth, proliferation, survival, and differentiation (Wieduwilt and Moasser 2008, Wee and Wang 2017). EGFR tyrosine kinase is stimulated by its ligand epidermal growth factor (EGF) (Purba, Saita et al. 2017, Hajdu, Varadi et al. 2020).

Activated EGFR tyrosine kinase stimulates intracellular signal transductions, which in turn promote transcription of growth related genes, and increase cell growth and proliferation (Wee and Wang 2017). Erlotinib, an FDA-approved EGFR kinase inhibitor was associated with strong clinical response in cancer (Johnson, Cohen et al. 2005). Erlotinib impeded EGFR to inhibit cell proliferation and induce cell death (Dermawan, Gurova et al. 2014). And from our previous finding's FOXQ1 overexpression-mediated ferroptosis is cell type dependent (Fig. 22A, B).

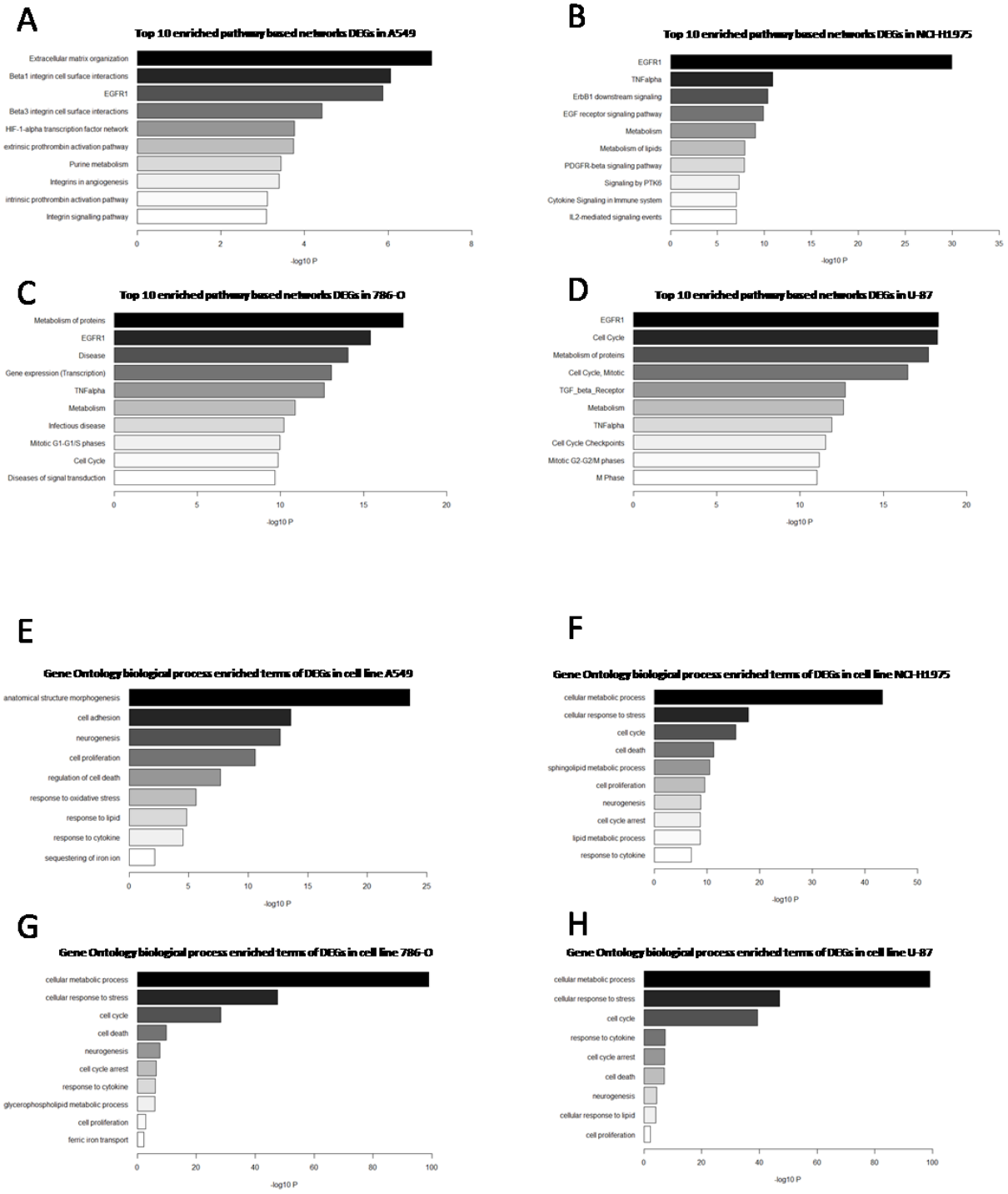

Figure 21: The 10 key pathway enrichment and gene ontology (GO) terms of differentially expressed genes (DEGs).

(A-D) The top 10 enriched pathways based on DEG network analysis, the bar plot shows the enrichment scores of the significant enrichment induced by FOXQ1 overexpression in A549 (Top left), NCI-H1975 cells (Top right), 786-O (Down left) and U-87 cells (Down right). **(E-H)** The 10 key Gene Ontology biological process terms in the identified DEGs, the bar plot shows the enrichment scores of the significant enrichment GO terms of A549 (Top left), NCI-H1975 cells (Top right), 786-O (Down left) and U-87 cells (Down right). The cut-off criteria, p-value ≤ 0.05, and $\log_2$FC ≥ 1.5 (FC, fold change) used respectively in identifying top 10 pathway-based networks and important biological functions of a specific gene.

To address whether EGFR inhibition may impact on cell proliferation, WT cell lines (A549, NCI-H1975, 876-O and U-87) were treated with erlotinib [10 µM] to inhibit EGFR (Fig. 22C). All the cells were incubated for 3 h with erlotinib [10 µM] and afterwards treated with increasing concentration of RSL3 and fixed concentration of Lip1 [250 nM].

