

**Development of novel *in vitro* and *in vivo* models for
determining primary events in HLRCC tumourigenesis**

A thesis submitted for the degree
of
Doctor of Philosophy in Clinical Medicine
Hilary Term 2012

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Abstract**Development of novel *in vitro* and *in vivo* models for determining primary events in HLRCC tumourigenesis**

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 Thesis submitted for degree of Doctor of Philosophy
 Nuffield Department of Clinical Medicine, University of Oxford
 Hilary Term 2012

Germline mutations of *fumarate hydratase* (FH), encoding an enzyme of the tricarboxylic acid (TCA) cycle, predispose affected individuals to hereditary leiomyomatosis and renal cell cancer (HLRCC). FH-deficient cells and tissues have been shown to accumulate fumarate, exhibit S-(2-succinyl) cysteine (2SC) protein modifications and to constitutively express hypoxia-inducible factor alpha (HIF-1 α and -2 α), under normoxic conditions. This thesis presents a phenotypic characterisation of Fh1^{-/-} mouse embryonic fibroblasts (MEFs), generated from previously reported conditional *Fh1* knockout mice, as a new *in vitro* system for investigating and identifying biochemical and metabolic pathways that are dysregulated as a result of Fh1 inactivation. These cell lines reproduced the aforementioned phenotypes, in addition to an observed shift from oxidative phosphorylation (OXPHOS) to glycolytic metabolism. Re-expression of either full length, mitochondrial-targeted FH (Fh1^{-/-} +FH) or cytoplasmic FH (Fh1^{-/-} +FH Δ MTS) in Fh1-deficient MEFs was sufficient to reduce intracellular fumarate and to correct for the dysregulation of the Hif pathway. These results were of particular interest as they demonstrated that normoxic stabilisation of Hif-1 α occurs independently of the persistent mitochondrial defect observed in Fh1^{-/-} +FH Δ MTS MEFs. These findings were corroborated *in vivo* following the development of transgenic mouse models, ubiquitously expressing either FH or FH Δ MTS in mice with targeted inactivation of Fh1 in renal tubular cells. Surprisingly, the cytoplasmic-restricted FH (FH Δ MTS) transgene was just as efficient as the transgenic mice expressing mitochondrial-targeted FH at rescuing the cystic phenotype associated with Fh1-deficiency in the kidneys. As the function of cytoplasmic FH has remained poorly understood, these results go some way to extricating a role for this isoform of FH. The results of this thesis demonstrate that these novel *in vitro* and *in vivo* models, used either alone or in combination, are a versatile and robust paradigm for studying altered cell metabolism in not only HLRCC but other diseases associated with metabolic dysregulation.

Declaration

I hereby declare that the thesis that I am submitting is wholly my own work, with the exception of those experiments acknowledged, both in the text and figure legends, to have been carried out by others.

Acknowledgements

In loving memory of my friend

Grace Fanning

11th April 1968-28th July 2011

Forever to be missed, but never to be forgotten

I dedicate my thesis not only to Grace but to my loving family and fiancé, Mark. I would like to take this opportunity to thank my parents, John and Mairéad, for their unfailing support and love. Dad, I am eternally grateful to you for your guidance and for your example of how to remain strong during challenging times. Mom, words cannot describe how inspired I am by you; thank you for grounding me all those times when I felt that I had lost my way. I thank my dear sister and travel companion, Aisling, for her wisdom, support and kindness, but most of all for her friendship and love. I would also like to thank my loving brother, Eamonn, for sharing his infectious enthusiasm for life and for always being there for me, cheering me on when I needed reminding of who I am and what I can achieve. I am especially grateful to Mark, my fiancé and best friend, for keeping me healthy and happy during my write up and for his love, patience and support. You are my rock in the storm; the butter to my bread and the breath to my life.

Many thanks to Dr. Patrick Pollard for his help and guidance over the past few years. Thanks also to Prof. Peter Ratcliffe for his supervisory input. I am grateful to you both for your support and the opportunity you bestowed upon me to work on such an interesting project. I would like to extend a special thanks to Dr. Julie Adam for being an amazing role model and for her patience and ability to challenge me, whilst

nurturing my confidence and scientific skills. I am truly grateful to have worked with such an inspirational scientist. To all the members of the Ratcliffe lab, past and present, thank you for your camaraderie and scientific input; especially to Norma for her fantastic sense of humour and to both Norma and Lina for kindly giving me a place to stay during my final experiments. Thanks also goes to those whom I have collaborated with over the years: Lisa Heather, Emma Carter, Alexander Zhdanov, Dmitri Papkovsky, Ben Davis, Houman Ashrafian, Emanuela Volpi, Violetta Steeples and Chiara Bardella - I am grateful to have learned from your example. I am also grateful to Sarah Noujaim for her support and cheerful nature and Ruth Porter for her IT skills.

I would also like to thank my friends (Aisling, Aileen, Bryon, Eileen, Emine, Fergal, Kate, Ita, Julie-Ann, Margo, Sarah, Lindsey, Michael, Sara, Simon, Matt and everyone else) for keeping me sane and for making sure that I never lost touch with my fun side. A special thank you goes out to David, for being so obliging when I bombarded him with chemistry questions, the Fanning family and also the extended Scarrott family (and future in-laws) for their support.

Finally, I would like to thank the Nuffield Department of Medicine, the Medical Research Council, Cancer Research UK and the Wellcome Trust for funding the research outlined in this thesis.

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Abbreviations

µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar
¹ H-MRS	¹ H-magnetic resonance spectroscopy
2-FDG	2-(¹⁸ F)-fluoro-2-deoxy-D-glucose
2HG	2-hydroxyglutarate
2n	Diploid
2-OG	2-oxoglutarate
2SC	S-(2-succinyl) cysteine
3-NPA	3-nitropropionic acid
4n	Tetraploid
6n	Hexaploid
8n	Octaploid
Å	Ångströms
Acetyl-CoA	Acetyl coenzyme A
Ad	Adenovirus
ADP	Adenosine 5'-diphosphate
ALKBH	Human homologue of AlkB
AMP	Adenosine 5'-monophosphate
AMPK	AMP-activated protein kinase
APKD	Autosomal polycystic kidney disease
APS	Ammonium persulphate
ARNT	Aryl hydrocarbon receptor nuclear translocator
A-site	Catalytic binding site A
ATP	Adenosine 5'-triphosphate
BAD	BCL2-antagonist of cell death
Bcl-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large

BHD	Birt-Hogg-Dubé
bHLH	Basic-helix-loop-helix
Bnip3	BCL2/adenovirus E1B 19kDa protein-interacting protein 3
Bp	Base pair
BSA	Bovine serum albumin
B-site	Catalytic binding site B
CAD	C-terminal transactivation domain
CAG	Chicken β -actin promoter/CMV early enhancer
CBP	cAMP response element-binding (CREB) binding protein
CCD	Charge-coupled device
CCRCC	Clear cell RCC
cDNA	Complimentary DNA
CDRCC	Collecting duct RCC
CH1	Cysteine/histidine rich-1
CLM	Cutaneous leiomyomata
CMV	Cytomegalovirus
CO ₂	Carbon Dioxide
CoASH	Coenzyme A
CRCC	Chromophobe RCC
C _T	Cycle threshold
C-terminal	Carboxyl-terminal
Cul2	Cullin-2
Cys	Cysteine
DAB	3, 3'-diaminobenzidine
Da	Daltons
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide

DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ECM	Extracellular matrix
ECMV	Encephalomyocarditis virus
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
<i>Egln3</i>	Egl nine homolog 3
EGTA	Ethylene glycol-bis(2-aminoethylether)-N. N, N', N' tetra acetic acid
EPAS1	Endothelial PAS domain protein 1
<i>EPO</i>	Erythropoietin
ER	Endoplasmic reticulum
Erk1/2	Extracellular-signal-regulated kinases 1/2
ES cells	Embryonic stem cells
ETC	Electron transport chain
EtOH	Ethanol
F ₁ F ₀ Complex	ATP Synthase
FACS	Fluorescence-activated cell sorting
FADH ₂	Flavin adenine dinucleotide, reduced form
FBS	Foetal bovine serum
FCCP	Carbonylcyanide-p-trifluoromethoxyphenylhydrazone
Fe ²⁺	Ferrous iron
Fh1	Murine orthologue fumarate hydratase
FHD	Fumarate hydratase deficiency
FH	Fumarate Hydratase
FIH	<u>F</u> actor <u>i</u> nhibiting <u>H</u> IF
FITC	Fluorescein isothiocyanate
<i>FLCN</i>	Folliculin
fmole	Femtomole
<i>Fum</i>	Yeast fumarate hydratase
G6P	Glucose-6-phosphate
Gal	Galactose

GFP	Green fluorescent protein
G	Gauge
Glu	Glucose
<i>Glut 1/Slc2a1</i>	Glucose transporter isoform 1
GPX	Glutathione peroxidase
GTP	Guanosine-5'-triphosphate
H&E	Haematoxylin and eosin
H ⁺	Hydrogen cation
H ₂ O ₂	Hydrogen peroxide
H ₂ O	Water
HBSS	Hanks buffered saline solution
HCl	Hydrogen chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hg	Mercury
HIF- α	Hypoxia-inducible factor alpha
<i>HK2</i>	Hexokinase 2
<i>HLRCC</i>	Hereditary leiomyomatosis and renal cell cancer
<i>HMOX1</i>	Haem oxygenase 1
HPRCC	Hereditary PRCC
HRE	Hypoxia-response element
HRP	Horse radish peroxidase
IDH	Isocitrate Dehydrogenase
IHC	Immunohistochemistry/immunohistochemical
IPAS	Inhibitory PAS
IRES	Internal ribosome entry site
IR	Ionizing radiation
IRP1	Iron regulatory protein 1
IRP2	Iron regulatory protein 2
ITS solution	Insulin, transferrin and sodium selenite solution
JmjC	Jumonji domain-containing
JMJD	Jumonji domain
KCl	Potassium chloride
kDa	Kilo Daltons

KEAP1	Kelch-like ECH-associated protein 1
Keap1	Murine kelch-like ECH-associated protein 1
KH ₂ PO ₄	Potassium dihydrogen phosphate
K _i	Dissociation constant
K _M	Michaelis constant
KOH	Potassium hydroxide
Ksp1.3/Cre	Kidney-specific cadherin (Cadherin 16) promoter
LDHA	Lactate dehydrogenase A
Ldha	Murine lactate dehydrogenase, isozyme a
Ldhb	Murine lactate dehydrogenase, isozyme b
<i>LKB1</i>	Liver kinase B1
LOH	Loss of Heterozygosity
mA	Milli Amp
MAPK	Mitogen activated protein kinase
MCUL	Multiple cutaneous and uterine leiomyomata
MEF	Mouse embryonic fibroblast
<i>MET</i>	Hepatocyte growth factor receptor gene
mFISH	Multiplex-fluorescence in situ hybridization
MG132	<i>N</i> -[(phenylmethoxy)carbonyl]- <i>L</i> -leucyl- <i>N</i> -[(1 <i>S</i>)-1-formyl-3-methylbutyl]- <i>L</i> -leucinamide
mg	Milligram
MLC2v	Cardiomyocyte-specific promoter of myosin light chain
mM	Millimolar
MOI	Multiplicity of infection
MOPS	Morpholino propanesulfonic acid
MPP	Mitochondrial processing peptidase
mRNA	Messenger RNA
MS	Mass spectrometry
MTS	Mitochondrial targeting sequence
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADPH	Reduced NADP

NADP	Nicotinamide adenine dinucleotide phosphate
NaOHEt	Sodium ethoxide
NF2	Neurofibromatosis 2
ng	Nanogram
Nm	Nanometer
nm	Nanomolar
Nqo1	NAD(P)H dehydrogenase quinone 1
Nrf2	Murine nuclear factor (erythroid-derived 2)-like 2
NRF2	Nuclear factor (erythroid-derived 2)-like 2
N-terminal	Amino-terminal
O ₂	Dioxygen
O ₂ ⁻	Superoxide
ODDD	Oxygen-dependent degradation domain
OD	Optical density
OH [·]	Hydroxyl free radical
ORF	Open reading frame
OXPHOS	Oxidative phosphorylation
P/O ratio	Ratio of phosphate incorporation into ATP per dioxygen molecules reduced to water
P3H	Collagen prolyl 3-hydroxylase
P4H	Collagen prolyl 4-hydroxylase
p53	Protein 53
PAGE	Polyacrylamide gel electrophoresis
PAS	<u>Per-Arnt-single-minded-protein</u>
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
<i>Pdk1</i>	Pyruvate dehydrogenase kinase isoenzyme 1
pEF	Eukaryotic translation elongation factor 1 alpha 1 promoter
PEI	Polyethylenimine
PET	Positron emission tomography
PFK1	Phosphofructokinase 1
PFKBP3	6-phosphofructo-2-kinase/bisphosphatase 3

PFU	Plaque-forming unit
PGM	Phosphoglycerate mutase
PHD	<u>P</u> rolyl <u>h</u> ydroxylase <u>d</u> omain
PI3-kinase	Phosphoinositide 3-kinase
<i>PKD1</i>	Polycystic kidney disease gene 1
<i>PKD2</i>	Polycystic kidney disease gene 2
PK-M1	Pyruvate kinase isozyme 1
PK-M2	Pyruvate kinase isozyme 2
PK	Pyruvate kinase
PRCC	Papillary RCC
pS6K	Phospho-p70-S6 kinase
pVHL	VHL protein
Q-PCR	Reverse-transcription quantitative PCR
Q	Ubiquinone
RB/RB1	Retinoblastoma
Rbx	RING-box protein
RCC	Renal cell cancer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Revolutions per minute
rtTA	Reverse tTA
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>SDHA</i>	Succinate dehydrogenase complex, subunit A
<i>SDHB</i>	Succinate dehydrogenase complex, subunit B
<i>SDHC</i>	Succinate dehydrogenase complex, subunit C
<i>SDHD</i>	Succinate dehydrogenase complex, subunit D
SDH	Succinate Dehydrogenase
SDS	Sodium dodecyl sulphate
S.D.	Standard deviation
SEM	Standard error of the mean
Seq	Sequence
S.E.	Standard error
shRNA	Small hairpin RNA

siRNA	Small inhibitory RNA
SOD	Superoxide dismutase
SSC	Saline-sodium citrate
SV40	Simian virus 40
TCA cycle	Tricarboxylic acid cycle
TEMED	N, N, N', N'-Tetramethylethylenediamine
TE	Tris/EDTA
TetR	Tetracycline repressor
TF	Transferrin
Tris	<i>Tris</i> -(hydroxymethyl)aminomethane
<i>TSC1</i>	Tuberous sclerosis gene 1
<i>TSC2</i>	Tuberous sclerosis gene 2
tTA	Tetracycline-controlled transactivator
TU	Transduction units
UAS	Upstream activator sequences
ULM	Uterine leiomyomata
UPR	Unfolded protein response
UTR	Untranslated region
UV	Ultraviolet
v/v	Volume for volume
<i>VEGFA</i>	Vascular endothelial growth factor A
<i>VHL</i>	von Hippel Lindau
V	Volt
w/v	Weight per volume

1 Chapter 1 Introduction

1.1 Fumarate Hydratase

Fumarate hydratase (FH), also referred to as fumarase, is a homotetrameric enzyme which catalyses the hydration of fumarate to malate in the tricarboxylic acid (TCA) cycle and is also involved in amino acid metabolism (Salway 1999 ; Nelson 2004). FH is highly conserved between species (Table 1.1).