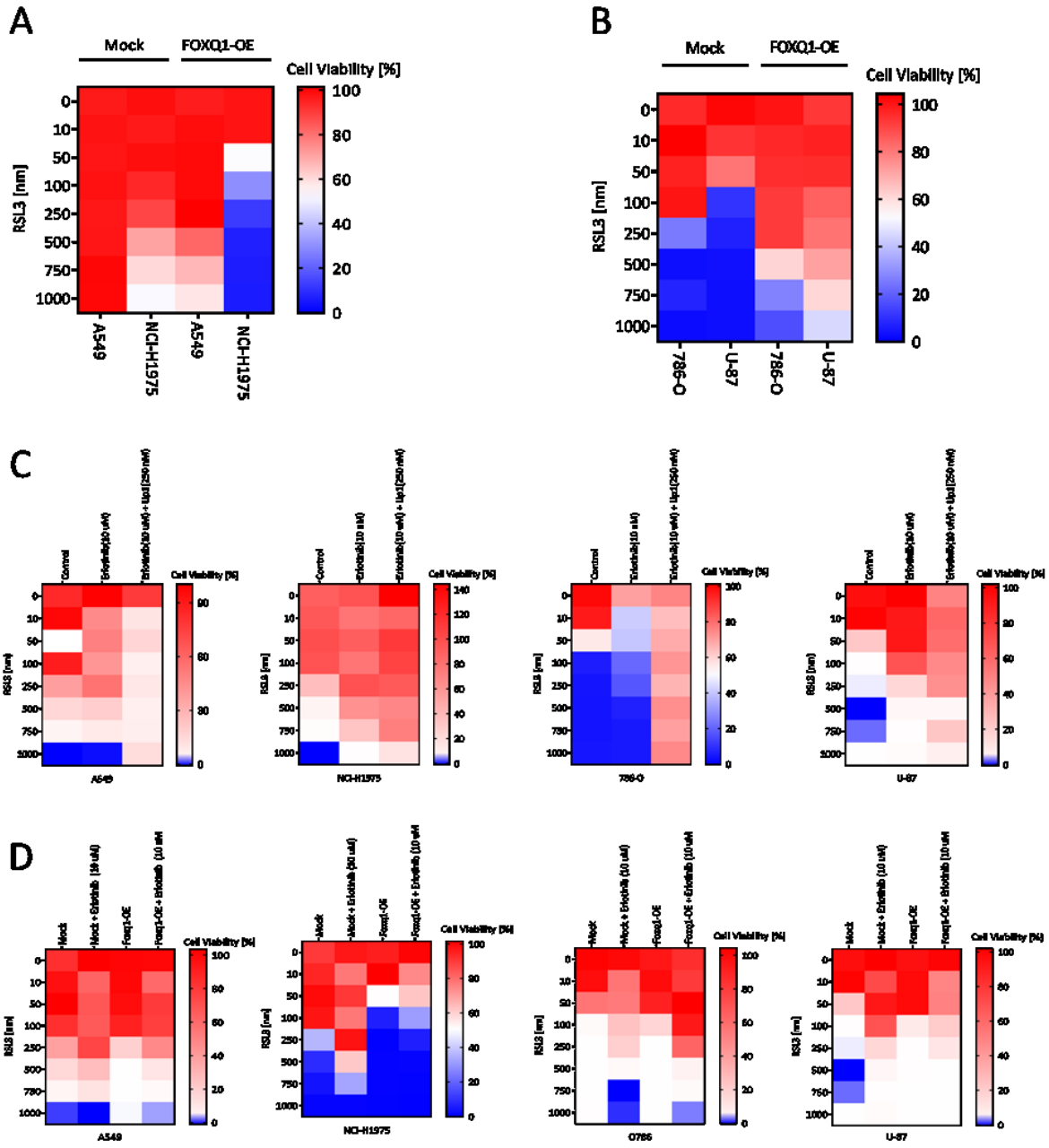

Figure 22: Functional enrichment analysis of DEGs revealed EGFR signalling pathway regulation

(A) Dose dependent toxicity of oxidative cell death induced by RSL3. Panel of lung cancer cells overexpressing FOXQ1 were treated with increasing concentration of RSL3 with or without Lip-1. Both A549 and NCI-H197s5 cell overexpressing FOXQ1 shows increased sensitivity to induced ferroptosis compared to the Mock. Cell viability was measured 24 h after treatment using the Aqua Bluer method. Data shown from three independent experiments represent the mean ±s.d. of n=3 wells of a 96 well plate, $p<0.001$ (two-way ANOVA). **(B)** Increased concentration of RSL3 failed to induce rapid lipid peroxidation in 786-O, and U-87 cell lines. Both the cell lines mediated resistance to ferroptosis. Cell viability was measured 24 h after treatment using the Aqua Bluer. Data shown represent the mean ±s.d. of n=3 wells of a 96 well plate, $p<0.001$ (two-way ANOVA) (N=3). **(C)** Panel of

cancer cells A549, NCI-H1975, 786-O and U-87 (WT) treated with fixed concentration of erlotinib [10 µM] and with increasing concentration of RSL3 with or without Lip-1. Cell viability was measured 24 h after treatment using the Aqua Bluer method. Data shown from three independent experiments represent the mean ±s.d. of n=3 wells of a 96 well plate, p<0.001 (two-way ANOVA). **(D)** A panel of cancer cells A549, NCI-H1975, 786-O and U-87 (Mock and FOXQ1-E) incubated with increasing concentration of RSL3 with or without Lip-1 and with fixed concentration of erlotinib [10 µM]. Cell viability was measured 24 h after treatment using the Aqua Bluer. Data shown from three independent experiments represents the mean ±s.d. of n=3 wells of a 96 well plate, p<0.001 (two-way ANOVA) from three independent experiments.

After 24 h of incubation with erlotinib and RSL3, cell viability assay was performed to analyse the RSL3-induced ferroptosis effect. The viability assay revealed that erlotinib did not regulate RSL3-induced cell death. This analysis is in agreement with previous descriptions of A549 and NCI-H1975 cells bearing genetic aberrations (EGFR, KRAS mutant) leading to erlotinib resistance (Li, Chan et al. 2021). After all, RNA-Seq data analysis identified that FOXQ1 overexpression significantly regulated EGFR signalling pathway (Fig 22B), but it was strongly urged whether EGFR inhibition impacts FOXQ1-mediated ferroptosis in the FOXQ1-OE cells. Intriguingly, following the protocol of previous experiments Mock and FOXQ1-OE cell lines (A549, NCI-H1975, 786-O and U-87) were incubated for 3 h with erlotinib [10 µM] and for 24 h with gradual concentration of RSL3 (Fig. 22D). Strikingly, very similar pattern of cell viability was observed in both Mock and FOXQ1-OE cell lines. Aqua Bluer assay revealed that EGFR inhibition did not produce a significant impact on ferroptosis regulation in both control and FOXQ1-OE cells. Taken together, enrichment pathway-based network DEGs analysis indicated that, FOXQ1 overexpression regulated EGFR signalling pathway in A549, NCI-H1975, 786-O and U-87 cell lines, but pharmacological inhibition of EGFR did not reverse the effect of FOXQ1 mediated ferroptosis in these cell lines.

4.9. Genome-wide CRISPR screening using a lentiviral guide RNA library

The functional role of ferroptosis in relation to diseases has become a hotspot of etiological research. Number of pathways and downstream genes modulating ferroptosis have been investigated but the functional changes and the underlying molecular mechanism of ferroptosis still need to be explored. CRISPR-mediated genome wide KO screens are a powerful approach to explore previously unknown molecular mechanisms, pathways and to discover novel genes contributing to the ferroptotic process (Li, Yu et al. 2018). To this end, a genome wide CRISPR/Cas9 knockout screen was performed to identify novel genes involved in ferroptosis suppression (Fig. 23). The breast cancer cell line BT474 was seeded at low

density (300,000 cells) onto 3 x 15 cm dishes. The lentiCRISPRv2 plasmid was used to deliver sgRNAs and Cas9 together and induce loss of function in the targeted genes on a genomic scale (Koike-Yusa, Li et al. 2014, Wang, Wei et al. 2014, Doench, Fusi et al. 2016).