Located on chromosome 1 band q42.1 (Craig et al. 1976; Launonen et al. 2001; Tomlinson et al. 2002) this nuclear-encoded gene consists of 10 exons, including the mitochondrial targeting sequence (MTS) which is encoded by exon 1 (also referred to in the literature as exon 0) (Nielsen et al. 1997). The protein product of *FH* is a 55 kDa subunit comprising of 510 amino acids. The active FH enzyme consists of a 220kDa homotetrameric protein that is both iron-independent and thermo-stable (Woods et al. 1988). There are two isoforms of FH. Both isoforms are transcribed from the same nuclear-encoded gene but are processed such that one isoform is targeted to the mitochondria whereas the other isoform is restricted to the cytoplasm. This common occurrence of single gene translation, but different subcellular localisation of the resulting protein, has been well documented and is referred to as dual localisation (Karniely and Pines 2005).

Dual localisation has been reported to be characteristic of a number of mitochondrial proteins (Dinur-Mills et al. 2008; Yogev and Pines 2011). Mitochondrial FH is targeted to the mitochondrion by an amino (N)-terminal mitochondrial targeting sequence (MTS; approximately 129 bp) wherein it functions as an enzyme of the TCA cycle. Cleavage of the MTS region restricts FH to the cytoplasmic compartment,

where it is involved in amino acid metabolism, scavenging fumarate from the urea cycle and converting cytoplasmic fumarate to malate (Ravdin and Crandall 1951; Ratner et al. 1953; Edwards and Hopkinson 1979; Akiba et al. 1984; Salway 1999 ; Nelson 2004). Moreover, a recent study of cytoplasmic fumarase in *Saccharomyces cerevisiae* (*S. cerevisiae*) has suggested that cytoplasmic fumarase translocates to the nucleus following DNA damage, thus changing the nuclear concentration of fumarate and activating the DNA damage response (Yogev et al. 2010). The exact mechanism through which human FH is localised to the cytoplasm remains to be determined, however yeast studies of the localisation of fumarase have proposed the following mechanisms: changes in targeting sequence accessibility (affecting protein folding); reverse translocation (protein is targeted to the mitochondria, processed by mitochondrial processing peptidase (MPP) after which a small population of the molecules are translocated back to the cytoplasm); availability of cytoplasmic or mitochondrial chaperones and correlation between the pattern of distribution and the rate of fumarase import into the mitochondria (Knox et al. 1998; Sass et al. 2003; Karniely and Pines 2005; Karniely et al. 2006; Yogev et al. 2011; Yogev and Pines 2011). It has also been reported that increased concentrations of succinate or inactivation of genes involved in the glyoxylate shunt (an anabolic pathway which produces malate from glyoxylate and acetyl-CoA.; not present in mammals) in yeast promotes localisation of fumarase to mitochondria, although the mechanism remains unknown (Regev-Rudzki et al. 2009). Establishing the mechanism(s) responsible for targeting FH to either the cytoplasmic or mitochondrial subcellular compartments is of importance as it will help to elucidate better the function of FH in diseases associated with FH-deficiency.

Homozygous or compound heterozygous *FH* mutations (inherited in an autosomal recessive manner) predispose affected individuals to a condition known as fumarate hydratase deficiency (FHD) which is characterised by severe neurological impairments (Gellera et al. 1990; Elpeleg et al. 1992; Bourgeron et al. 1994). In the event that individuals with heterozygous germline *FH* mutations acquire a mutation in the remaining wildtype allele (referred to as the Knudson ‘two hit’ hypothesis (Knudson 1971)) they are predisposed to a rare and aggressive form of cancer known as hereditary leiomyomatosis and renal cell cancer (HLRCC) (Kiuru et al. 2001; Launonen et al. 2001; Tomlinson et al. 2002; Toro et al. 2003). *FH* is therefore described as a tumour suppressor gene, although the exact mechanism through which it carries out this function has yet to be determined. The role of the FH enzyme in cancer and disease will be described in greater detail in section 1.1.

Table 1.1 Pairwise Alignment of FH(from HomoloGene, www.ncbi.nlm.nih.gov/homologene)

Species	Gene	Identity (%)	
		Protein	DNA
H.sapiens	FH		
vs. P.troglodytes	FH	99.4	99.3
vs. C.lupus	FH	95.7	91.6
vs. B.taurus	FH	94.7	91.2
vs. M.musculus	Fh1	93.5	87.6
vs. R.norvegicus	Fh1	93.1	85.7
vs. G.gallus	FH	87.6	79.2
vs. D.rerio	fh	82.7	71.0
vs. D.melanogaster	l(1)G0255	77.1	65.7
vs. A.gambiae	AgaP_AGAP001884	78.1	64.6
vs. C.elegans	fum-1	73.7	65.8
vs. S.pombe	fum1	69.2	60.6
vs. S.cerevisiae	FUM1	67.2	61.3
vs. K.lactis	KLLA0D00902g	68.3	63.1
vs. E.gossypii	AGOS_ACR013C	67.0	58.6
vs. M.grisea	GeneID:2682673	67.8	60.7
vs. N.crassa	NCU10008	67.1	59.0
vs. A.thaliana	AT5G50950	70.4	66.1
vs. A.thaliana	FUM1	69.9	65.8
vs. O.sativa	Os03g0337900	68.9	63.9

1.1.1 FH structure

Crystal structure analysis of *Escherichia coli* (*E. coli*) fumarase highlights three regions important for FH enzyme activity: The central domain, which allows interaction between the four individual monomers and the two carboxylic acid substrate-binding sites, the catalytic binding site A (A site) and the proposed non-

catalytic binding site B (B site) (Weaver et al. 1997; Alam et al. 2005; Picaud et al. 2011). Analysis of the human FH crystal structure (Figure 1.1) revealed that three of the four monomers contribute to the formation of the FH A site which is located ~12 ångströms (Å) away from the B site, the latter of which has been proposed to regulate secondary binding (Picaud et al. 2011). Contrary to previous belief, it has recently been suggested that the B site may in fact have the capacity for catalytic activity and as such may act as an extension of the A site (Picaud et al. 2011). A concavity on the surface of the enzyme (~20Å in depth) forms one of four ‘fumarase folds’ or A/B sites responsible for substrate binding and product release. Each of these A/B sites is lined with fifteen charged residues (10 negatively charged and 5 positively charged) of which seven are conserved throughout the fumarase group of enzymes so far identified (Liang et al. 1998; Rose and Weaver 2004). These residues ensure recognition and presentation of the correct substrate (fumarate) to the active sites thus converting fumarate to malate by facilitating the addition of a hydroxyl group and hydrogen atom to fumarate and the release of H₂O and H⁺. Mutations of A and/or B sites have been shown to disrupt FH enzyme function as is evident by the prevalence of these mutations around highly conserved regions of *FH* (Alam et al. 2005). The impact of FH mutations on cell metabolism and cancer formation will be discussed in further detail in subsequent sections within this chapter.

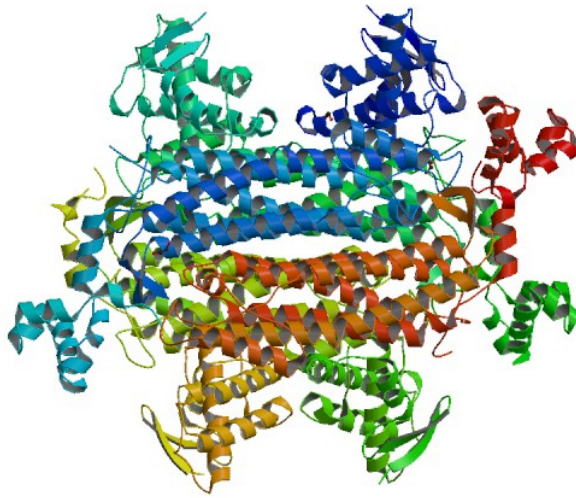


Figure 1.1 FH Structure

Crystal structure of human FH (Protein Data Bank (PDB) ID: 3E04; DOI: 10.2210/pdb3e04/pdb (Kavanagh To Be Published)).

1.1.2 *FH and the Tricarboxylic acid cycle*

The TCA cycle is comprised of a series of oxidation-reduction reactions which at the end of a single cycle produces eight molecules of nicotinamide adenine dinucleotide (NADH + H⁺), two molecules of flavin adenine dinucleotide (FADH₂; hydroquinone form), two molecules of guanosine-5'-triphosphate (GTP) and six molecules of carbon dioxide (CO₂). In addition to the two molecules of NADH + H⁺ and two molecules of adenosine 5'-triphosphate (ATP) produced during glycolysis, aerobic catabolism of one glucose molecule produces thirty eight ATP molecules (Figure 1.3).

This process is an important source of energy, required for facilitating anabolic reactions within the cell. Of the thirty ATP molecules produced by the TCA cycle, four molecules are generated by two molecules of FADH_2 and a further two molecules of ATP are generated by two GTP molecules. The remaining twenty four molecules of ATP are generated by the movement of eight $\text{NADH} + \text{H}^+$ through the electron transport chain (ETC) to O_2 (electron acceptor), resulting in a proton-motive force across the mitochondrial membrane which activates the F_1F_0 complex (ATP synthase) (Salway 1999 ; Lodish 2000; Nelson 2004). These ATP values are calculated using integral (whole number) values, based on the P/O (phosphate incorporation into ATP/dioxygen molecules reduced to water) ratios, which is equivalent to two ATP molecules per molecule FADH_2 and three ATP molecules per molecule of NADH (Salway 1999). GTP is energetically comparable to ATP. In practice however, the net production of ATP is lower than thirty eight molecules due to the translocation of two protons (from NADH and H^+ generated by glycolysis) from the cytoplasm to the mitochondria which results in the dissipation of mitochondrial membrane potential and thus loss of ATP.

Aside from energy production, the TCA cycle contributes metabolic intermediates crucial to pathways such as nucleotide biosynthesis, amino acid metabolism, fatty acid synthesis and the pentose 5'-phosphate pathway (Salway 1999 ; Lodish 2000; Nelson 2004). Under conditions of low oxygen availability a shift from aerobic respiration (i.e. TCA cycle coupled to the ETC and oxidative phosphorylation [OXPHOS; process of generating ATP from oxidation of metabolites]) to anaerobic respiration (i.e. glycolysis only; process of converting glucose to pyruvate) will occur in affected cells. As glycolysis is only capable of producing two ATP molecules

(NADH + H⁺ produced during glycolysis cannot generate ATP under anaerobic conditions), this negatively impacts energy metabolism within the cell and consequently alters the regulation of metabolic pathways that are reliant on TCA cycle intermediates, such as fatty acid biosynthesis and amino acid metabolism. Under these circumstances the cell sustains anabolic processes, such as fatty acid biosynthesis, by utilising the pentose 5'-phosphate pathway (using glucose 6-phosphate from glycolytic metabolism as a substrate) and the catalytic activities of malic enzyme (facilitates cytoplasmic conversion of malate to pyruvate) for their ability to generate NADPH which has the energy equivalent of four molecules ATP (Salway 1999).

Anaerobic conditions are not the only source capable of diminishing TCA cycle activity. Mutations or deleterious modifications of any of the eight enzymes responsible for catalytic conversion of TCA cycle intermediates (Figure 1.2), have been reported as having serious clinical consequences (Rustin et al. 1997). For example, loss of function mutations or deletions of succinate dehydrogenase (*SDH*) or *FH* are associated with rare but distinct tumourigenic phenotypes (discussed in section 1.1) and as such have been classified as tumour suppressors (Baysal et al. 2000; Astuti et al. 2001 ; Launonen et al. 2001; Tomlinson et al. 2002; Lehtonen et al. 2004; Bardella et al. 2011). Loss of function of either of these enzymes prevents the TCA cycle from completing a full cycle beyond the point at which the enzyme is lost, thus resulting in an accumulation of TCA cycle intermediates, in particular the substrates of the mutated/deleted enzyme (i.e. succinate and fumarate).

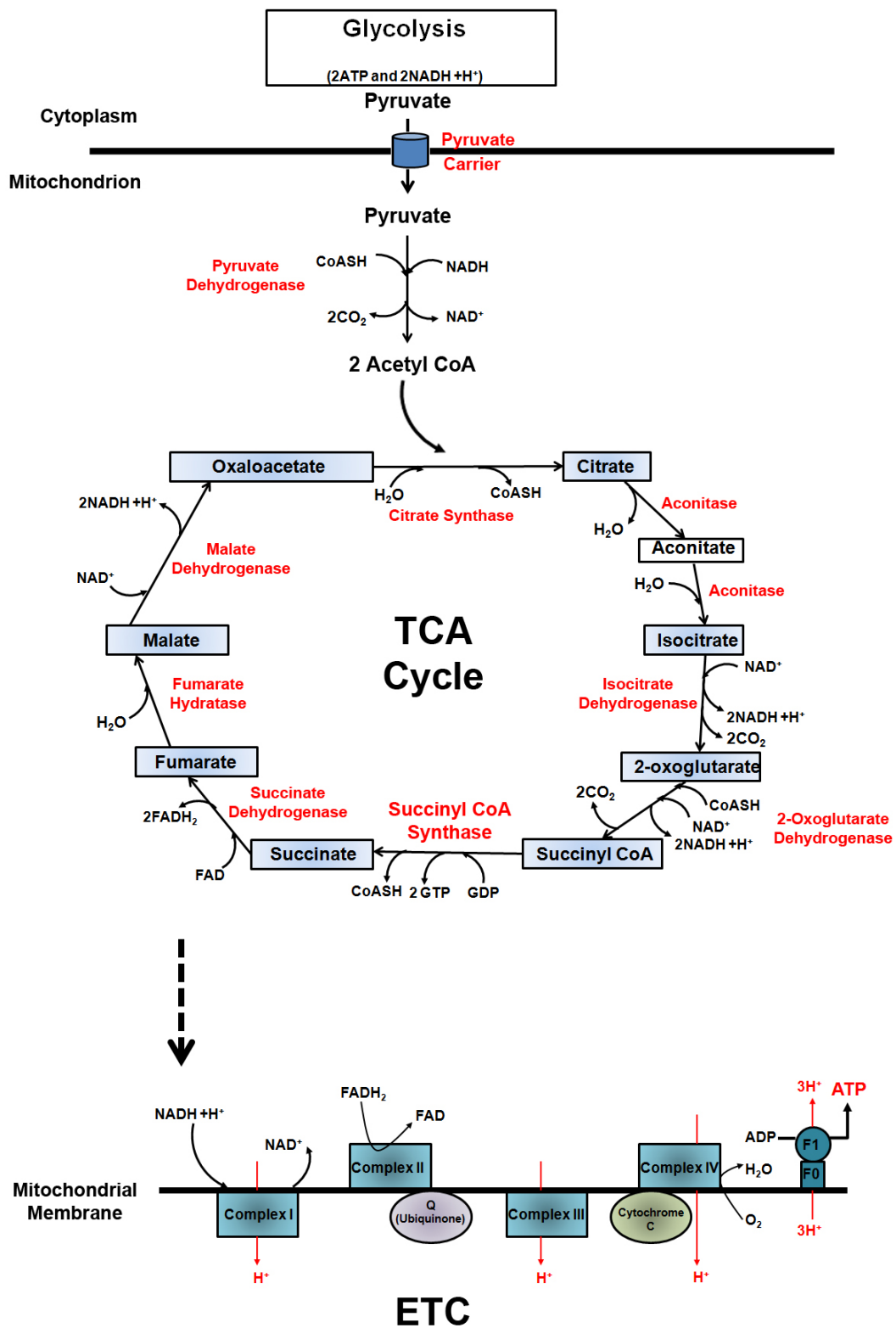


Figure 1.2 TCA Cycle and ETC

An overview of the TCA cycle and the ETC.