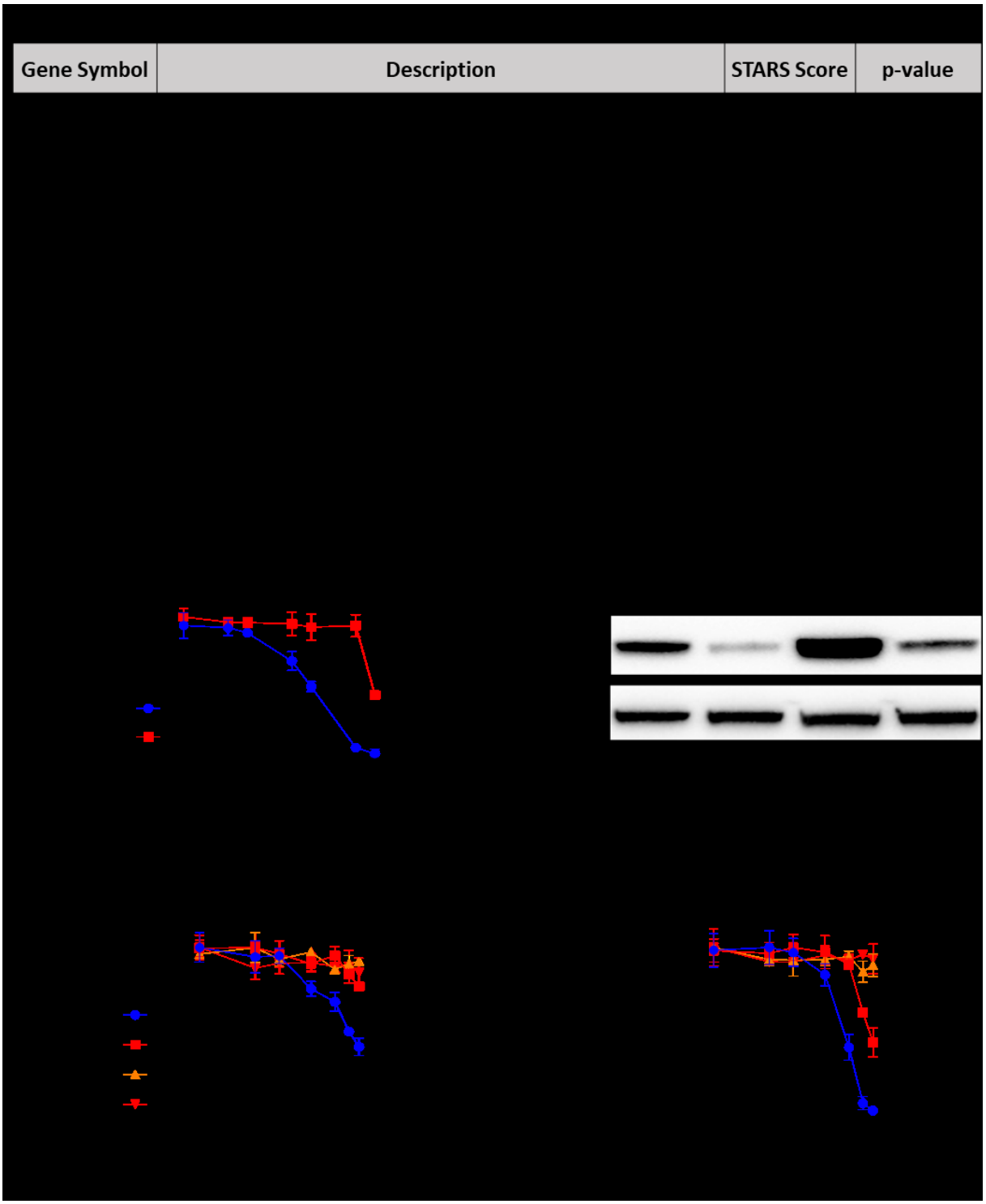

Figure 23: Genome-wide CRISPR/Cas9 screening identifies novel ferroptosis players.

(A) Top 10 highly targeted transcripts top to bottom PPARD, PTEN, FOXC1, LRRC29, ACSL4, POLE4, PRSS37, TM4SF19, PILRA, and AVPR1A by STARS gene-ranking algorithm for genetic perturbation screens (Broad

Institute). Obtained data files sorted into excel sheet to compare to the reference library (#73179 Addgene) and display sgRNAs top hits with a corrected p-value (false discovery rate (FDR)) from the selected cell pool. **(B)** Dose-dependent cytotoxicity of RSL3. BT474-WT cells were treated with increasing concentration of RSL3 with or without Lip-1. Cell viability was assessed 24 h after treatment using Aqua Bluer. Data shown represent the mean ± s.d. of n = 3 wells of a 96-well plate from one representative of three independent experiments, $p<0.001$ (two-way ANOVA). **(C)** Immunoblot analysis of A549, NCI-H1975 (WT and PTEN knock out) using antibodies against PTEN and gene expression was normalised to the reference gene *VCP*. **(D-E)** Dose-dependent cytotoxicity of RSL3. A549 and NCI-H1975 (WT, PTEN-KO) cells were treated with increasing concentration of RSL3 with or without lip-1. PTEN-KO cell lines exhibit increased resistance to ferroptosis induced by RSL3. Cell viability was assessed 24 h after treatment using Aqua Bluer method. Data shown from three independent experiments represent the mean ± s.d. of n = 3 wells of a 96-well plate (two-way ANOVA).

Pseudo-typed (Moloney Murine Leukemia Virus) lentiviral particles of the CRISPR/CAS9 library (Brunello library puro, 73179 Addgene) from Broad GPP comprising about 76441 sgRNAs with an average target of 4 sgRNAs targeting each human gene were produced in HEK293T cells. Breast cancer BT474 cells were transduced under biosafety level S2 conditions at MOI of 0.3 to minimize superinfection of cells (Doench, Fusi et al. 2016, Sanson, Hanna et al. 2018). After 48 h of infection cells were treated with different concentration of RSL3 100 nM, 250nM, and 500 nM (3x for each RSL3 concentration) and without RSL3 (3x, control) followed for about total of 7 days.

After that genomic DNA was harvested from the RSL3 250 nM treated and untreated (control) cell pool and the isolated DNA was amplified by PCR using primers embedded with two different barcodes. The PCR products were pooled equimolarly and sent for next generation sequencing (NGS) for sequencing on an Ion Torrent P1 chip (PrimBio Research Institute, LLC, Exton, PA). The data was provided in the form of FASTQ files already sorted by the different barcodes. Subsequently the necessary sgRNA information form the raw sequencing FASTQ file was extracted from single reads. Results were analysed using the spliced transcripts alignment to a reference (STAR) (Dobin, Davis et al. 2013). The top 10 hits with highest STAR score (PPARD, PTEN, FOXC1, LRRC29, ACSL4, POLE4, PRSS37, TM4SF19, PILRA, AVPR1A) identified in the RSL3 treated cell pool are represented in Fig. 23A. Peroxisome proliferator-activated receptor delta (*PPARD*) and Phosphatase and tensin homolog (*PTEN*) identified were considered as the most promising candidate gene to be further validated. A dose-dependent cytotoxicity of RSL3 induced ferroptosis in BT474 cells was performed (Fig. 23B). The cell viability was assessed 24 h after treatment with increasing concentration of RSL3 with or

without lip-1. Obtained data suggested that BT474-WT cells were sensitive to RSL3 induced ferroptosis. Moreover, *PTEN* is a multi-exon gene, therefore two pLentiCRISPRv2 plasmids (CAS9 + sgRNA guide) were used to target *PTEN*. The lung cancer cell lines (A549 and NCI-H1975) were transduced with a pLentiCRISPRv2 plasmid expressing a sgRNA-blast targeting exon 1 and sgRNA-puro targeting exon 5 of the *PTEN* gene, while non-targeting gRNA was used as control. Selection was followed by cell passaging for multiple time. Protein expression analysis with a monoclonal antibody against *PTEN* was the 1st approach used to confirm the successful KO (Fig. 23C). A significant reduction in the protein expression was observed in the cell receiving the *PTEN* targeting guides (*PTEN*-KO) compared to the control cells. For further confirmation, a dose-dependent cytotoxicity of RSL3 induced ferroptosis was performed in A549 and NCI-H1975 cell lines (Fig. 23D-E). The cell viability was assessed 24 h after treatment with increasing concentration of RSL3 with or without lip-1. *PTEN*-KO cells were more resistant to ferroptosis compared to non-targeted control cells which were very sensitive to RSL3 induced ferroptosis (Fig. 23D, E). Together, my CRISPR screening approach highlighted the role of *PTEN* as a potential anti-ferroptotic gene, while the underlying mechanism needs to be investigated in the future.

5. DISCUSSION

5.1. Genome-wide screening to identify novel ferroptosis player

Ferroptosis is an iron dependent necrotic form of cell death characterised by oxidative stress (Dixon, Lemberg et al. 2012). Glutathione peroxidase 4 (GPX4) effectively reduce peroxidase phospholipids to the corresponding alcohol to restrain lipid peroxidation. GPX4 is the key regulator of ferroptotic cell death by reducing phospholipids (PL-OOH), while its deficiency triggers lipid metabolic pathways for the enhanced production of polyunsaturated fatty acids (PUFAs) (Imai and Nakagawa 2003, Friedmann Angeli, Schneider et al. 2014, Yang and Stockwell 2016) (Yang and Stockwell 2016). Acyl-CoA Synthetase Long Chain Family Member 4 (ACSL4) has a preferential role in activating long-chain polyunsaturated fatty acids for phospholipid biosynthesis (Doll, Proneth et al. 2017, Kagan, Mao et al. 2017). ACSL4 has substrate preference for polyunsaturated fatty acids like arachidonic acid (AA) [5 µM] and plays a crucial role by shaping the cellular lipidome (Doll, Proneth et al. 2017, Kuwata and Hara 2019). Our group successfully generated an ACSL4 knocked-out cell line (Pfa1-*ACSL4*-KO) (Pfa1 = TAM inducible Gpx4 -/- MEFs) and the cells were found to be very much resistant to ferroptosis. ACSL4 depletion suppresses ferroptosis triggered by the GPX4 inhibitor RSL3, while exogenous supplementation of AA sensitized *ACSL4*-KO cells to ferroptosis (Fig. 6B, C). Owing to the function of regulating PUFAs, ACSL4 determines a cell's sensitivity or resistance to ferroptosis (Doll, Proneth et al. 2017). Despite of all the previous investigations, we sought to gain insights into the molecular mechanisms of ferroptosis induced by GPX4 inhibition along with the supplements of AA. In this study, a genome wide CRISPR/Cas9 KO screening has been applied on *ACSL4*-KO cells with exogenous supplementation of AA [5 µM] to identify new potential ferroptotic players (Fig. 8D). In this approach, Pfa1-*ACSL4*-KO cells were transduced at MIO of 0.3 with a pseudotype dependent lentiviral particles of the CRISPR/CAS9 library (Brie #73632 Addgene) (~4 sgRNAs per gene) to achieve targeted genome-wide mutagenesis (Cannon, Sew et al. 2011, Doench, Fusi et al. 2016, Loo, Gatchalian et al. 2020). Additionally, I made second human genome-wide CRISPR/Cas9 knockout screen to identify novel genes involved in ferroptosis suppression (Fig. 23). In the second screening, I transduced human breast cancer cell line BT474 cells at MIO of 0.3 with *Brunello library* puro (Brunello #73179 Addgene) an average target of 4 sgRNAs/gene (Sanson, Hanna et al. 2018).

In the first screen, genes Acyl-CoA Synthetase Long Chain Family Member 3 ACSL3 and Forked head box 1 (FOXQ1) were considered as most promising hits of the sequenced sgRNAs in the selected cell population (Fig. 8E). In our group, Doll et all, 2017, and other studies have previously explained that ACSL3 is a close structural homolog of ACSL4 that preferentially activates and uses arachidonic fatty acids efficiently but ACSL3 overexpression did not sensitize cells to ferroptosis (Doll, Proneth et al. 2017, Radif, Ndiaye et al. 2018). Besides, to identify the role of ACSL3 in ferroptosis, research is under process by our group. In the second screen Phosphatase and Tensin homolog (PTEN) identified was considered as the most promising candidate gene to be further validated (Fig. 23A). To validate the identified candidate gene PTEN from my second screen, I used CRISPR/Cas9 technology knock out method to nullify PTEN in multiple cancer cell lines. Previously PTEN has been discovered as tumor suppressor (Li and Sun 1997, Guzeloglu-Kayisli, Kayisli et al. 2003). PTEN regulates cellular glucose and fatty acid metabolism (Chen, Chen et al. 2018). It's also known to regulate PI3K/AKT signalling pathways that are vital for cell growth and survival (Oda, Stokoe et al. 2005, Georgescu 2010). Some studies have reported that PTEN inhibits the PI3K-AKT-mTOR pathway and induces ferroptosis (Tan, Kong et al. 2022), while others have described hyperactive alteration of PI3K-AKT-mTOR signalling, rendering protection against oxidative stress and ferroptosis (Yi, Zhu et al. 2020, Lan, Yao et al. 2022, Wang, Wang et al. 2022). I used a two guides' system (sgRNA-PTEN-KO) carrying different antibiotic resistant genes to knockout *PTEN* in BT474, A549 and NCI-H1975 cells. Transduction of the cells was followed by specific antibiotic selection, while generation of *PTEN*-KO cell lines was confirmed by Western blot using antibody against PTEN. Immunoblot analysis showed the downregulation of endogenous PTEN in both A549 and NCI-H1975 cell lines, except for the BT474 cells (Fig 24C-). Cell viability assay identified that *PTEN*-KO cells were resistant to RLS3-induced ferroptosis (Fig. 23D, E). Further investigations will be continued to uncover the direct molecular mechanisms of PTEN involved in regulating ferroptosis.

However, the main part of dissertation was to investigate the molecular mechanism of FOXQ1 mediating ferroptosis in both MEFs and human cell lines. FOXQ1 is a member of FOX transcription factors, a family that contains a very large range of divergent members of TFs (Weigel and Jackle 1990, Jackson, Carpenter et al. 2010, Kaneda, Arao et al. 2010, Brown and Webb 2018). To evaluate the role of FOXQ1 transcript in ferroptosis, I focused on the molecular characteristics and transcriptional activities of FOXQ1. FOXQ1 encodes a forkhead

box transcripts related proteins and regulates various biological process, including cell proliferation, cell cycle regulation and cellular senescence (Li, Zhang et al. 2016, Zhang, Wang et al. 2016).

I used publically available Cancer Therapeutics Response Portal (CTRP) to identify the additional features of FOXQ1 associated with response to the ferroptosis compounds (Basu, Bodycombe et al. 2013, Rees, Seashore-Ludlow et al. 2016, Viswanathan, Ryan et al. 2017). CTRP obtained data analysis identified average high expression of FOXQ1 in over 870 cancer cell lines and correlated with cellular resistance to the ferroptosis inducing compounds RSL3, Erastin, and ML210 (Fig. 9A). Though this observation is in contrary of what was found in our first CRISPR screen. CRTP results instead correlated to RSL3 and erastin compounds sensitivity. As from the screen, FOXQ1 was observed as most promising hit of the sequenced sgRNAs in the selected (Pfa1) cell pool. Gene knockout correlates with compound activity and high expression correlates with resistance. But still the correlation of FOXQ1 with ferroptosis is very interesting. CRTP suggests candidate dependencies associated with common features including cell line selection, growth conditions, gene expression and data analysis that may contribute to differences in results (Liberzon, Subramanian et al. 2011, Basu, Bodycombe et al. 2013, Rees, Seashore-Ludlow et al. 2016, Viswanathan, Ryan et al. 2017). I believe that by analysing correlation of FOXQ1 with small molecules can be used to identify new potential therapeutic targets for cancer inhibition.

To better understand the implication of FOXQ1 in response to the ferroptosis inducing compounds, it was necessary to analyse the range of expression and mediated ferroptosis effect across many cell lines. FOXQ1 expression in Pfa1 cells was conformed via immunoblot analysis using antibody against FOXQ1 (Fig. 9C). While a codon optimized FOXQ1-Flag overexpression was confirmed via Western Blot analysis by using antibodies against FOXQ1 and flag and also by qPCR using primers hybridized only to overexpressed FOXQ1-Flag (Fig. 9C-E). Expression level of the genes that play crucial role in ferroptosis pathway such as GPX4, ACSl4 and AIFM2 were also examined in both control and FOXQ1-OE cells via immunoblot and qPCR. When I compared the degree of proteins and relative genes expression, no significant difference was observed in the FOXQ1-OE cells compared to Mock (Fig. 9C-E). Pfa1 WT cells are known to be sensitive to oxidative cell death induced by Gpx4 inhibition (Friedmann Angeli, Schneider et al. 2014). In the present study I observed that stable overexpression of

FOXQ1-Flag in Pfa1 WT cells increased sensitivity to ferroptosis induced by RSL3 (Fig. 9F). And herein, Lip-1 was used to determine whether ferroptosis is engaged in the cell death induced by RSL3 (Fan, Pang et al. 2021).

Several transcription factors like TP53, NFE2L2/Nrf2, and SP1, have been identified in shaping ferroptosis sensitivity (Dai, Chen et al. 2020, Liu, Zhang et al. 2020). Although, the mechanism of activation is either transcription-dependent or transcription-independent (Dai, Chen et al. 2020). FOX transcripts such as the FOXO have an important role in the transcription of certain genes like *FasI* or *BCL2L11* and thereby participate in the activation of the caspase cascade that in turn induces cell death (Brunet, Sweeney et al. 2004, Carter and Brunet 2007). Some studies have reported that FOXQ1 regulates the expression of various downstream genes (*Cdh1* and *p21* gene) that are crucial for cell survival (Larue and Bellacosa 2005, Kaneda, Arao et al. 2010, Ray, Ryusaki et al. 2021). From our findings, it can be stated that FOXQ1 is the only member of FOX transcriptions family that mediated ferroptosis sensitivity in Pfa1 cells, even though the molecular mechanisms still need to be exploited in detail.