1.1.3 The 'Warburg Effect' and metabolic transformation in cancer

In 1927 Otto Warburg was the first scientist to describe the link between altered metabolism and tumourigenesis (Warburg et al. 1927) with a subsequent study demonstrating a metabolic shift from OXPHOS to aerobic (normal oxygen tension) glycolysis in cancer cells (Warburg 1956). Subsequently, this hypothesis is referred to as the 'Warburg effect'. The 'Warburg effect' is characterised by an increase in glucose uptake and lactate production and secretion during aerobic glycolysis (Feron 2009) (Figure 1.3).

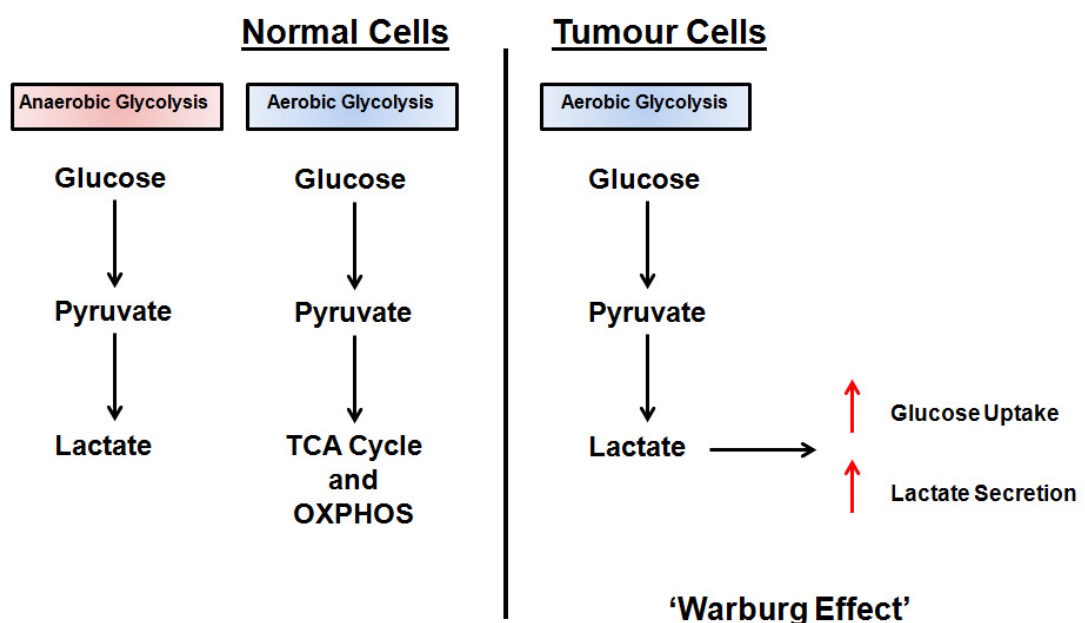


Figure 1.3 The Warburg Effect

Summary of the Warburg Effect showing increased uptake of glucose and lactate secretion in tumour cells compared to healthy cells, under normal oxygen conditions.

Recently there has been a resurgence of support for the ‘Warburg effect’ following increased interest in metabolic dysregulation in cancer (Eng et al. 2003; Gottlieb and Tomlinson 2005; Kaelin 2009; Tennant et al. 2009; Weinberg and Chandel 2009; Koppenol et al. 2011). Metabolic transformation is now considered to be one of the hallmarks of cancer along with the six classic hallmarks which are as follows: 1) resistance to cell death (e.g. inactivation of the pro-apoptotic Bcl-2 regulatory proteins); 2) sustaining proliferative signalling (e.g. mitogen activated protein (MAP)-kinase and phosphoinositide 3(PI3)-kinase signalling pathways); 3) evading growth suppressors (e.g. retinoblastoma (RB) and p53 tumour suppressors); 4) activating invasion and metastasis (e.g. inhibition of neurofibromatosis 2 (NF2) and liver kinase B1 [LKB1]); 5) enabling replicative immortality (e.g. increased telomerase activity) and 6) inducing angiogenesis (e.g. vascular endothelial growth factor A (VEGFA) induction) (Hanahan and Weinberg 2011).

A metabolic shift from OXPHOS to aerobic glycolysis is less economical with respect to ATP production, however, cancer cells which comply with the Warburg hypothesis are still capable of sustaining proliferation under these conditions (Vander Heiden et al. 2009). This may be achieved by utilising an alternative pathway for sustaining anabolic process in the absence of adequate ATP supplies, such as feeding intermediates into the pentose phosphate shunt. This pathway produces NADPH and ribose-5'-phosphate which can then be used for driving anabolic processes such as lipid, cholesterol and nucleic acid synthesis (Salway 1999). Furthermore, dysregulation of four enzymes of the glycolytic pathway have been identified as upholding tumourigenesis: Glucose-6-phosphate (G6P), Phosphofructokinase 1 (PFK1), phosphoglycerate mutase (PGM) (the only glycolytic enzyme not activated

by HIF) and pyruvate kinase (PK) (Tennant et al. 2009). Over-expression of G6P is associated with increased motility in a wide range of tumour cells (Maruyama et al. 1995; Niizeki et al. 2002; Yanagawa et al. 2004) whereas expression of PFK1 is controlled in a more complex manner *via* the increased levels of 6-phosphofructo-2-kinase/bisphosphatase 3 (PFKBP3) present in tumours (Atsumi et al. 2002; Minchenko et al. 2002). PGM expression is controlled by the p53 tumour suppressor and is the only glycolytic enzyme that is not a target of the HIF pathway (Kondoh et al. 2005; Yeung et al. 2008). The embryonic splice variant of PK (PK-M2) is found active in tumours rather than PK-M1 which is normally expressed in healthy adult tissues (Yamada and Noguchi 1999; Christofk et al. 2008).

Increased glycolytic activity is not a feature unique to the Warburg effect but also occurs as a consequence of activation of the hypoxia inducible factor (HIF) pathway. While the Warburg effect is characterised by aerobic glycolysis, activation of glycolysis by the HIF pathway generally occurs under hypoxic (anaerobic) conditions. However, in the case of FH-deficiency, the source of glycolytic activation becomes more complicated as the HIF pathway is also activated under normoxic conditions in this setting (to be discussed in further detail in section 1.2) (Ashrafian et al. 2010; O'Flaherty et al. 2010; Yogev et al. 2010; Frezza et al. 2011). Therefore, it is difficult to determine which pathway is responsible for metabolic dysregulation in an FH-deficient background. This situation calls into question whether the Warburg effect is merely a product of HIF activation (although this has been shown not to be the case for PGM activation (Yeung et al. 2008)), the latter of which also has an active role in cancer development. Until further investigations are carried out to address the Warburg/HIF conundrum, the dispute over which pathway is responsible for tumour

initiation and metastasis, if indeed they exist independently of one another, remains unresolved. These concerns aside, the pathways outlined above, that have been implicated in metabolism transformation, remain attractive targets for therapeutic interventions in cancer (Tennant et al. 2010). In further support of the relevance of the Warburg effect in cancer metabolism, Warburg's observation of increased glucose uptake by cancer cells has been successfully utilised for clinical application as it has enabled tumours to be visualised using positron emission tomography (PET) to show uptake of the glucose analogue 2-(^{18}F)-fluoro-2-deoxy-D-glucose (2-FDG) (Pauwels et al. 2000).

1.2 FH and Disease

1.2.1 FH-deficiency

Autosomal recessive inheritance of biallelic *FH* mutations leads to FHD (OMIM 136850) which is clinically characterised by severe neurological impairment, brain malformation, seizures, severe encephalopathies and hypotonia (low muscle tone) (Gellera et al. 1990; Elpeleg et al. 1992; Bourgeron et al. 1994; Shih 2006 [Updated 2009]). Most individuals with FHD do not survive beyond early childhood (< 2-5 years) however those that do survive suffer from profound psychomotor impairment (Shih 2006 [Updated 2009]). Persistent fumaric aciduria (build up of fumaric acid in the urine) is also a feature of fumarate hydratase deficiency (FHD) and is closely linked to elevated cellular levels of TCA cycle intermediates such as 2-oxoglutarate (2-OG), succinate, malate and citrate (Gellera et al. 1990; Elpeleg et al. 1992).

Of the known FHD-associated mutations the majority are either missense mutations (single nucleotide mutations which result in an amino acid change) or in-frame deletions (alters the codon reading frame thus affecting protein function), which may be homozygous (same mutation on both alleles) or heterozygous compound (different mutation on each allele) (Tomlinson et al. 2002). Unlike leiomyomatosis-associated *FH* mutations (discussed in section 1.1), FHD patients retain detectable, albeit diminished, levels of FH enzyme activity. In confirmation of this, a case study published by Gellera *et al.* demonstrated that cultured fibroblasts from a 7-year old FHD patient showed 30 and 50% lower FH activity, in isolated mitochondria and cytoplasmic fractions respectively, when compared to healthy fibroblasts taken from the parents (Gellera et al. 1990; Bourgeron et al. 1994). It has yet to be reported that FHD patients are susceptible to developing leiomyomata or renal cell cancer (Tomlinson et al. 2002).

1.2.2 Kidney cancer

Kidney cancer, or renal cell carcinoma (RCC), is a relatively rare form of cancer with only 1% of men and 0.5% of women in Western Europe estimated to be at risk (van Spaendonck-Zwarts et al. 2012). RCCs are classified according to five histological subtypes: papillary (PRCC), clear cell (CCRCC), chromophobe (CRCC), collecting duct (CDRCC) and unclassified RCCs (Kovacs et al. 1997). PRCCs can be further sub-typed into type 1 and type 2 PRCCs. Type 1 and type 2 PRCCs have distinct histologies with type 1 PRCC tumour cells classified as having pale cytoplasm with numerous macrophages, an example of which is hereditary papillary renal cell

carcinoma (HPRCC), whereas the cytoplasm of type 2 PRCCs show an increased volume and eosinophilic content (e.g. HLRCC) (Delahunt et al. 2001).

Aside from HPRCC and HLRCC, other hereditary renal cancer syndromes include Birt-Hogg-Dubé (BHD) syndrome and Von Hippel-Lindau (VHL) disease which also present with early-onset tumour formation (Maher 2011). While these diseases have a common tissue origin, they are very distinct with respect to their phenotypes and the causal genes involved in pathogenesis (Linehan et al. 2010). For example, individuals with germline mutations of the *VHL* gene on chromosome 3 present with renal cysts and CCRCC (Crossey et al. 1993; Latif et al. 1993a; Richards et al. 1993; Yao et al. 1993; Rankin et al. 2006; Frew et al. 2008). Mutation of *VHL* prevents its protein product, pVHL, from forming a crucial complex responsible for regulation of hypoxia-inducible factor alpha (HIF- α) (to be discussed in detail in section 1.2) resulting in stabilisation of HIF, in particular HIF-2 α (Krieg et al. 2000; Mandriota et al. 2002; Kondo et al. 2003). While HLRCC tumours show similar dysregulation of the HIF pathway, though mainly of the HIF-1 α protein, they differ from von Hippel-Lindau disease in that *FH* is the causal gene and, while renal cyst development is associated with both conditions, HLRCC tumours are predominantly type 2 PRCCs (discussed in detail section 1.1.4 below).

FH is not the only TCA cycle gene associated with tumourigenesis. The succinate dehydrogenase (*SDH*) tumour suppressor genes, *SDHA*, *SDHB*, *SDHC* and *SDHD* encode four protein subunits which form the SDH enzyme complex. Mutation of the *SDH* genes is closely linked not only to familial paragangliomas (neuroendocrine tumours of the head and neck) but also pheochromocytomas (neuroendocrine tumours

of the adrenal glands) (Baysal et al. 2000; Niemann and Muller 2000; Astuti et al. 2001; Burnichon et al. 2010). The role of *SDH* and other metabolic gene mutations in cancer will be discussed in section 1.1.4.

In a non-TCA cycle context, the hepatocyte growth factor receptor (*MET*) gene is mutated in hereditary PRCC cancers, including a small subset of sporadic type 1 PRCCs, with morphologically distinct papillary or tubulopapillary features (Schmidt et al. 1997; Lubensky et al. 1999; Schmidt et al. 1999). It has also been reported that loss of FH function results in transcriptional activation of MET oncogene in order to uphold transformation and tumourigenesis (Costa et al. 2010). Individuals carrying germline mutations of either tuberous sclerosis gene 1 (*TSC1*) or 2 (*TSC2*), encoding hamartin and tuberin proteins respectively, commonly develop angiomyolipomas (benign vascular tumours) however some instances of clear cell renal cancer and other tumour types have also been reported (Bjornsson et al. 1996). Lastly, a study of families with members affected by hereditary BHD syndrome showed that > 90% of these individuals were identified as having a mutated copy of the folliculin (*FLCN*) gene on chromosome 17, which encodes the folliculin protein (Schmidt et al. 2001). BHD patients present with fibrofolliculomas (benign skin tumours), pulmonary cysts and chromophobe kidney tumours (Pavlovich et al. 2002; Zbar et al. 2002). A summary of the different kidney diseases and causal genes can be seen in Table 1.2.