5.2. FOXQ1 mediated sensitivity to ferroptosis is cell context dependent

The FOX family exhibits functional diversity in key biological processes, including oxidative stress and apoptosis but in many cases their particular function and the underline mechanism of action are not completely understood (Benayoun, Caburet et al. 2011, Golson and Kaestner 2016). Therefore, I aimed to better examine the expression and potential roles of FOXQ1 in different cell lines. Although the importance of FOXQ1 in development and progression of cancers is well established, its potential role in the oxidative cell death needs to be clarified (Liu, Wu et al. 2017, Elian, Are et al. 2021). Here, eight different cell lines were selected for a quick and accurate means of observing the expression level of FOXQ1, and the protein expression was examined by Western Blot analysis. Eventually, a marked difference in the expression of FOXQ1 was observed in between the different cell lines (Fig. 10A). Thus, parallel exploration of the FOXQ1 proteins expression in multiple cell lines will likely reveal the transcriptional regulation of FOXQ1 in different cell lines.

Ferroptosis is a unique type of cell death characterized by accumulation of lipid peroxides within the cells. RSL3 and erastin can be used to induce ferroptosis in cancer cells respectively

(Dixon, Lemberg et al. 2012, Doll, Freitas et al. 2019, Zheng, Sato et al. 2021). Despite of the prior findings, I intended to treat different human cancer cell lines with ferroptosis compounds (RSL3 and erastin) in order to determine the concentration dependent effects of these compounds (Fig. 10a, 10b). In addition, I used Lip-1, a potent pharmacological inhibitor of ferroptosis to determine whether ferroptosis is engaged in the cell death process induced by RSL3 and erastin (Zilka, Shah et al. 2017, Fan, Pang et al. 2021, Zhang, Chen et al. 2021). I used twelve different cancer human cell lines to induce ferroptosis via both RSL3 and erastin (Fig. 10a, 10b). After analysing the viability assay, I observed a variable ferroptosis effect in each cell line separately. Though all the cell lines incubated with same increasing concentration of RSL3 and erastin and for equal duration of time, still the sensitivity to ferroptosis between the cell lines was different. I assume that the differences in the induced ferroptosis is possibly due to the different pattern of genes activation or mechanism of action with respect to the induced cell death in each cell line. In addition, as has been stated previously, RSL3 and erastin induce cell death with a different molecular mechanism. RSL3 inhibition of GPX4 leads to the generation of toxic lipid radicals that are lethal to the cell, while erastin by inhibiting the cystine/glutamate antiporter depletes GSH (an essential cofactor of GPX4) and also causes ROS accumulation (Dixon, Lemberg et al. 2012, Magtanong, Ko et al. 2016).

To explore the function of FOXQ1 in multiple cells lines further, I generated a ready to use plasmid cloned with FOXQ1-Flag. The aim of these experiments was to identify for the first time a comprehensive role of FOXQ1 in ferroptosis. It is known that TFs regulate genes expression in a controlled way. A TF recognizes and binds to a segment of DNA in the promotor and influences the transcription of the specific gene (Inukai, Kock et al. 2017). Many TFs are known to facilitate the transcription of hundreds of genes, while some regulates few selective ones (Iyer, Horak et al. 2001, Swift and Coruzzi 2017).

The successful overexpression of plasmid cloned with FOXQ1-Flag was conformed to specific antibiotic selections and western blot analysis using antibody against flag (Fig. 11A). And specifically designed qPCR primers were used to quantify FOXQ1-Flag overexpression in multiple cell lines (Fig. 11B). FOXQ1 is a TF, therefore I was curious if it could regulate the expression of other ferroptotic genes. After Western blot analysis I found that the protein expression of key ferroptotic genes (*GPX4*, *GPX1*, *XCT* and *ACSL4*) were also regulated in the FOXQ1-OE cells compared to control (Mock). Earlier studies have shown that FOX factors are

involved in regulating various signalling pathways in pathological and physiological conditions, but in many cases the molecular mechanisms are poorly understood (Benayoun, Caburet et al. 2011, Golson and Kaestner 2016). Recently it has been identified that, post-translational modifications, such as phosphorylation, acetylation, and ubiquitination of the transcription factors O (FOXOs) modulate its functional activity, which in turn regulates the expression of the targeted genes (Fu and Tindall 2008, Obsil and Obsilova 2008, Boccitto and Kalb 2011). I observed that FOXQ1 overexpression upregulated the protein expression GPX4, GPX1, XCT and ACSL4 in lung cancer cell line but the degree of the increased expression was not equal (Fig. 11A). On the other hand, when I analysed the protein expression GPX4, GPX1, XCT and ACSl4 in kidney and glioblastoma cells, the observed effect was contrary to that in lung cancer cells. FOXQ1 overexpression in 786-O, U-87 and U-138 downregulated the protein expression of GPX4, GPX1, XCT and ACSl4 compared to control (Fig, 13A). In some other cell lines like U-251, U-373 and MDA-MB346, FOXQ1 did not induce any significant effect by regulating the protein expression of aforementioned genes (Fig, 14A). Another study suggested that FOXQ1 regulates the expression of Muc5ac in different tissues, but the question has remained whether FOXQ1 directly activates the transcription of Muc5ac, or acts through a distant enhancer (Verzi, Khan et al. 2008). I therefore cannot exclude the possibility that FOXQ1 directly or indirectly regulates the expression of certain genes. Future studies might resolve the underlying molecular mechanisms of FOXQ1 modulating the expression of key ferroptotic genes in different cell lines.

Cell viability assays may increase the probability to determine the function of FOXQ1 regulating ferroptosis in different cell lines. Here, I found that stable overexpression of FOXQ1-Flag mediated ferroptosis in cells is contextual. FOXQ1-Flag-OE increase sensitivity in lung cancer cells to both RSL3- and erastin-induced ferroptosis (Fig. 11C, D). Other cell lines examined in this research exhibited different degree of FOXQ1 mediated ferroptosis. Especially. Kidney and some glioma cells were found to suppress ferroptosis with overexpression of FOXQ1 (Fig. 12C, D). I also analysed some other cell lines cloned with FOXQ1-Flag, but the induced ferroptosis effect was very low compared to the initial results of our experiments (Fig. 13B). Many studies have identified FOXQ1 regulating the expression of different genes (Candelario, Chen et al. 2012, Zhang, Cao et al. 2022). In this study, I also found that FOXQ1 regulates variably a number of key ferroptotic genes. But our group for the first time pointed out the important role of FOXQ1 in ferroptosis (cell viability experiments),

though to identify the exact molecular mechanism of FOXQ1-mediated ferroptosis a detailed investigation has to be continued.