Table 1.2 Summary of a selection of genes involved in kidney disease

Gene	Disease	Phenotype
<i>FH</i>	- FHD - HLRCC (Type 2 PRCC)	- Severe neurological impairment, brain malformation, seizures, severe encephalopathies, fumaric aciduria and hypotonia - Multiple cutaneous and uterine leiomyomatosis, renal cysts and papillary RCC
<i>FLCN</i>	BHD	Fibrofolliculomas, pulmonary cysts and chromophobe kidney tumours
<i>MET</i>	Type 1 PRCC	Distinct papillary or tubulopapillary features
<i>SDH</i>	- HPGL - PCC	- Rare head and neck tumours, carotid body tumours, no evidence for increased catecholamine secretion - Adrenal chromaffin tumours with increased catecholamine secretion and hypertension
<i>TSC1</i> <i>TSC2</i>	Angiomyolipomas	Benign renal tumours, with some reported instances of CCRCC, and association with tuberous sclerosis
<i>VHL</i>	CCRCC	Renal cysts and clear cell RCC

1.2.3 Hereditary leiomyomatosis and renal cell cancer

The Knudson ‘two hit’ hypothesis describes the loss of heterozygosity (LOH) (i.e. loss of function of one allele when the other allele is already inactive) of a tumour suppressor gene as one of the primary events contributing to the onset of cancer

(Knudson 1971). *FH* has been classified as a tumour suppressor gene as individuals carrying a heterozygous *FH* germline mutation are susceptible to developing hereditary leiomyomatosis and renal cell cancer (HLRCC) in the event that they acquire a second ‘hit’ on the remaining wild-type allele (OMIM 605839) (Kiuru et al. 2001; Launonen et al. 2001; Tomlinson et al. 2002; Toro et al. 2003). HLRCC is a condition classified as a type 2 PRCC and occurs as a result of biallelic inactivation of *FH*. Compared to type 1 PRCCs, type 2 PRCCs are considered to be more aggressive in terms of higher incidences of metastasis, secondary tumours and a lower survival rate (Klatte et al. 2009). Furthermore, inactivation of *FH* has also been reported in patients with cutaneous and uterine leiomyomata (MCUL; OMIM 150800) or RCC only (Alam et al. 2003; Ahvenainen et al. 2008).

Genetic studies carried out in the early 2000s led to the identification of the gene locus for HLRCC predisposition (Kiuru et al. 2001; Launonen et al. 2001; Tomlinson et al. 2002; Toro et al. 2003). Genome-wide linkage analysis mapped the gene to the long arm of chromosome 1 band q42.1 which encodes the *FH* gene (Launonen et al. 2001). Moreover, the same location was implicated in predisposition to multiple cutaneous and uterine leiomyomatosis (Alam et al. 2003). From these findings it was apparent that inactivation of *FH* via LOH was the common causal factor in both conditions. In contrast to FHD, the majority of *FH* mutations associated with HLRCC are missense mutations which are clustered around the regions encoding the A/ B sites (Tomlinson et al. 2002; Alam et al. 2003). Data gathered from the 144 families participating in case studies worldwide identified 81 germline mutations, four whole gene deletions, one exon deletion and one exon duplication of *FH* (Gardie et al. 2011) (Appendix Table 1.1). The majority of studies on *FH* mutations in familial

MCUL/HLRCC have been carried out in Finland, United States of America and the United Kingdom, with some case studies also documented in the Netherlands and Asia (Tomlinson et al. 2002; Toro et al. 2003; Alam et al. 2005; Wei et al. 2006; Makino et al. 2007; Soni et al. 2008; Stewart et al. 2008; Smit et al. 2011). To date, only one mutation of the MTS region has been reported in the literature and while no defined function has been established, it has been proposed that it may have a role in abolishing protein translation (Ahvenainen et al. 2008).

HLRCC is a rare cancer syndrome characterised by early onset, usually between 20 and 40 years of age, and clinical presentation of multiple benign smooth muscle tumours of cutaneous and uterine origin, renal cysts and aggressive type 2 papillary carcinoma, although the latter only occurs in a small subset of affected individuals (Tomlinson et al. 2002; Toro et al. 2003; Chan et al. 2005; Lehtonen et al. 2006; Gardie et al. 2011). Cutaneous leiomyomata (CLM) lesions form clusters of papules in a segmental or diffuse pattern on the extremities, such as the arms and legs, and also on the trunk; these lesions are often painful to touch and sensitive to changes in temperature (Badeloe et al. 2006; Stewart et al. 2006). The risk of cutaneous leiomyomata (CLM) is 70% in female carriers of *FH* mutations compared to 100% in male carriers and is generally considered to be the first point of presentation and clinical diagnosis of individuals affected by HLRCC (Ahvenainen et al. 2008). Uterine leiomyomata (ULM), also referred to as fibroids, originate in the myometrium and are characterised by large, round bundles of smooth muscle in a whorled pattern lying in the uterine wall, endometrium or serosal layer of the uterus (Reed et al. 1973; Stewart 2001). Symptoms include menorrhagia (excessive menstrual bleeding), metrorrhagia (irregular uterine bleeding) and abdominal pain. Treatment of ULM

varies from prescription of oral contraceptive, myomectomy or hysterectomy in severe cases. It is difficult to diagnose HLRCC using ULMs as a clinical marker as sporadic formation of fibroids is a common occurrence in the female population especially those aged 45 year and onwards (Cramer and Patel 1990). However, women with *FH* mutants have an >80% risk of developing and receiving treatment for ULMs before the age of 40 years (Smit et al. 2011).

In 2-16% of HLRCC patients, loss of *FH* results in aggressive type 2 papillary or collecting duct renal tumours and in some cases renal cysts (Tomlinson et al. 2002; Toro et al. 2003; Chan et al. 2005; Lehtonen et al. 2006; Gardie et al. 2011). These renal cysts differ from cysts associated with autosomal polycystic kidney disease (APKD) in that they arise from inactivation of different causal gene(s). Individuals with APKD harbour germline mutations of polycystic kidney disease genes 1 or 2 (*PKD1* and *PKD2*, respectively), encoding the proteins polycystin 1 (PC1) and polycystin 2 (PC2). Loss of function of the remaining wildtype *PKD1/2* allele results in profound renal cyst development in affected individuals (Harris and Torres 2009). In contrast to other papillary RCCs such as HPRCs, which occur bilaterally (in both kidneys), HLRCC kidney tumours are often unilaterally expressed (Linehan 2009)

1.2.4 TCA cycle enzymes with tumour suppressor activities

While *FH* behaves as a “classic” suppressor gene with respect to the Knudson ‘two hit’ hypothesis, it differs from other more traditional tumour suppressor genes, such as the *p53*, retinoblastoma (*RB1*) and *VHL* genes, in that it encodes a crucial TCA cycle enzyme. This indicates that altered metabolism may be just as important in cancer as

regulation of the cell cycle and apoptotic pathways (Eng et al. 2003; Hanahan and Weinberg 2011). However, it remains to be determined whether it is the dysregulation of metabolism that promotes tumourigenesis or the process of tumourigenesis itself that results in dysregulated metabolism. In the case of *FH* inactivation, it may be that both scenarios occur simultaneously. In further support of the emerging role of metabolic regulation in cancer, another TCA cycle enzyme, SDH, has also been identified as having a tumour suppressor function (Baysal et al. 2000; Niemann and Muller 2000; Astuti et al. 2001; Kaelin 2009; Burnichon et al. 2010; Bardella et al. 2011) Although these two genes encode TCA enzymes, they result in distinctly different clinical outcomes when they are mutated (Gottlieb and Tomlinson 2005; Pollard et al. 2005; Linehan et al. 2010).

Germline mutation of SDH is associated with the onset of the rare and inherited familial cancers: paragangliomas and pheochromocytomas (Baysal et al. 2000; Astuti et al. 2001). It is the only enzyme of the TCA cycle to form a membrane-bound complex (complex II) of the ETC. In this capacity it acts as an electron donor by transferring electrons from the reduction of FAD to FADH₂, generated in the process of succinate oxidation to fumarate, to ubiquinone (Q) bound to the inner mitochondrial membrane. From here the electrons flow through to complex IV of the ETC where the generation of ATP from ADP occurs following reduction of molecular oxygen to water (Salway 1999).

Another metabolic enzyme with oncogenic activities is isocitrate dehydrogenase (IDH) which catalyses the conversion of isocitrate to 2-OG, with NADH/NADPH and CO₂ formed as by-products (Salway 1999). Three isoforms of IDH have been

identified in distinct cellular compartments: IDH1 is located in the cytoplasmic/peroxisomal compartments whereas IDH2 and IDH3 (involved in the TCA cycle) are mitochondrial. These isoforms are encoded by genes located on chromosomes 2, 15 and 15 respectively. Only germline mutations of *IDH1* and *IDH2* have been reported to have a link with predisposition to gliomas and acute myeloid leukemia (Parsons et al. 2008; Yan et al. 2009; Frezza et al. 2010), the latter of which has only been shown to affect a small subset of patients (Balss et al. 2008; Mardis et al. 2009; Yan et al. 2009; Marcucci et al. 2010; Weller et al. 2011). Mutations of *IDH3* in cancer have yet to be reported (Yan et al. 2009). It was suggested that IDH enzymes were tumour suppressor genes following a report that dominant negative mutations of *IDH1* (arginine (R)132) and *IDH2* (R172) resulted in loss of IDH function (Zhao et al. 2009). However, as mutations were observed only at these specific residues and only on one allele of *IDH1/2* it was proposed that these enzymes undergo a gain-of-function which has oncogenic potential (Frezza et al. 2010). It has been proposed that the IDH1 oncogenic pathway may be related to an increase in a reduced form of 2-OG, 2-hydroxyglutarate (2HG) (Dang et al. 2009). Although the mechanism through which 2HG accumulation mediates a tumourigenic response has yet to be fully understood, it has been proposed that 2HG binds to and subsequently inhibits the prolyl hydroxylase domain (PHD) enzymes (Xu et al. 2011; Koivunen et al. 2012) in a manner similar to succinate and fumarate (MacKenzie et al. 2007). However, a recent study has contradicted the findings of Xu *et al* by showing that PHD 1 and PHD 2, but not PHD 3, are activated by 2HG (Koivunen et al. 2012). Therefore 2HG has not only been identified as an onco-metabolite but it also has an important analogy with fumarate.

1.3 *FH and the hypoxia inducible factor response pathway*

1.3.1 *The hypoxia inducible factor*

Fluctuations in cellular and tissue oxygen levels can have a significant impact on health and disease in aerobic organisms (Bertout et al. 2008; Mole and Ratcliffe 2008) and therefore oxygen sensing in the cell is tightly regulated by post-transcriptional and post-translational modifications. Hypoxic induction of gene expression in a variety of tissues has been attributed to the activities of the HIF- α transcription factor (Forsythe et al. 1996; Semenza 1996; Semenza et al. 1997; Iyer et al. 1998; Kaelin and Ratcliffe 2008). The discovery of HIF- α as a master regulator of the hypoxic response pathway was made following investigations into the regulation of the erythropoietin (*EPO*) gene which increases oxygen delivery *via* erythropoiesis upon activation (Pugh et al. 1991; Semenza and Wang 1992; Maxwell et al. 1993; Semenza et al. 1994; Mole and Ratcliffe 2008)).

HIF is a heterodimeric protein consisting of an α -subunit and a ubiquitously expressed β -subunit (also referred to as the aryl hydrocarbon receptor nuclear translocator (ARNT) (Reyes et al. 1992)). Each subunit contains an N-terminal basic-helix-loop-helix (bHLH) motif and a central PAS domain (named after the three proteins in which it occurs: period circadian protein [(Per)-Arnt-single-minded protein] which are responsible for facilitating protein-protein interactions (Wang et al. 1995; Semenza et al. 1997). Two further HIF proteins have been described, HIF-2 α (encoded by the endothelial PAS domain protein 1 (*EPAS1*) gene) and HIF-3 α . HIF-2 α has been shown to be capable of dimerising with HIF-1 β and to promote transcription of downstream target genes (Hu et al. 2003; Raval et al. 2005). It has recently been

reported that renal tubular expression of HIF-2 α occurs as a result of *VHL* inactivation and is implicated in fibrosis and cyst development in this setting (Schietke et al. 2012). Conversely, HIF-3 α (also known as inhibitory PAS, IPAS) (Gu et al. 1998) has been proposed to negatively regulate HIF-1 α (Hara et al. 2001; Makino et al. 2001). Unlike HIF-1 α , which protects cells under severe or prolonged hypoxia, HIF-3 α has been suggested to protect cells under conditions of moderate hypoxia (Heidbreder et al. 2003).

1.3.2 Regulation of HIF- α

Homeostatic regulation of cellular oxygen is modulated by the transcriptional activities of HIF-1 which is stabilised under hypoxia and degraded under normoxic conditions. Normoxic degradation of HIF-1 α occurs at a post-translational level following hydroxylation of key proline residues (Pro402 and Pro564 in humans and Pro402 and Pro577 in mice) in the oxygen-dependent degradation domain (ODDD) of the HIF-1 α subunit (Huang et al. 1997; Pugh et al. 1997; Salceda and Caro 1997; Huang et al. 1998; Epstein et al. 2001; Ivan et al. 2001; Jaakkola et al. 2001; Masson et al. 2001; Yu et al. 2001). Hydroxylation of these prolyl residues is mediated by a family of 2-OG-dependent dioxygenase enzymes, known as PHD 1, 2 and 3 (Bruick and McKnight 2001; Epstein et al. 2001; Masson et al. 2001) (Figure 1.4). The PHD enzymes are themselves oxygen sensors as they have a considerably low affinity for molecular oxygen ($K_m = 230\text{-}250\text{ }\mu\text{M}$) (Schofield and Ratcliffe 2004). In order to carry out their enzymatic function of donating one oxygen atom to the hydroxyl group on the modified substrate (HIF- α), the PHDs require molecular dioxygen and 2-OG as co-substrates and ferrous iron (Fe^{2+}) and ascorbate as co-factors (Hausinger 2004;

Berra et al. 2006; Clifton et al. 2006). The remaining oxygen atom is utilized by the PHDs in the catalytic decarboxylation of 2-OG into succinate (Kivirikko and Myllyharju 1998; Gottlieb and Tomlinson 2005). The prolyl hydroxylases PHD1, PHD 2 and PHD 3 are differentially expressed in various tissues and differ in their function of regulating HIF (Appelhoff et al. 2004).

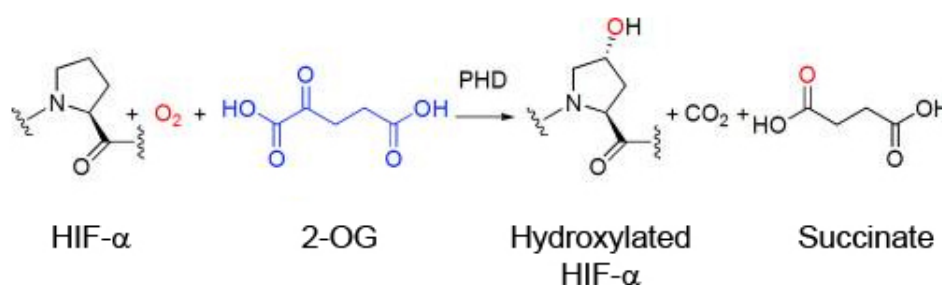


Figure 1.4 Hydroxylation of Hif via PHD enzyme activity

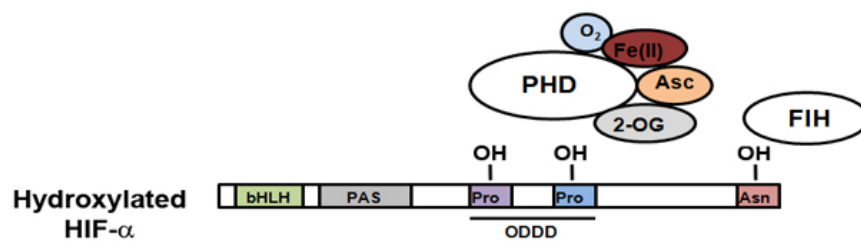
Adapted from McDonough *et al* (McDonough et al. 2006), this figure illustrates the chemical reaction involved in the PHD-dependent catalytic modification of HIF- α to 4-hydroxyproline and conversion of 2-OG to succinate.

Prolyl hydroxylation of HIF-1 α enables it to interact with, and undergo polyubiquitination by, the VHL tumour suppressor E3 ubiquitin ligase complex which comprises of the VHL protein (pVHL), Elongin B, Elongin C, Cullin-2 (Cul2) and RING-box protein (Rbx) (Maxwell et al. 1999; Hon et al. 2002; Min et al. 2002; Kim and Kaelin 2003). Ubiquitinated HIF-1 α is thus targeted for proteolysis by the ubiquitin-proteasome pathway (Cockman et al. 2000; Epstein et al. 2001; Masson et al. 2001; Maxwell et al. 2001). Hydroxylation of the HIF-1 α Carboxy(C)-terminal transactivation domain (CAD) at asparagine residue Asn803 (Asn813 in mice) is catalysed by another 2-OG dioxygenase enzyme known as factor inhibiting HIF (FIH)

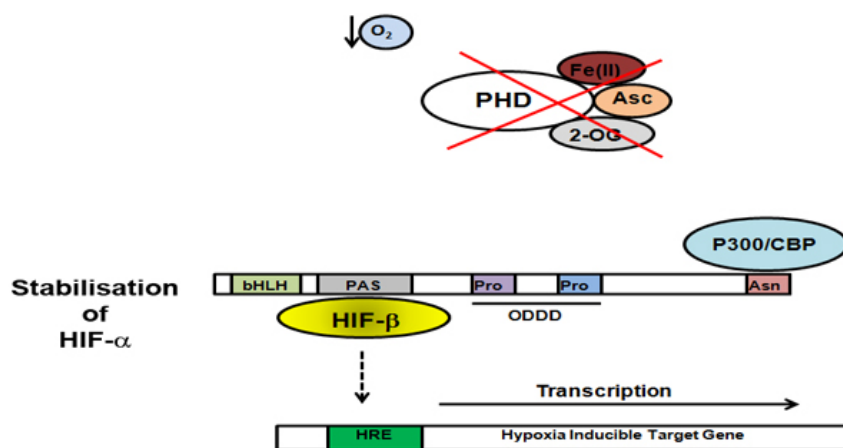
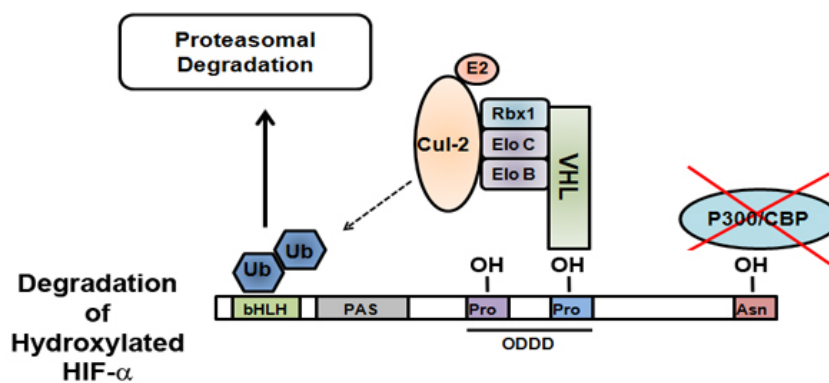
(Hewitson et al. 2002; Lando et al. 2002; Lando et al. 2002), although this enzyme is less closely related to its PHD counterparts (Stolze et al. 2004; Pouyssegur et al. 2006; Kaelin and Ratcliffe 2008). In the presence of asparaginyl hydroxylation, the cysteine/histidine rich-1 (CH1) domain of p300 is unable to interact with and facilitate transcriptional activation of HIF-1 α (Lando et al. 2002; Schofield and Ratcliffe 2004; Kaelin 2005), thus providing a further means of regulating HIF-1 α expression independently of prolyl hydroxylation. See Figure 1.5 for a schematic representation of HIF pathway regulation.

When oxygen supplies drop below a tolerable physiological threshold ($\leq 2\%$ O₂) the PHDs are inactivated due to insufficient supply of dioxygen and consequently the proline residues in the ODDD of HIF-1 α are not hydroxylated (Wang and Semenza 1995). As a result, HIF-1 α is stabilised and the transcriptional co-activator, p300/CREB binding protein (p300/CBP), is recruited to Asn803 in the C-terminal activation domain (CAD) (Dames et al. 2002; Freedman et al. 2002), following which HIF-1 α is translocated to the nucleus. Once in the nucleus HIF-1 α forms a dimer with the HIF-1 β subunit enabling the complex to bind to specific DNA sequences in the 3' promoter region of HIF-1 target genes. These DNA binding regions, approximately 50-nucleotides in length, are referred to as hypoxia-response elements (HREs) which contain a 5'-NCGTG-3' sequence (Semenza 1996). Following binding of the HIF-1 α/β heterodimer to the HRE, transcription of HIF target genes is enabled, many of which are associated with glucose uptake, glycolysis, angiogenesis and metastases (Shweiki et al. 1992; Wenger 2000; Seagroves et al. 2001; Pennacchietti et al. 2003). In further support of the sensitivity of HIF to cellular oxygen and its ability to mediate

a hypoxic response, a study measuring the expression of stabilised HIF proteins in the presence of decreasing oxygen, ranging from 5% hypoxia to anoxia (0% O₂), showed a direct correlation between hypoxia and increased HIF expression {Wang, 1995 #1616; Jiang, 1996 #1448}. Moreover, this response to decreasing oxygen levels precedes diminishing supplies in cellular ATP stores (Hochachka et al. 1996) therefore enabling the cells to utilize glycolysis as an alternative metabolic pathway to OXPHOS and maintain ATP storage and production.



Normoxia



Hypoxia

Figure 1.5 Regulation of the Hypoxia Inducible Factor Pathway

Figure 1.5 Regulation of the Hypoxia Inducible Factor Pathway

Regulation of the HIF pathway under normoxic and hypoxic conditions (adapted from (Mole and Ratcliffe 2008)). Under normoxic conditions, active PHD enzymes hydroxylate the proline (Pro) residues on the ODDD of HIF- α whereas FIH hydroxylates the asparaginyl (Asn) residue. The hydroxylated Pro residues are recognised by the VHL-dependent E3 ligase complex which target the hydroxylated HIF for proteasomal degradation. Recruitment of the transcription co-activator p300/CBP to the Asn residue is prevented following hydroxylation by FIH. Under hypoxic conditions, dioxygen molecules are unavailable as a co-substrate for the PHDs and consequently HIF- α is not targeted by the VHL-dependent E3ligase degradation pathway. Furthermore, the p300/CBP complex can promote transcriptional activation of HIF in the absence of asparaginyl hydroxylation. The stabilised HIF- α subunit then forms a heterodimeric complex with the HIF- β subunit which is capable of binding to the hypoxic response elements (HREs), thus promoting transcription of HIF target genes.

1.3.3 HIF and cancer

HIF-activation of angiogenic genes such as *EPO* and *VEGF* stimulates the synthesis of new blood cells and angiogenesis, respectively. Activation of these pathways enables cells to overcome reduced oxygen tensions, especially where the diffusion distance between cells and the nearest blood supply is greater than the optimal distance of 1-2 mm³ (Folkman 1990). This is especially relevant for cells located in the hypoxic core of solid tumours. Over-expression of HIF-1 α , and also some HIF-2 α , induced genes in cancer is associated with more aggressive tumour types whereby cells gain selective advantage for clonal selection by evading hypoxia-induced cell death (Brizel et al. 1996; Airley et al. 2001; Harris 2002; Brown and Wilson 2004; Quintero et al. 2004). While the upregulation of HIF in the hypoxic core of a solid tumour is not surprising, another feature of HLRCC is the stabilisation and upregulation of HIF under normoxic conditions. This occurrence is referred to as a 'pseudo-hypoxic drive' as it activates a hypoxic response in the cell in the absence of a hypoxic stimulus (Gottlieb and Tomlinson 2005; Pollard et al. 2005). Accumulation of normoxic HIF subsequently promotes transcriptional activation of HIF-target genes which are involved in numerous metabolic pathways such as aerobic glycolysis (e.g. glucose transporter isoform 1 (*Glut 1*), hexokinase 2 (*HK2*), lactate dehydrogenase A (*LDHA*)), haem catabolism (haem oxygenase 1 (*HMOX1*)), erythropoiesis (e.g. *EPO*), angiogenesis (e.g. *VEGF*) and iron metabolism (e.g. Transferrin (*TF*)) (Lee et al. 1997; Pouyssegur et al. 2006; Kim et al. 2007; Semenza 2007; Mole and Ratcliffe 2008; Xie et al. 2009). Many of the genes involved in the metabolic pathways listed are currently being targeted for therapeutic treatment of numerous cancer types (Vander Heiden 2011).

A close affiliation between stabilisation of HIF-1 α and poor prognosis has been reported, in part due to the hypoxic regions of solid tumours being more resistant to chemotherapeutic interventions (Wilson et al. 1989; Grau and Overgaard 1992; Wouters et al. 2007).

1.3.4 Fumarate-mediated activation of HIF-1 α

As referred to above, a marked increase in HIF activity and expression of its downstream target genes have been shown to be a characteristic feature in human tumours with *FH* deletions or mutations (Isaacs et al. 2005; Pollard et al. 2005a; Pollard et al. 2005b). The proposed hypothesis for the stabilisation of HIF in FH-deficiency is that loss of FH results in cellular accumulation of fumarate. As the chemical structure of fumarate is similar to that of 2-OG (See Chapter 3, section 3.1, Figure 3.1) it was shown by Koivunen *et al* that fumarate is capable of competitively binding to and significantly inhibiting PHD 1, PHD 2 and PHD 3 (K_i values of 80, 60 and 50 μ M, respectively) (Koivunen et al. 2007). These findings were corroborated in an independent study using a 2-OG turnover assay as a read-out of fumarate-mediated inhibition of the PHDs (Hewitson et al. 2007). Conversely, fumarate is a weak inhibitor of FIH (Koivunen et al. 2007). It has been reported previously from in vitro studies that stabilisation of the HIF pathway occurs as a result of fumarate accumulation following Fh1-deficiency (Figure 1.6) (Pollard et al. 2005; Ashrafian et al. 2010; O'Flaherty et al. 2010). Given the extensive implications for the effects of transcriptional activation of HIF- α on numerous pathways involved in cell regulation, its relevance in FH-associated cancer is not negligible. Since the HIF hydroxylases are localised to the cytoplasm and nucleus, but not the mitochondria (Metzen et al.

2003), there may be an important role for cytoplasmic rather than mitochondrial FH in regulating activation of the HIF-1 α transcription factor. Fumarate-mediated activation of the HIF pathway is discussed in further detail in Chapter 3, section 3.1.

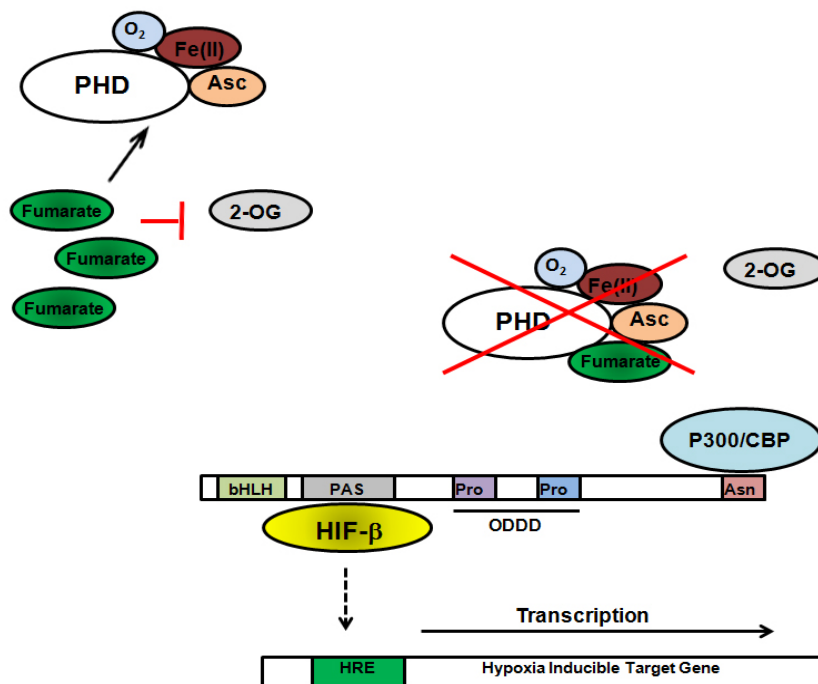


Figure 1.6 Fumarate-mediated inhibition of Hif- α

This figure illustrates the proposed pathway for fumarate-dependent stabilisation of HIF- α by outcompeting 2-OG for substrate binding to the PHDs, resulting in PHD inactivation, under normoxic conditions. As a result of fumarate-mediated inactivation of the PHDs, unhydroxylated HIF- α subunits are not targeted for proteasomal degradation by the VHL-dependent E3 ligase pathway, thus leads to transcriptional activation of HIF target genes.