Several studies have identified the involvement of TFs to induce or inhibit programmed cell death. Some transcription factors such as overexpression of TIEG trigger apoptotic cell death via decreasing the protein expression of anti-apoptotic gene Bcl-2 (Chalaux, Lopez-Rovira et al. 1999). Other TFs like E2F-1 has both the pro-apoptotic and anti-apoptotic activities in multiple cell lines. E2F-1 is crucial for cell proliferation and its depletion induces cell cycle arrest (Field, Tsai et al. 1996, Hunt, Deng et al. 1997). Furthermore, increased activity of many transcription factors such as SIRT1, SP1, FKHR-L1, c-Fos, REST induces apoptosis via cell cycle arrest or with the activation of pro-apoptotic stimuli (Qin, Livingston et al. 1994, Preston, Lyon et al. 1996, Dijkers, Birkenkamp et al. 2002, Deniaud, Baguet et al. 2006, Wang, Chen et al. 2006, Zhang, Li et al. 2016). Here I found that breast cancer cell lines MCF7 and MDA157 could not exhibit the lentiviral expression of FOXQ1-Flag (Fig. 14). Both cell lines stopped proliferation and lost viability within few days after continued selection with specific antibiotic. All the cell lines including MCF7 and MDA157 were cultured and transduced by following the same protocol and under similar circumstances. Schmidt et al have described a similar effect with overexpression of FOXO1 and FOXO3a (Ramaswamy, Nakamura et al. 2002, Schmidt, Fernandez de Mattos et al. 2002, Fu and Tindall 2008, Ho, Myatt et al. 2008). Based on their observations, FOXO1 and FOXO3a promote cell cycle arrest by repressing CDK4 activity. From the observed results, I hypothesise that in some cell lines the stable overexpression of FOXQ1 influenced cell cycle arrest and induced cell death in contrast to other TFs such as FKHR-L1, FOXOs, Sp1 and SIRT1 (Field, Tsai et al. 1996, Dijkers, Birkenkamp et al. 2002, Deniaud, Baguet et al. 2006, Wang, Chen et al. 2006). To particularly analyse the effect of FOXQ1 activity, I generated cell lines (A549_pSlik_FOXQ1-OE, MCF7_pSlik_FOXQ1-OE and MDA157_pSlik_FOXQ1-OE) overexpressing a doxycycline inducible form of FOXQ1-Flag (Fig. 14). Finally, all the infected cell lines robust the doxycycline dependent controlled expression of FOXQ1. Although, the FOXQ1-mediated ferroptosis effect was stronger in the cells expressing stable FOXQ1 (A549-FOXQ1-OE) compared to doxycycline dependent controlled expression (A549_pSlik_FOXQ1-OE). On the other hand, in MCF7_pSlik_FOXQ1-OE and MDA157RSL3_pSlik_FOXQ1-OE the RSL3-induced ferroptosis effect was slightly suppressed. However, our results indicate that the mechanism by which FOXQ1 overexpression halt cell proliferation in breast cancer cells appears to be distinct from the

ones with previously described TFs. Therefore, further investigations using the inducible form of FOXQ1-Flag will be required to determine the underlying mechanism and molecular targets involved.

5.3. *FOXQ1*-KO induces lethal effect

Gene knockout (KO) is a critical and irreversible method for identifying the functions and impact of that particular gene (Doudna and Sontheimer 2014, Ishibashi, Saga et al. 2020). A limited knowledge and yet no knockout model has been published regarding the pathological functions of FOXQ1. FOXQ1 has been involved in cell cycle regulation, cell signalling, and tumorigenesis (Pei, Wang et al. 2015, Zhang, Wang et al. 2016). Several studies have reported that inhibition of FOXQ1 induces cell death (Pei, Wang et al. 2015, Zhang, Ma et al. 2015, Zhang, Wang et al. 2016). I applied CRISPR/Cas9 sgRNA dependent technology to introduce genetic interruption of FOXQ1 in MEFs, in order to establish a model that should be used for further in vitro functional studies (Cong, Ran et al. 2013, Mali, Yang et al. 2013).

For this investigations, the first strategy was to apply sgRNA for targeting FOXQ1. For this purpose I designed two individual sgRNAs with least potential off-target effects to knockout the single-exon *FOXQ1* gene. I applied lentiCRISPR cloned sgRNAs on both Pfa1 and Pfa1-*ACSL4*-KO cell lines to target *FOXQ1* (Fig. 15B, C) (Doll, Proneth et al. 2017). As FOXQ1 was identified in CRISPR/cas9 screen of Pfa1-*ACSL4*-KO in the presence of AA, so to avoid misconception, I also applied *FOXQ1*-sgRNAs into the cells supplemented with AA (Fig. 15B, C). Cells infected with *FOXQ1*-sgRNAs, continued with selection at a constant concentration of specific antibiotic, stopped proliferation compared to control and lost cell viability after 3 days. Yet the observed results were contrary to our expectations from the screen (Fig. 15), but still very interesting because no FOXQ1 null model has been published so far. A similar protocol was followed for human cells as well. Knocking out *FOXQ1* halted cell proliferation and inhibited cell viability (Fig 17B, C). Taken together, it can be hypothesized, that a lacking of FOXQ1 induces cell cycle arrest and cell death. Additionally, apoptosis inhibitor zVAD and ferroptotic cell death inhibitor Lip-1 failed to rescue for long time (Li, Yao et al. 2019, Fan, Pang et al. 2021).

It is known that TFs regulates important developments in the cells, therefore the deletion of a particular TF exhibits profound impact in the cells (Huilgol, Venkataramani et al. 2019, Goos, Kinnunen et al. 2022). In many cases, the absence of even a single Fox TF is lethal (Frischer, Hagen et al. 1986). Others have demonstrated that, FOX proteins play pleiotropic roles in cell cycle control, proliferation, cell differentiation and various key signalling pathways. Deregulation of any FOX gene has consequences in cancer (Lee and Young 2013, Todeschini, Georges et al. 2014, Bhagwat and Vakoc 2015, Vishnubalaji, Hamam et al. 2016, Laissue 2019). However, the particular mechanism of FOX family deregulations seems to be context dependent in cancer (Jiramongkol and Lam 2020). I found for the first time, that FOXQ1 depletions arrest G1/S phase in both MEFs and human cells (Fig. 15D). Flow cytometry analysis revealed that *FOXQ1* targeted with *FOXQ1*-sgRNA contained high number of analysed cells in S phase as compared to control cells.

A similar effect has been reported with analysis of FOXM1. FOXM1 an another member of FOX proteins which controls cell cycle regulations, and its depletion induces apoptosis (Laoukili, Stahl et al. 2007). For cells with FOXM1 deficiency reduced expression of genes that are potent for cell cycle regulation has been reported. Suppression of FOXM1 dependent genes resulted in G2-arrest and cell death (Wang, Chen et al. 2005, Wonsey and Follettie 2005, Laoukili, Stahl et al. 2007, Vishnubalaji, Hamam et al. 2016). Moreover, both FOXO and FOXM1 TFs are key drivers of the cell cycle, by controlling the expression of cell cycle genes, and therefore acting as direct targets for CDK/cyclin complexes (Laoukili, Stahl et al. 2007, Ho, Myatt et al. 2008, Marais, Ji et al. 2010, Palmer and Kaldis 2020). Other studies have already indicated the role of FOXQ1 knockdown both in *in vitro* and *in vivo* experiments. According to these investigations, FOXQ1 inhibition reduces cell proliferation, migration and reduces xenograft tumor growth and angiogenesis in CRC cells (Kaneda, Arao et al. 2010, Weng, Okugawa et al. 2016, Tang, Zheng et al. 2020).

Form these identifications I hypothesized that targeting FOXQ1 may arrest cell cycle or it may impact the expression of partner protein that is essential for cell proliferation (Fig. 16A). Therefore, to nullify FOXQ1 and to analyse the impact of FOXQ1 depleted on cell cycle regulatory genes, I infected human cell lines both WT and FOXQ1-OE with FOXQ1-sgRNA (Fig. 16B, C). As expected, cell proliferation was inhibited and cell viability was lost after few days of the infection and continued antibiotic selection. Though the cell death ratio was slightly

different across the cells lines, probably because of the different cell proliferation rate of each cell line. Interestingly cell cycle regulatory genes CDKs were downregulated in cells infected with FOXQ1-sgRNA in an earlier investigation (Lim and Kaldis 2013).

Similar regulatory effects of FOXO gene in cancer cells has been reported by other studies. Though the effect is highly dependent on cell and tissue context, leading to different activation of genes (Ho, Myatt et al. 2008). For example, deregulated E2F-1 activity gives rise to reduce proliferative and apoptotic signals (Field, Tsai et al. 1996). Cell cycle regulators such as cyclin D1 and CDK2 or CDC2 are the transcriptional targets of E2F and pRB-related proteins (Dalton 1992, Furukawa, Terui et al. 1994). While FOXO is known to cumulate the inhibition of E2F and influence changes in the factors associated with cell cycle transition from a normal state to an oncogenic state (Dalton 1992, Furukawa, Terui et al. 1994, Ramaswamy, Nakamura et al. 2002, Araki, Nakajima et al. 2003). Here**,** I found that FOXQ1 depletion leads to downregulation of CDK4, CDk6 and Cyclin D (Fig. 17), enhancing cell cycle arrest (Fig. 18) and inducing cell death, which is also consistent with the previous observation that FOXQ1 inhibition induced apoptosis (Pei, Wang et al. 2015, Zhang, Ma et al. 2015, Zhang, Wang et al. 2016).

Other findings demonstrated that FOXQ1 expression is essential to maintain cell motility and proliferation in cancer cells. (Gao, Shih Ie et al. 2012). Obtained results from our research suggest that FOXQ1 overexpression mediates ferroptosis in lung cancer cells but its deregulation might be beneficial to inhibit tumour cell growth. So whether FOXQ1 interaction directly or indirectly thereby has a negative effect on the expression of CDKs requires further investigation. The ability to genetically impair FOXQ1 or to design pharmacological compounds that manipulate FOXQ1 protein might have beneficial therapeutic effects in the treatment of diseases.

5.4. FOXQ1 mediates EGFR signalling pathway

This study was performed via using high-throughput RNA-Seq technology to analyse variations in the expression profile of ferroptosis related genes and to identify genes that were differentially expressed (p-value ≤ 0.05) in FOXQ1-OE cells in comparison to control (Mock) cells (Fig 20). RNA-seq offers the possibility to identify unknown transcripts (Wang, Gerstein et al. 2009, Martin and Wang 2011). Based on RNA-Seq analysis, we identified the signature

of the 300 ferroptosis related genes that are differentially expressed in multiple cancer cell lines. In FOXQ1-OE cells, we identified important genes and their functional interactions and how they were associated with the FOXQ1 transcription factor (Carlsson and Mahlapuu 2002, Myatt and Lam 2007, Li, Zhang et al. 2016, Bach, Long et al. 2018). Interestingly, our analysis revealed significant overrepresentation of genes and pathways relevant to cellular metabolic and metabolism process, cellular response to stress, cell cycle regulation and cell death (Fig. 19B, C).

We further analysed the regulation of genes involved in pathways related to oxidative stress, cell cycle regulation and protein/lipid metabolism (Fig. 19). In accordance with these findings, we observed 300 DEGs regulated mutually in all five cancer cell lines. After all, it is worth to mention that 18 top upregulated DEGs could represent a compensatory mechanism mediated by FOXQ1 (Fig. 19B). Iron plays an essential role in several biological processes such as cellular proliferation, cell cycle regulation and DNA repair, while iron deficiency induces G1/S arrest (Kulp, Green et al. 1996, Huang 2003, Nurtjahja-Tjendraputra, Fu et al. 2007, Zhang 2014). Numerous proteins are involved in DNA repair that utilise iron as a cofactor (Zhang 2014). Ferritin is a ubiquitous intracellular iron storage polymeric protein that consists of two distinct subunits of the heavy and light type (Harrison and Arosio 1996, MacKenzie, Iwasaki et al. 2008, Wang and Pantopoulos 2011, Arosio, Elia et al. 2017). Upon hierarchical clustering analysis of 18 upregulated DEGs in human cell lines overexpressing FOXQ1, ferritin heavy chain 1 (FTH1) and ferritin light chain (FTL) both are the key subunits of ferritin and are significantly upregulated in the FOXQ1-OE cells (Fig. 19B) (Hu, Zhou et al. 2021).

Studies have shown that increased expression of ferritin suppresses ferroptosis, while its degradation increases the intracellular iron level that results in ROS production via Fenton reaction (Gkouvatsos, Papanikolaou et al. 2012, Gao, Monian et al. 2015, Hou, Xie et al. 2016). FTL as cofactor of iron metabolism plays a crucial role in ferroptosis as well as in apoptosis (Fan, Yamada et al. 2009, Wang, Qiu et al. 2021). Transcriptional inhibition or gene silencing of *FTL* effectively suppressed proliferation and increased the sensitivity of cancer cells to ferroptosis (Li, Cao et al. 2020, Kambara, Amatya et al. 2022, Ke, Wang et al. 2022). Further, the results of in vivo studies suggest that FTH1 depletion leads to iron over absorption and induction of ferroptosis (Tao, Wu et al. 2014, Sun, Ou et al. 2016). However, other studies have shown a contrary effect of FTH1. A study showed that FTH1 overexpression may

compensate oxidative damage by preventing mitochondrial dysfunction and suppressing erastin-induced ferroptosis (Urrutia, Aguirre et al. 2017, Du, Wang et al. 2019, Feng, Schorpp et al. 2020, Pedrera, Espiritu et al. 2021). In our study, FOXQ1 overexpression significantly increased the expression of both FTL and FTH1 (Fig. 19C). Though the underlying mechanism remains to be uncovered, these findings point towards a context-dependent cell cycle regulation via FOXQ1 in different cell lines. TF-regulated expression of ferritin subunits has been also outlined by other studies. NRF2, a key transcription factor responsible for balancing the oxidative stress, is known to be required for regulating the transcription of both FTH1 and FTL. However, the specific role of both subunits in ferroptosis needs to be further investigated (Pietsch, Chan et al. 2003, Kensler, Wakabayashi et al. 2007, Hou, Xie et al. 2016, Kasai, Mimura et al. 2018, Kerins and Ooi 2018, Dodson, Castro-Portuguez et al. 2019, Anandhan, Dodson et al. 2020, Jaganjac, Milkovic et al. 2020).

Finally, a number of genes were analysed via immunoblotting to better understand the process of gene regulation (Fig. 20A). Genes involved in ferroptosis and cell cycle process were predicated as the most differentially regulated in all cell lines. Further, we identified in the volcano plot a number of highly regulated ferroptosis-associated genes (Fig. 20B-E). The top and mutually regulated genes like GPX4, XCT and AIFM2 are well-known players of ferroptosis which have been verified by our group and others (Friedmann Angeli, Schneider et al. 2014, Doll, Proneth et al. 2017, Doll, Freitas et al. 2019, Ma and Zhang 2019, Dai, Zhang et al. 2020, Song, Wang et al. 2020, Ma, Du et al. 2022, Wang, Zhang et al. 2022). Notably, protein level and mRNA expression of the aforementioned genes were not always consistent in the analysed cell lines, a possible speculation can be that gene expression is subject to post-translational regulation (Landini, Trbojevic-Akmacic et al. 2022).

Additionally, the GO and enrichment pathway analysis on functionally grouped modules in FOXQ1-OE cells revealed that the upregulated genes were mainly involved in cell receptors, response to cytokines, and cell cycle arrest (Fig. 21). Genes most strongly regulated in overexpressed cells were found to be key drivers involved in the differential regulation of the EGFR signalling pathway. However, the question arises how FOXQ1 overexpression regulates the cell cycle progression driven by EGFR (Zhu, Liu et al. 2001, Carrasco-Garcia, Saceda et al. 2011, Lim, Jeon et al. 2015, Jackson and Ceresa 2016). It is reasonable to assume that genes regulated via FOXQ1 within a cell may require a rapid and flexible strategy of diversifying the

response to the differential expression. Our analysis demonstrated how much outline can be generated as FOXQ1 overexpression regulates EGFR signalling pathway. Many studies have reported that in different cancer cells the regulatory function of EGFR changes from cell proliferative to growth inhibitory and apoptotic effects in metastatic cancer (Lim, Jeon et al. 2015, Jackson and Ceresa 2016). Others have reported that DNA damage triggers EGFR signalling, which in turn induces p53 pathway and arrests cell cycle to modulate DNA repair (Ali, Brown et al. 2018, Volman, Hefetz et al. 2022). Inhibition of EGFR mostly causes cell cycle arrest in G1 prior to DNA repair (Zhu, Liu et al. 2001, Carrasco-Garcia, Saceda et al. 2011).