1.3.5 The effects of FH inactivation on Reactive Oxygen Species generation

Another mechanism through which loss of FH may lead to HIF-1 α stabilisation is via elevated cellular reactive oxygen species (ROS) levels. ROS are defined as molecules containing reactive oxygen species, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl free radicals [$OH^\cdot$]. Under conditions of oxidative stress, such as exposure to ionizing radiation (IR), ultraviolet (UV) light, disruption to complexes I and IV of the ETC, temperature fluctuations and diminished oxygen supply, levels of cellular ROS increase (Chandel and Schumacker 2000). This increase in ROS production can result in activation of pathways regulated by oxidative stress such as inflammation, HIF-1 α stabilisation, autophagy and induction of apoptosis (Chandel et al. 2000; Avery 2011; Heyman et al. 2011; Li et al. 2011; Morgan and Liu 2011).

Disruption to normal TCA cycle function and subsequent dependency on glycolytic metabolism has also been indicated to elevate ROS levels in FH-deficient cells (Sudarshan et al. 2009). This disruption to the TCA cycle depletes the supply of electrons (donated by $NADH + H^+$ molecules) normally required to generate an electrochemical proton gradient across the electron transport chain (ETC), which activates oxidative phosphorylation of ADP to ATP, thus resulting in altered cell energy metabolism (Gottlieb and Tomlinson 2005). The ETC (which is composed of four protein complexes: complexes I-IV) is the primary source of mitochondrial ROS in the cell as electrons can leak out in the process of being passed from one complex to the next and as a result electrons are free to react with O_2 to produce O_2^- (Loschen et al. 1974; Boveris and Cadenas 1975; Liu et al. 2002). Superoxide dismutases 1-3 (SOD1-3) then convert the O_2^- to H_2O_2 and O_2 (Boveris et al. 1972; Chance et al.

1979). There are many oxidoreductases that act as scavengers for ROS located within subcellular compartments such as the mitochondria (e.g. superoxide dismutase SOD 2), peroxisomes (e.g. catalase) and cytoplasm (e.g. glutathione peroxidase (GPX) 1), including those that are ubiquitously expressed throughout the cell (e.g. SOD1). These oxidoreductases are responsible for repairing oxidative damage and regulating homeostatic levels of cellular ROS as part of the antioxidant response (Sies 1997).

Enhanced mitochondrial ROS has been attributed to a positive feedback-loop which both activates and is a product of HIF- α -stabilisation (Chandel et al. 1998; Chandel et al. 2000; Chandel and Schumacker 2000; Guzy et al. 2005; Mansfield et al. 2005; Solaini et al. 2010). However, this paradigm remains in dispute (Hagen et al. 2003; Tuttle et al. 2007) as ROS generated *via* cytoplasmic processes, such as the unfolded protein response (UPR), the endoplasmic reticulum (ER) oxidoreductases and NADH oxidase, must also be taken into consideration (Kultz 2005; Santos et al. 2009). Moreover, it remains a challenge to distinguish which mechanism is the primary instigator as neither the HIF, nor the ROS pathways, function independently of the other.

1.4 The role of HIF-independent 2-oxoglutarate- dependent dioxygenases in FH-deficiency

In addition to the HIF hydroxylase enzymes there is an extensive family of approximately 60-70 known 2-OG-dependent oxygenases (Loenarz and Schofield 2008). These oxygenases catalyse other biological oxidations such as histone and

nucleic acid demethylation via Jumonji domain-containing (JmjC) histone demethylases (Loenarz and Schofield 2008) and ALKBH (human homologue of AlkB) DNA-repair enzymes, which raises the possibility that fumarate accumulation might also perturb epigenetic regulation and other functions of these enzymes by the same mechanism.

Other potentially interesting candidate 2-OG oxygenases for fumarate-mediated inhibition are the collagen prolyl hydroxylases. Collagen prolyl 3-hydroxylase (P3H) activity, together with prolyl 4-hydroxylase (P4H) and other post-translational modifications such as lysyl hydroxylation and glycosylation, are fundamental for collagen synthesis (see review by (Myllyharju and Kivirikko 2004)). It has been published that the P3H2 member of the P3H family may be responsible for the hydroxylation of collagen IV (key component of basement membranes and formation of the extracellular matrix [ECM]) (Tiainen et al. 2008), which is abundant in the kidneys. Of further interest is the emerging evidence for the regulation of HIF by P4H family members (Bruick and McKnight 2001). Thus, there may be reason to consider the case for fumarate-mediated inhibition of the collagen hydroxylases, subsequently disrupting the extracellular matrix (ECM) and promoting tumour metastasis.

1.5 Loss of FH and the consequent impact on HIF-independent pathways

1.5.1 Fumarate-mediated protein succination

Succination is a spontaneous, irreversible chemical reaction that occurs between fumarate and proteins containing sulfhydryl cysteine residues leading to the formation

of stable S-(2-succinyl) cysteine (2SC) modifications (Alderson et al. 2006; Frizzell et al. 2011) . This reaction is referred to as a Michael addition which involves a 1,4-nucleophilic addition of a carbanion to a α - β unsaturated carbonyl compound (Sykes 1992; Little 2004) (Figure 1.7). Accumulation of cellular fumarate has been shown to correlate directly with an increase in 2SC proteins during maturation of 3T3-L1 fibroblasts to adipocytes (Nagai et al. 2007). Nagai *et al.* showed that a >5-fold increase in fumarate during adipogenesis produced >10-fold increase in 2SC proteins in adipocytes compared to undifferentiated fibroblasts grown in conditions of high (30mM) glucose. In light of the close association between fumarate and 2SC, investigations were carried out to determine whether this fumarate-dependent protein modification could also be a specific biomarker for HLRCC, a condition where inactivation of FH has been shown to result in elevated levels of fumarate (Pollard et al. 2005). Using a 2SC-specific antibody (a kind gift from Dr. Norma Frizzell), immunohistochemical (IHC) analysis of FH-deficient tumours and cysts showed a striking direct relationship between the *FH* inactivation and an increase in 2SC proteins, which was absent in non-HLRCC tumours and normal tissue controls (Bardella et al. 2011). These findings were further validated using mass spectrometry (MS) which identified succinated proteins by detection of a 116 Da molecular weight shift. It has thus been shown that 2SC is a highly specific biomarker for HLRCC. Moreover, these 2SC modifications may help to identify other cysteine-rich regulatory proteins targeted by fumarate for succination and their associated pathways, which may contribute to the early stages of tumourigenesis.

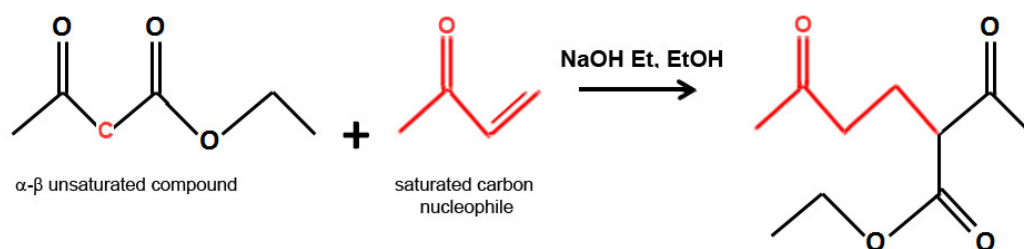


Figure 1.7 Michael Addition

An example of a Michael addition illustrating the 1, 4- addition of ethyl acetoacetate (carbanion nucleophile) to 3-Buten-2-one (α - β unsaturated carbonyl compound), in the presence of sodium ethoxide (NaOHEt) and ethanol (EtOH) as reaction bases (Example adapted from http://www.chem.shef.ac.uk/level-3/project-2001/michael_reaction.html). NaOHEt exists as Na^+ and OEt^- of which the OEt^- is capable of deprotonating ethyl acetoacetate between the two carbonyl groups (carbon highlighted in red) resulting in a loss of one hydrogen atom, which confers a negative charge to this carbon to form an enolate ion, and generation of the EtOH base. The enolate ion then attacks the electrophilic alkene of the 3-Buten-2-one which accepts a hydrogen atom from EtOH to form the final compound shown on the right.

1.5.2 Fumarate-mediated activation of the Nrf2 pathway

As previously mentioned 2SC modifications may prove beneficial in identifying signalling pathways involved in FH-deficient diseases. Two independent studies have recently identified cysteine residues within the Kelch-like ECH-associated protein 1 (KEAP1 (human) or murine Keap1) which are subject to fumarate-mediated 2SC modifications (Adam et al. 2011; Ooi et al. 2011). Succination of cysteine residues Cys38, Cys151, Cys241, Cys288, Cys319, and Cys613 was shown to prevent Keap1 from binding to Nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Cys151 and

Cys288 have previously been shown to regulate Keap1 (Nguyen et al. 2009). Succinated Keap1 is unable to bind to and promote degradation of Nrf2 and consequently unbound Nrf2 is able to activate the Nrf2-mediated antioxidant response within Fh1-deficient cells (Adam et al. 2011; Ooi et al. 2011). Moreover, this was shown by Adam *et al.* to occur independently of the mitochondrial defect associated with Fh1-deficiency (Adam et al. 2011). The dysregulation of the KEAP1/NRF2 pathway was found in both sporadic and hereditary type 2 PRCC but not in CCRCC therefore potentially enabling more targeted and specific clinical treatments to be devised for HLRCC (Ooi et al. 2011).

1.5.3 Glutamine-dependent reductive carboxylation

Cells harbouring mutations in key metabolic enzymes and/or respiratory defects must utilise alternative pathways to supply metabolite precursors for the synthesis of macromolecules and other anabolic processes required to survive. One such example is the utilisation of glutamine-driven reductive carboxylation. Reductive carboxylation supplies acetyl-coenzyme A for lipid biogenesis and four-carbon TCA cycle intermediates through carboxylation of 2-oxoglutarate (Mullen et al. 2012). It has recently been suggested that this pathway is upregulated in the FH-deficient UOK262 cancer cell line and that reductive carboxylation may be required for malate anaplerosis, which may explain why malate levels are not ablated in the absence of FH activity (Unpublished observation, Dr. Christian Frezza, The Beatson Institute, Glasgow).

1.5.4 Induction of haem oxygenase 1 in *Fh1*-deficient cells

Another hypothesis for the ability of FH-deficient cells to survive in the absence of a functional TCA cycle is *via* activation of the haem biosynthesis/degradation pathway (Frezza et al. 2011). It has been proposed that *Fh1*-deficient renal cells are able to utilize this pathway to relieve the elevated fumarate levels by generating haem from the carbons of TCA cycle intermediates, such as fumarate. Haem can then undergo degradation to bilirubin, which correlates with an increase in glutamine uptake, and thus be excreted from the cell. In support of this, transcription of haem oxygenase 1 (*Hmox1*) is found to be markedly increased in *Fh1*^{-/-} renal cells (Frezza et al. 2011). *Hmox1* is responsible for catalytic conversion of haem to biliverdin, which is then catalysed by biliverdin reductase b to bilirubin (Frezza et al. 2011). In the study by Frezza *et al*, targeted deletion of *Hmox1* was found to induce synthetic lethality in *Fh1*^{-/-} cells (Frezza et al. 2011) and therefore remains a candidate pathway for therapeutic intervention in FH-associated HLRCC. In further support of these findings, elevated mRNA levels of *Hmox1* were also observed in an independent study of fumarate-dependent activation of the Nrf2-mediated antioxidant response (Adam et al. 2011).

1.6 *In vivo* models for the study of FH-deficiency

A common approach to understanding and eliciting the function of a gene of interest is to investigate the effects of gene inactivation, modification or deletion. This can be achieved *in vivo* using conditional gene targeting procedures, such as site-specific DNA recombination or transcriptional transactivation, to generate transgenic mice

models (Orban et al. 1992; Lewandoski 2001). The first transgenic mouse lines emerged in 1981, demonstrating that stable expression of a foreign gene (also known as a transgene), in either a ubiquitous or a tissue-specific manner, could be achieved *in vivo* (Costantini and Lacy 1981; Gordon and Ruddle 1981; Harbers et al. 1981; Wagner et al. 1981). Incorporation of a transgene into the germline allows for expression in future progeny. Two methods are used for producing transgenic mice: 1) Blastocyst injection of embryonic stem (ES) cells (harvested at E3.5) harbouring a DNA expression vector, including promoters and enhancers sequences, required for integration of the gene of interest into the host genome and 2) injection of the DNA vector into the male pronucleus of a freshly fertilized egg (Tecott 2002). The advantage of using the former method is that ES cells expressing the desired DNA sequence can be screened prior to injection into the blastocyst, thus improving the rate of contribution of the transgene-positive cells to the resulting adult chimera.

The ability to turn genes on and off using transgenic mice (mice expressing foreign DNA) has been utilised in a plethora of studies for over 30 years (Gordon et al. 1980; Gordon and Ruddle 1981). Some of the current methods available for conditional targeting of genes *in vivo* are as follows: Transcriptional transactivation *via* TetR- and Gal4-based systems or site-specific DNA recombination *via* Cre/loxP-, combined Cre/loxP and TetR-systems and Flp/FRT-based systems (Lewandoski 2001). The TetR system (Gossen and Bujard 1992) is a popular method for controlled gene transcription transactivation. A fusion sequence consisting of a VP16 transactivation domain and *E. coli* tetracycline repressor (TetR) protein recognises and binds to the *tet* operon of the transgene thus facilitating its transcription. This system has been adapted to incorporate two levels of transcriptional control. In the first, and original,

system the tetracycline-controlled transactivator (tTA) is prevented from binding to the target DNA in the presence of an inducer, such as doxycycline ('tet-off'). Conversely, in the second system, the reverse tTA (rtTA) binds the target DNA in the presence of the inducer ('tet-on') (Gossen et al. 1995). The GAL4-based system involves *S. cerevisiae* derived Gal4 (transcriptional activator) binding to upstream activator sequences (UASs) to mediate transcription of Gal4-responsive genes (Ornitz et al. 1991). This Gal4/UAS system has been used in murine studies to bypass embryonic-lethal phenotypes (Rowitch et al. 1999). The Flp/FRT system is a eukaryotic homolog of Cre/loxP for tissue-specific gene targeting and therefore works on the same principle as the Cre/loxP system, described below (Stricklett et al. 1999). The first Flp/FRT transgenic mice developed did not produce consistent recombination results due to the instability of the Flp enzyme at 37°C (Buchholz et al. 1996). A new thermo-stable variant of Flp (Flpe) has now been integrated for use in this system which is comparable with the activities of Cre *in vivo* (Rodriguez et al. 2000).