To analyse the cell proliferative effect of EGFR in multiple cancer cells, I used tyrosine kinase inhibitor erlotinib (Schettino, Bareschino et al. 2008) (Fig. 22). Erlotinib has been identified to inhibit EGFR, activate cell death pathway, and induce apoptotic cell death (Ling, Lin et al. 2008, Dermawan, Gurova et al. 2014, Lee, Noh et al. 2021). From our findings, erlotinib treatment did not regulate RSL3-induced ferroptotic cell death in A549 and NCI-H1975 cells. However, it did suppress slightly RSL3-induced cell death in 786-O and U-87 cells (Fig. 22C). Both A549 and H1975 cell lines bear mutated *PIK3CA* and *KRAS,* implying that the cell lines does not rely on EGFR signalling (Li, Chan et al. 2021). Due to the mutation in *KRAS* that is present in this cell line, it could be possible that the cells are not greatly affected by erlotinib (Kobayashi, Ji et al. 2005, Ikediobi, Davies et al. 2006, Godin-Heymann, Bryant et al. 2007, Regales, Gong et al. 2009, Liu, Lee et al. 2012, Matsushima, Ohtsuka et al. 2014). EGFR downstream genes ERK1/2, and mTOR are crucial in different biological processes, including cell proliferation (Wong, Bigner et al. 1987, Brunet, Bonni et al. 1999, Dibble and Cantley 2015). FOXQ1 overexpression upregulates phosphorylation of FAK/PI3K/AKT in HCT116 and LOVO cells, while FOXQ1 depletion lowers FAK/PI3K/AKT phosphorylation (Liu, Wu et al. 2017). Tyrosine kinase inhibitors restrain EGFR phosphorylation, which impacts ERK and AKT phosphorylation, while AKT inhibits FOXO1 activity by phosphorylation (Li, Zhou et al. 2013). Here the question raised whether EGFR inhibition can influence FOXQ1 mediated ferroptosis effect in the experimental cell lines.

Unfortunately, an FOXQ1-meditated effect was not retrieved by the EGFR inhibitor erlotinib in both control and FOXQ1-OE cell lines (Fig. 22D). Though, our obtained results are contrary to FOXO3 activity. Earlier Studies have shown that EGFR pathway downregulates FOXO3 activity leading to cell cycle arrest, indicating that EFGR-based cell proliferation is dependent

on the loss of FOXO3 activity (Sunters, Madureira et al. 2006, Zeng, Samudio et al. 2006, Snoeks, Weber et al. 2008, Snoeks, Weber et al. 2009). But another study has reported, similar to our findings, that erlotinib does not inhibit cell growth in both A549 and H1975 cells (Kobayashi, Ji et al. 2005, Godin-Heymann, Bryant et al. 2007, Regales, Gong et al. 2009, Matsushima, Ohtsuka et al. 2014). Our present work discovered that FOXQ1 expression represents one of the main factors determining EGFR-based cell proliferation. However, the observations of our experiments were slightly variant. Possibly the cell type-specific proliferative rate or experimental conditions influenced the results, therefore, further investigations in different cell lines are required to authenticate our findings.

Understanding the mechanisms underlying FOXQ1-mediated ferroptosis and its implications for the treatment of cancer can provide insights for the identification of new therapeutic targets, e.g. for the treatment of cancer. Future experimental validation should be aimed at identifying conditions as well as biological processes in which the FOXQ1 and EGFR pathway is involved. In order to validate RNA-Seq results, I used qPCR analysis (Yuan, Reed et al. 2006, Li, Zhang et al. 2019). To conform the expression profile of EGFR signalling pathway from RNA-Seq results, all the downstream genes of EGFR pathway were analysed from both control and FOXQ1-OE cells. Expression profiles of the selected genes acquired by qPCR were consistent with expression patterns identified by RNA-Seq. Hence, the qPCR results can be considered as technical validation of the analysed DEGs. Regardless of the aforementioned results for validations, there are still some limitations in our study. Our RNA-Seq analyses are not free from potential random errors and false positive DEGs. The results of our data, particularly on FTL/FTH1, are based on bioinformatics prediction without subsequent experimental validation. Therefore, further studies and practical approaches are needed to experimentally prove our findings in more detail.

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AFFIDAVIT

I hereby declare that the dissertation titled “Identifying Forkhead Box Q1 as a novel regulator of ferroptosis” is my own, original work undertaken in fulfilment of the requirements for the doctoral degree. I have made no use of sources, materials or assistance other than those specified in my thesis.

I have not employed the services of an organization that provides dissertation supervisors in return for payment or that fulfils, in whole or in part, the obligations incumbent on me in connection with my dissertation.

I have not submitted the dissertation, either in the present or in a similar form, as part of another examination process.

I have not yet been awarded the desired doctoral degree nor have I failed the last possible attempt to obtain the desired degree in a previous doctoral program.

I am familiar with the public available Regulations for the Award of Doctoral Degrees of the Medical Faculty at the University of Rostock. I am also aware of the consequences of filing a false affidavit.

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ACKNOWLEDGEMENT

First and foremost, I want to express my deepest appreciation to my project supervisors Prof. Dr. med. Alexander Storch, Director of the Department of Neurology at Rostock University Medical Center, and Prof. Dr. med. Uwe Walter, Deputy Director of the Department of Neurology, for giving me the opportunity to do my PhD studies. Their unwavering guidance, invaluable insights, and constant encouragement throughout the entire research process have been instrumental in shaping this work. This PhD student project was mainly funded by the European Union within the European Innovation Council Pathfinder III program "Network of Centres of Excellence in Neurodegeneration (CoEN) 2017", as part of the PRION-IRON consortial project (lead investigator: Prof. Dr. David Devos, Lille, France), partnered by the Deutsches Zentrum für Neurodegenerative Erkrankungen (DZNE; grant No. CoEN4007 raised by Prof. Storch and Prof. Walter). My doctoral thesis advisor, Prof. Dr. Uwe Walter, provided me with additional financial support through his third-party funds.

I am immensely grateful to my lab supervisor Dr. rer. nat. Marcus Conrad, Director of the Institute for Metabolism and Cell death (MCD), Helmholtz Center Munich, for his excellent guidance and outstanding support. His dynamism, vision, sincerity and motivation have inspired me a lot. His way of motivating people is invigoration and set a very enjoyable work atmosphere.

I am very thankful to Dr. rer. nat. Sebastian Doll (same institute), for his supervision during my research and thesis. It was a great privilege to work and study under his guidance. I am grateful for what he has offered me. He was a fountain of inspiration in discussions and I envy his perception of science. His reading and critique of my thesis draft is greatly appreciated.

Most importantly, I thank Dr. Dietrich Trümbach for helping with the RNA-Seq data analysis. His expertise and collaboration has been instrumental in the success of this research.

I would like to express special thanks to my colleagues at MCD Dr. Bettina Proneth, Dr. Katalin Buday, Svenja Lorenz and all other lab members who have offered their support, engaged in meaningful discussion, and provided a stimulating academic environment that has positively influenced my research.

I am very thankful to Prof. Dr. Andreas Hermann, Director of the Translational Neurodegeneration Section "Albrecht-Kossel", Department of Neurology, Rostock University Medical Center, who made way for the thesis writing during my current appointment in his research lab.

Most importantly, I deeply thank my friends and family particularly my elder brother for their love, caring, all the sacrifices for my education and supporting every step of this journey. Their belief in my abilities and their constant motivation have set the stage for my scientific carrier. I love you all!

Finally, I would like to acknowledge the countless individuals who participated in this study, providing their time, insights, and cooperation. Their collective efforts have played an integral role in the completion of this thesis, and I am grateful for their assistance.

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In Preparation	Downstream effect of FOXQ1 on Gene Expression Impaired in Cancer Cells M. Ismail, S. Doll, B. Proneth, A. Storch, U. Walter, D. Trümbach, M. Conrad
Conference	ENCALS meeting 2023, Bercelona, Spin, 11 – 15 July 2023. Schilling Symposium, Universität Rostock, Germany, March 14th 2023 Scientific Illustration Workshop Munich, 14th - 15th September 2018. 27th Conference of the European Cell Death Organization in Dresden, Germany, Sep 25 – 27 2018. 9th Student Symposium on Molecular Medicine on the topic of Perspectives of Trauma Research.
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