In light of the *in vivo* studies described in Chapter 4, emphasis will be placed on the Cre/loxP system. Cre/loxP recombination is a robust method for conditional (controlled) gene expression (Lakso et al. 1992; Rajewsky et al. 1996; Nagy 2000; Dragatsis and Zeitlin 2001; Lewandoski 2001). The use the Cre/loxP system for genetic manipulation *in vivo* has increased in recent years following the first publication of a tissue-specific knockout of a gene in a whole organism (Gu et al. 1994). The Cre/loxP system works on the principle that a Cre-recombinase enzyme, derived from bacteriophage P1, recognises and binds to a 34bp sequence motif referred to as a loxP site (Hoess et al. 1982; Abremski et al. 1983; Hamilton and

Abremski 1984). The gene of interest can be modified to integrate loxP sites flanking either the entire gene or particular exons essential for gene function. Upon binding to the loxP sites, Cre-recombinase initiates homologous recombination (the exchange of nucleotides between similar DNA molecules at identical locations) at these specific sites and excises the DNA sequence between the two loxP sequences (Sauer and Henderson 1988; Sauer and Henderson 1989; Sauer and Henderson 1990; Orban et al. 1992; Watson 2004). Following recombination of two loxP sites, a single loxP site remains behind in the open reading frame (ORF; contains no stop codon) of the gene sequence which may have phenotypic consequences if it is not tolerated by the host genome (Grieshammer et al. 1998). Further versatility is afforded by this system as the loxP sites can be inserted in either the same or opposite orientations, resulting in gene deletion or gene inversion, respectively (Abremski et al. 1983; Hamilton and Abremski 1984). In order to facilitate the targeted knockout (or expression of an inserted gene) of a loxP-flanked gene *in vivo*, loxP-transgenic mice must be crossed with a Cre-expressing transgenic mouse line in a ubiquitous or cell-type specific manner (Rajewsky et al. 1996; Utomo et al. 1999). Alternatively, expression of a loxP-flanked gene can also be induced in a cell-type specific and time controlled manner using tetracycline inducible system (Furth et al. 1994; Schultze et al. 1996). This is referred to as a combined Cre/loxP and TetR system, as previously described.

A study by Pollard *et al*, illustrated that inactivation of *Fh1* (mouse orthologue of human FH), using a Cre/loxP conditional gene targeting system, contributed to progressive renal cyst development and activation of the Hif (mouse orthologue of human HIF) pathway (Pollard et al. 2007). Prior to the development of the *in vivo* models described in Chapter 4, the *Fh1*-conditionally targeted knockout mice were

the most versatile *in vivo* model systems available for studying the phenotypic effects of Fh1-deficiency and understanding the role of Fh1 in both the cystic and HIF phenotypes associated with HLRCC (Pollard et al. 2005; Pollard et al. 2007). Expression of a Cre recombinase driven by the kidney-specific cadherin (Cadherin 16) promoter (Ksp1.3/Cre) facilitates the excision of loxP-flanked *Fh1* exons 3 and 4 (encoding the active site of Fh1) thus resulting in gene inactivation exclusively in renal tubular epithelial and collecting duct cells of *Fh1^{fl/fl}* or *Fh1^{fl/+}* mice expressing the Ksp1.3/Cre allele (Shao et al. 2002a; Shao et al. 2002b; Pollard et al. 2007). As Ksp-cadherin mRNA is not detectable in the metanephros of the developing mouse kidney until day E14.5 (Shao et al. 2002; Shao et al. 2002), the problem of early lethality (E6.0 (Pollard et al. 2007)) associated with embryonic deletion of *Fh1^{-/-}* is circumvented as a result of the Ksp1.3/Cre driven Cre-recombinase being expressed at a later stage in embryonic development. Chapter 4 describes in detail the development of a new *in vivo* model system for investigating the role of the two Fh1 isoforms (mitochondrial and cytoplasmic), in protecting the kidney against the cystic phenotype associated with loss of Fh1 and better understanding the tumour suppressor function of Fh1. The human FH (full length, mitochondrial FH) and FH Δ MTS (cytoplasmic-restricted FH lacking its N-terminal mitochondrial targeting sequence) transgenes were individually expressed in *Fh1^{fl/fl}* or *Fh1^{fl/+}* mice harbouring a Ksp1.3/Cre-expressing allele to compare the phenotypes of the two cohorts and to study the effects of compartmentalised re-expression of FH in an Fh1-deficient background.

1.7 *In vitro* systems for the study of FH-deficiency

In the last 5 years a number of diverse cell models have emerged for the study of FH-deficiency. These *in vitro* models can facilitate a range of investigations into the metabolic and/or biochemical pathways that are dysregulated during the early stages of HLRCC. Prior to the establishment of these models the only available system for investigating the cellular effects of FH-deficiency *in vitro* involved the use of pharmacological inhibitors of FH, such as 3-nitropropionic acid (3-NPA), and transient knockdown of *FH* using siRNA. Cell lines from a HLRCC syndrome patient include UOK262 renal cells from an abdominal lymph-node metastasis and more recently another UOK cell line, UOK 268, has been described which was cultured from a metastatic renal tumour (Yang et al. 2010; Tong et al. 2011). NCCFH-1 is a recently established cell line originating from a lung metastasis of a 19-year old patient diagnosed with type 2 PRCC (Observations by Dr. Victoria Perrier-Trudova; Unpublished data). These cells have phenotypes that are characteristic of those associated with HLRCC such as accumulated cellular fumarate, stabilisation of normoxic HIF and markedly increased glycolytic activity glucose-dependency phenotype associated with HLRCC. However, one limitation that must be considered with the use of these cell lines is that they are established cancer cells that have been subjected to secondary biochemical changes and are likely to have acquired multiple mutations. The culturing of cells from primary renal tumours presents further difficulties due to the high number of necrotic cells and phagocytes, including other inflammatory response markers and also a lack of cell-type homogeneity (Ekwall 1990). For these reasons these cell lines are not ideal for identifying and investigating the pathway(s) affected during the primary stages of FH-associated tumourigenesis.

Murine cell models derived from Fh1-conditional knockout mice (Pollard et al. 2007) (see below, section 1.6, for details of this model system) are, however, useful systems for investigating the effects of *Fh1* inactivation on homeostatic regulation of specific metabolic and molecular pathways in a single cell type. The use of *in vitro* models enables candidate pathways to be identified prior to testing in an *in vivo* system where interactions between multiple cell types and organ systems may result in subtle molecular and/or metabolic alterations, important in initiating the early stages of a disease phenotype, being masked. To date, two cell lines derived from the Fh1-conditional knockout mice have been described; an Fh1^{-/-} renal cell line (Frezza et al. 2011) and an Fh1^{-/-} mouse embryonic fibroblast (MEF) cell line (O'Flaherty et al. 2010). Both of these cell lines have loxP sites flanking *Fh1* exons 3 and 4. Exogenous addition of a bacteriophage P1 Cre-recombinase, packaged in an adenovirus expression vector for genomic integration and expression, to immortalized Fh1 MEFs promotes recombination of the LoxP sites *in vitro*. The loss of exons 2 and 3 results in termination of *Fh1* transcription at exon 1 and thus inactivation of the gene's function to encode Fh1 protein.

1.8 Objective of this DPhil project

The aim of this project was to develop both *in vitro* and *in vivo* models that could be used, either in combination or in isolation, to identify candidate metabolic and/or biochemical specific signalling pathways involved in the primary stages of HLRCC tumourigenesis. Chapter 2 describes the work undertaken to generate immortalised

Fh1^{-/-} MEFs derived from Fh1 conditional mice and the characterisation of these cell lines against their wildtype counterparts (Fh1^{+/+} MEFs). Genetic complementation of the Fh1^{-/-} MEFs with either human mitochondrial FH (Fh1^{-/-} +FH MEFs) or cytoplasmic FH (Fh1^{-/-} +FHΔMTS MEFs) is described in Chapter 3. The purpose of developing these cell lines was to study the Fh1^{+/+}, Fh1^{-/-}, Fh1^{-/-} +FH and Fh1^{-/-} +FHΔMTS MEFs in combination to better understand the role of cytoplasmic versus mitochondrial FH in dysregulation of the HIF and respiratory pathways in Fh1-deficiency. In order to determine whether the findings of the *in vitro* MEF model could be recapitulated in an *in vivo* environment and, moreover, whether ubiquitous re-expression of either FH or FHΔMTS could ablate the cystic phenotype associated with Fh1-deficiency, a mouse model was designed and generated. Chapter 4 details how Fh1-conditional knockout mice were crossed with chimeras designed to express either a mitochondrial or cytoplasmic V5-tagged FH transgene and the resulting progeny were subsequently crossed with Ksp 1.3/Cre transgenic mice to excise *Fh1* in a kidney-specific manner.

It is predicted that the results of either individual or combined studies using these *in vivo* and *in vitro* models may help determine a function for FH in the early stages of tumourigenesis in HLRCC.

2 Chapter 2 Generation and characterisation of Fh1-deficient
murine embryonic fibroblasts

*Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts***2.1 Introduction**

The TCA cycle is crucial for maintaining a sufficient supply of GTP, FADH₂ and NADH + H⁺ which can be used to generate ATP and NADPH molecules for energy-demanding processes, such as amino acid and lipid biosynthesis that are essential for cell growth and function (Vignais 1976; Salway 1999 ; Nelson 2004). To date, mutation or deletion of genes encoding two TCA cycle enzymes, *SDH* and *FH*, has been linked to tumour formation and has thus led to their classification as tumour suppressor genes (Baysal et al. 2000; Tomlinson et al. 2002; Toro et al. 2003; Pollard et al. 2005; Brandon et al. 2006; King et al. 2006; Kaelin 2009). As previously described in Chapter 1, loss of *FH* function has been shown to predispose individuals to HLRCC (Tomlinson et al. 2002; Toro et al. 2003). There is substantial evidence to suggest that accumulation of cellular fumarate, resulting from the loss of FH, competes with 2-OG for binding to the 2-OG-dependent HIF PHDs, thus preventing hydroxylation of proline regions in the oxygen dependent degradation domain (ODDD) of HIF and subsequent degradation by the ubiquitin-ligase pathway (Ivan et al. 2001; Isaacs et al. 2005; Ashrafian et al. 2010; O'Flaherty et al. 2010). This is relevant as stabilisation of HIF, in particular HIF- α , under aerobic conditions has been observed in many tumour types (Harris 2002; Semenza 2003; Isaacs et al. 2005; Pollard et al. 2005; Pollard et al. 2005; O'Flaherty et al. 2010). Moreover, the loss of FH prevents the TCA cycle from going to completion thus promoting a switch from OXPHOS to aerobic glycolysis (referred to as the 'Warburg effect', as discussed in Chapter 1), which can be measured as an increase in glucose uptake and secretion of lactate following the catalytic conversion of pyruvate to lactate by lactate dehydrogenase (Warburg et al. 1927; O'Flaherty et al. 2010).

Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts

There is however emerging evidence for other fumarate-regulated pathways, independent of the HIF pathway, contributing to the onset of tumourigenesis such as stabilisation of the Nrf2-mediated antioxidant response (Adam et al. 2011; Ooi et al. 2011), the DNA damage response (Yogev et al. 2010) or haem biosynthesis (Frezza et al. 2011). Of further interest is the emerging role of a fumarate-mediated chemical reaction (a Michael Addition) whereby fumarate interacts with the thiol groups of key cysteine residues within target proteins to form a S-(2-succinyl) cysteine (2SC) modification (Bardella et al. 2011; Frizzell et al. 2011). This process, referred to as succination, has been shown to occur in Fh1-deficient murine renal cysts and also in human HLRCC tumours but not in healthy tissues, thus demonstrating its use as a highly specific clinical biomarker for conditions of FH-deficiency (Bardella et al. 2011). Recently, three papers have reported stabilisation of Nrf2 following succination of cysteine residues important for the regulation of KEAP1 activity by oxidant stress in response to fumarate accumulation (Nguyen et al. 2009; Adam et al. 2011; Ooi et al. 2011).

In order to better elucidate the early events and signalling pathways that contribute to the onset of FH-associated tumourigenesis an appropriate and robust model system is required as the condition is relatively rare with currently 100 families worldwide reported as being affected, of which only 2-16% present with aggressive renal cancer (Kiuru et al. 2001; Launonen et al. 2001; Tomlinson et al. 2002; Toro et al. 2003; Chan et al. 2005). At the time of submission of this work another murine and two human cellular models of FH-deficiency emerged; a mouse renal cell line derived from a *Fh1* conditional knockout mouse (Frezza et al. 2011), one human cell line of

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metastatic origin removed from a patient with a recurring cancer (UOK 262 cells) (Yang et al. 2010) and the other a spontaneously immortalised primary renal tumour cell line derived from a HLRCC patient (Tong et al. 2011). In 2005, an Fh1 conditional knockout mouse was developed for the study of Fh1 deficiency *in vivo*, as described in Chapter 1 (Pollard et al. 2007). This model incorporates two loxP sites flanking the DNA sequences of exons 3 and 4 of both *Fh1* alleles with homologous recombination of the loxP sites, driven by a tissue-specific Cre recombinase (Ksp 1.3/Cre), facilitating the excision of the flanked exons and thus inactivation of *Fh1* (Pollard et al. 2007). The background and use of the Cre/loxP system is discussed in greater detail in Chapter 1 and Chapter 3.

At the commencement of this thesis, published work predominantly focused on the use of either siRNA (Sudarshan et al. 2009; Xie et al. 2009) or pharmacological compounds, such as the irreversible inhibitor of succinate dehydrogenase and fumarate hydratase, 3-nitropropionic acid (3-NPA), to inhibit FH activity *in vitro* (Zeevalk et al. 1995; Binienda et al. 2001; Isaacs et al. 2005; Selak et al. 2005; Hewitson et al. 2007).

The development of an Fh1-deficient MEF line was desirable as, in the absence of cell models with endogenous knockout of *Fh1* or *FH*, such a cell line was considered to be an effective tool for the study of early biochemical, metabolic and signalling events which might contribute to FH-dependent pathogenesis. Both primary and immortalized MEFs have been extensively used as cellular models for elucidating a plethora of cellular phenotypes, contributing not only to our knowledge of cell growth but also of cell cycle regulation, transformation, tumourigenesis (Sun and Taneja

Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts

2007), immunology (Yan et al. 2010) and ageing (Karatza et al. 1984) to list but a few areas of research.

This chapter describes the generation and characterisation of MEF cell lines with the aim of examining early events and changes in cell metabolism and cell signalling pathways in an Fh1-deficient background. Characterisation of these MEF lines was carried out with respect to their Fh1 activity, metabolite levels as measured by ^1H -magnetic resonance spectroscopy (^1H -MRS), respiratory defects, influence of Fh1-deficiency on cell growth compared with Fh1 wildtype cells and knockdown of Ldha in Fh1-deficient MEFs in order to assess the effect of combined Fh1- and Ldha-deletion on cell lethality as is detailed in the results section below.

2.2 Results

2.2.1 Breeding strategy for generating mouse embryonic cell lines

Fh1 mice, with loxP sequences flanking exons 3 and 4 (Pollard et al. 2007), were intercrossed for the purpose of generating progeny with the following *Fh1* phenotypes: *Fh1*^{+/+} (wildtype), *Fh1*^{fl/+} (heterozygous) and *Fh1*^{fl/fl} (homozygous). Pregnant females were sacrificed and embryos dissected from the uterus at day 14.5 (E14.5) with MEFs generated as outlined in the Materials and Methods. Tissue samples were taken from each embryo and genomic polymerase chain reaction (PCR) carried out to confirm the genotypes (*Fh1*^{+/+}, *Fh1*^{fl/+} or *Fh1*^{fl/fl}) of the MEFs. Subsequently, the MEF lines were either maintained as a primary culture (where

Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts

specified) or immortalized following transfection with a pBabe-puro retroviral vector encoding simian virus 40 (SV40) Large T antigen (Gjoerup et al. 2007) as detailed below.

2.2.2 Immortalisation of Fh1 MEF lines and in vitro knockdown of Fh1 in primary and immortalised Fh1 MEFs

To establish immortalized MEFs, cells at passage 2 were infected with a pBabe puro SV40 Large T antigen (pBabe-puro construct was a kind gift from Prof. P. Jat, University College London) in the presence of 5µg/ml Polybrene. Infected cells were grown in medium supplemented with 2µg/ml Puromycin for two weeks to select for immortalised cells after which time approximately 40% of the initial cell population evaded cell death. Complete cell death occurred in uninfected control cells grown in the presence of 2µg/ml Puromycin. *In vitro* recombination of the loxP sites in both primary and immortalized Fh1^{fl/+} and Fh1^{fl/fl} MEFs was facilitated by infecting the cells for 72 hours with an adenovirus IRES-EGFP reporter plasmid encoding a bacteriophage P1 Cre recombinase (AD-Cre-GFP). The pIRES-EGFP vector harbours an internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) that enables translation of both the Cre recombinase gene and the enhanced green fluorescent protein (EGFP) gene by a single bicistronic mRNA, such that the translation efficacy of the gene of interest can be measured on the basis of EGFP expression. However, differences in the efficiency of IRES-dependent expression of two genes (i.e. EGFP (or GFP as it will be referred to for the remainder of the text) and the target gene) have been reported (Rees et al. 1996; Mizuguchi et al. 2000). In particular the study by Mizuguchi *et al.* showed that expression of the IRES-

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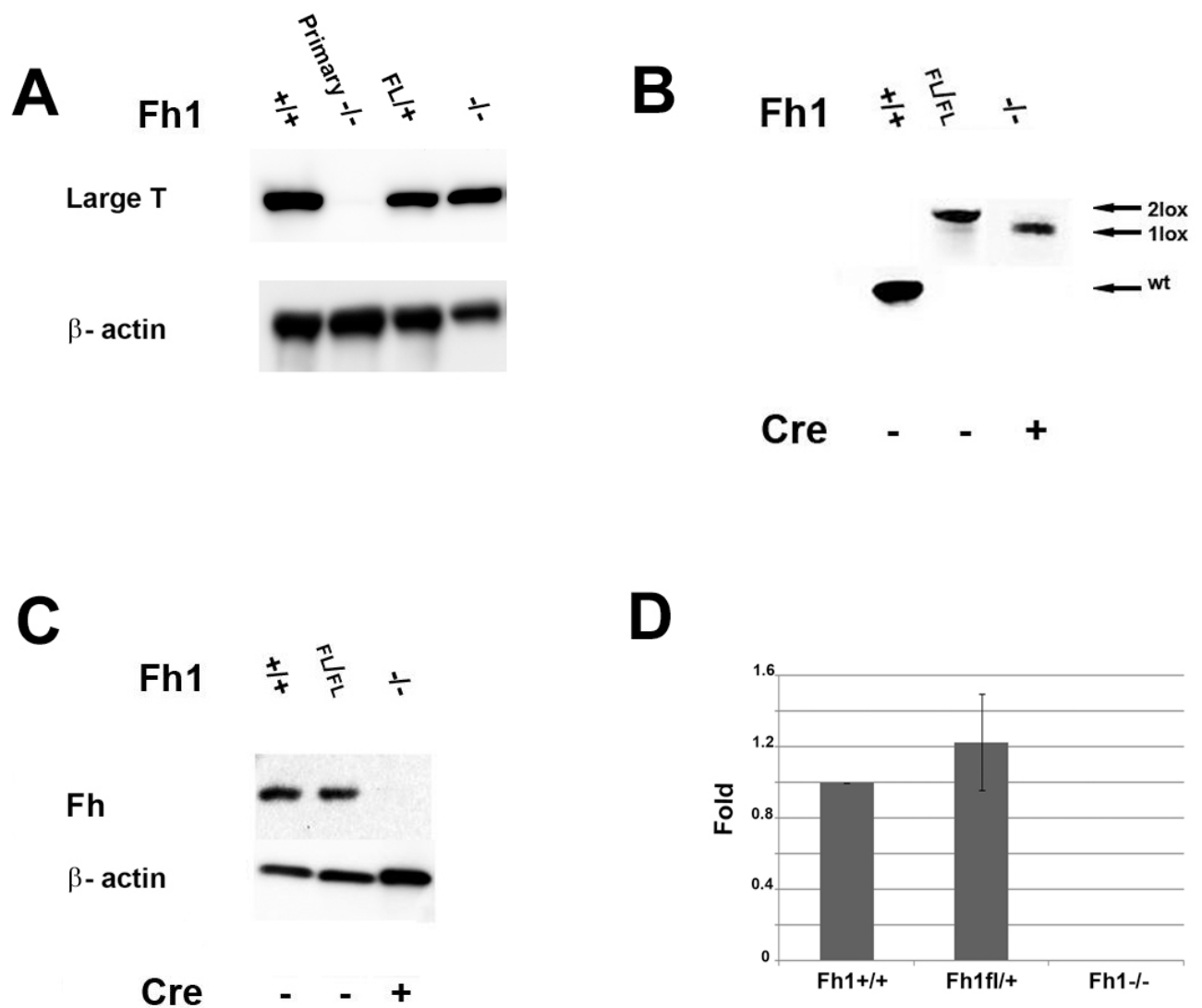
dependent second gene ranged from 6 to 100% (in most cases between 20 and 50%) that of the first gene (Mizuguchi et al. 2000). Therefore care should be taken regarding the decreased capacity of IRES-dependent downstream gene expression and also in determining which gene should be positioned as the first or second gene in a bicistronic vector. In light of this GFP expression should not be used as the sole means of quantifying the infection efficacy of the AD-Cre-GFP vector and should be measured in parallel with immunoblot analysis of the protein levels of the target gene.

Initial infections were carried out using 1×10^8 PFU/ml AD-Cre-GFP and analysed for recombination by genomic PCR 72 hours post-transfection. Immunoblot analysis of cellular extracts taken from immortalized MEFs confirm the presence of Large T protein, with non-immortalized Fh1^{-/-} MEFs used as a negative control (Figure 2.1A). While GFP expression was suboptimal with only approximately 10% of the infected cell population positive for GFP, knockout of the *Fh1* allele in the presence of translated Cre recombinase was confirmed by genomic PCR 72 hours post-transfection (representative PCR results shown in Figure 2.1B). As GFP expression was not a reliable method for selecting cells expressing Cre recombinase, a serial dilution of heterogeneous pools of immortalized MEFs was carried out to isolate colonies of a single cell origin and immunoblot and quantitative (Q)-PCR analyses performed to determine the presence or absence Fh1 protein and mRNA, respectively (Figure 2.1C and D, respectively).

As the MEFs were to be utilized for long term experimental analysis it was not feasible to continue growing the primary MEF lines in culture. While primary cell lines are more representative of the *in vivo* functions of the tissues from which they

Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts

originate they can, however, undergo spontaneous immortalisation or senescence making them less reliable for studies requiring multiple passages and long-term culturing. Therefore, for the remainder of the text it is to be assumed that all experiments were carried out using the immortalized MEF lines, unless stated otherwise.

Generation and characterisation of *Fh1*-deficient murine embryonic Fibroblasts**Figure 2.1 Validation of *Fh1*^{-/-} MEFs**

(A) Immunoblot analysis confirming the immortalisation of *Fh1*^{+/+}, *Fh1*^{fl/+} and *Fh1*^{-/-} MEFs as indicated by the presence of Large T antigen. An *Fh1*^{-/-} primary (non-immortalised) MEF line was used as a negative control. (B) PCR analysis of genomic DNA confirming excision by Cre recombinase of the floxed alleles in *Fh1* deficient (*Fh1*^{-/-}) MEFs. (C) Immunoblot analysis confirming the loss of Fh1 protein and (D) the absence of Fh1 mRNA, as shown by Q-PCR, in *Fh1*^{-/-} MEFs. Results are shown as mean $\pm$ SD.

*Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts**2.2.3 Assessment of karyotype and chromosomal stability of Fh1 MEFs*

While humans are diploid (2n) organisms, polyploidy (defined as having more than two sets of each chromosome) is a common occurrence in disease states such as cancer (Storchova and Pellman 2004). Polyploidy can also occur following immortalisation of cell lines and in some cases specialised cells, such as megakaryocytes, are maintained in a state of polyploidy in order to produce larger quantities of protein {Martinez, 2003 #1491; Watson, 2004 #1492}.

Karyotypic analysis of the Fh1 MEFs was carried out by multiplex-fluorescence *in situ* hybridization (mFISH) (performed by Dr Emanuela Volpi, Cytogenetics Core Facility, WTCHG, Oxford). This technique enables chromosomal changes to be identified by hybridising or ‘painting’ metaphase chromosomes with a mixture of coloured fluorescent probes (21XMouse Mouse mFISH by MetaSystems) (Speicher et al. 1996; Eils et al. 1998; Raap 1998; Lodish 2000; Anderson 2010). Fh1 MEFs were analysed at passage number 10 and for each Fh1 cell line approximately 20 metaphases were imaged (Figure 2.2B(i) shows a representative metaphase spread for each MEF line) and analysed with 24 colour mFISH. The occurrence of spontaneous polyploidy was observed in all the cell lines analysed with the extent of polyploidy (multiple copies of each chromosome) ranging from tetraploidy (4n)/near-tetraploidy (4n+) (present in all cell lines) to hexaploidy (6n)/near-hexaploidy (6n+) (Fh1fl/+ and Fh1+/+ MEFs) and octaploidy (8n)/near-octaploidy (8n+) (Fh1+/+ MEFs) (Figure 2.2A).

Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts

Failure of fused sister chromatids to segregate during anaphase results in the formation of a chromatin bridge, also referred to as an anaphase bridge (McClintock 1941). The appearance of anaphase bridges during mitosis is indicative of chromosomal instability and there is increasing evidence that both of these occurrences are strongly associated with tumourigenesis in both humans (Fouladi et al. 2000; Gisselsson 2003; Montgomery et al. 2003; Hoffelder et al. 2004) and mice (Artandi et al. 2000; Rudolph et al. 2001). In order to assess the status of chromosomal stability in the Fh1 MEFs, DAPI staining and imaging of metaphase chromosome preparations were carried out by Dr. Volpi. Indications of chromosomal instability were found in all of the Fh1 MEF lines analysed as was evident from the presence of nuclear blebs and anaphase bridges (Figure 2.2B(ii)). Interestingly, compared to chromosome preparations from either Fh1^{fl/+} or Fh1^{+/+} MEFs, numerous micronuclei (containing either whole chromosomes or fragments of chromatids) were visible in Fh1^{-/-} chromosome preparations. This finding may be indicative of increased DNA damage or mutations in Fh1^{-/-} MEFs which may be consistent with the reported role of FH in initiating the DNA damage response (Yogev et al. 2010). These findings are not unexpected among immortalised cell lines such as the Fh1 MEFs, which were transformed with a SV40 Large T antigen proto-oncogene.

As Fh1^{fl/+} MEFs were shown not to be significantly different from Fh1^{+/+} MEFs with respect to Fh1 mRNA expression (Figure 2.1D) and protein levels (see Appendix Figure 2.1) and chromosomal stability (Figure 2.2B(ii)), it was considered sufficient to continue experimental analysis using only one 'wildtype' control cell line (Fh1^{+/+} MEFs) for comparison with the Fh1-deficient cells.

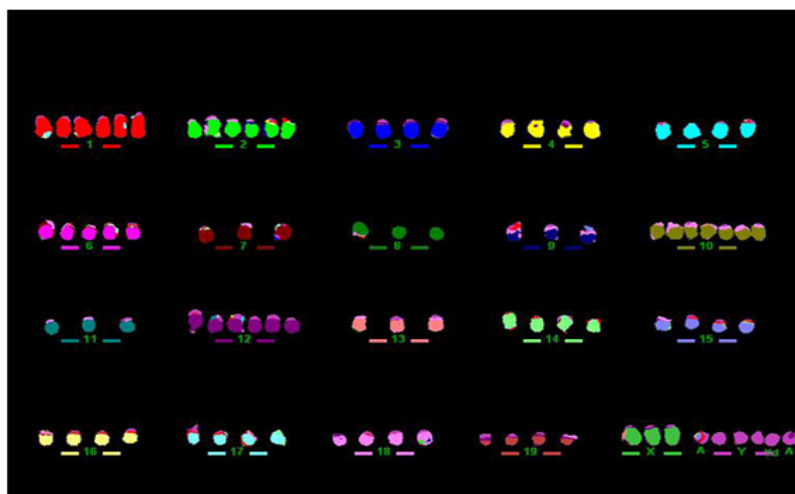
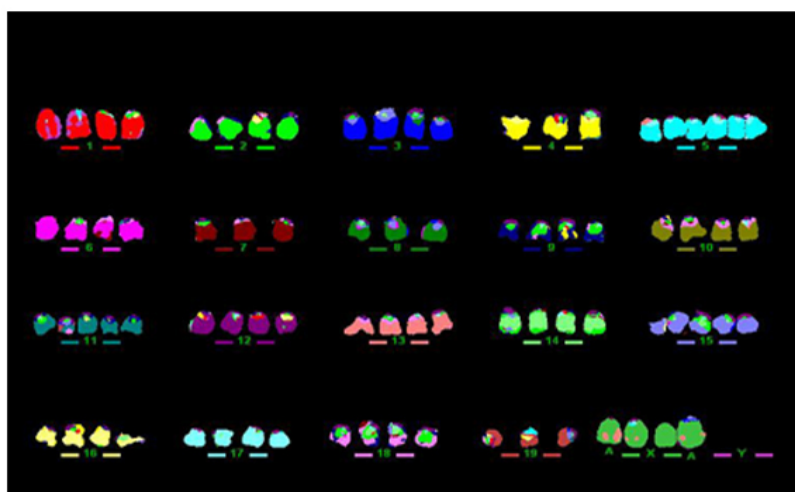
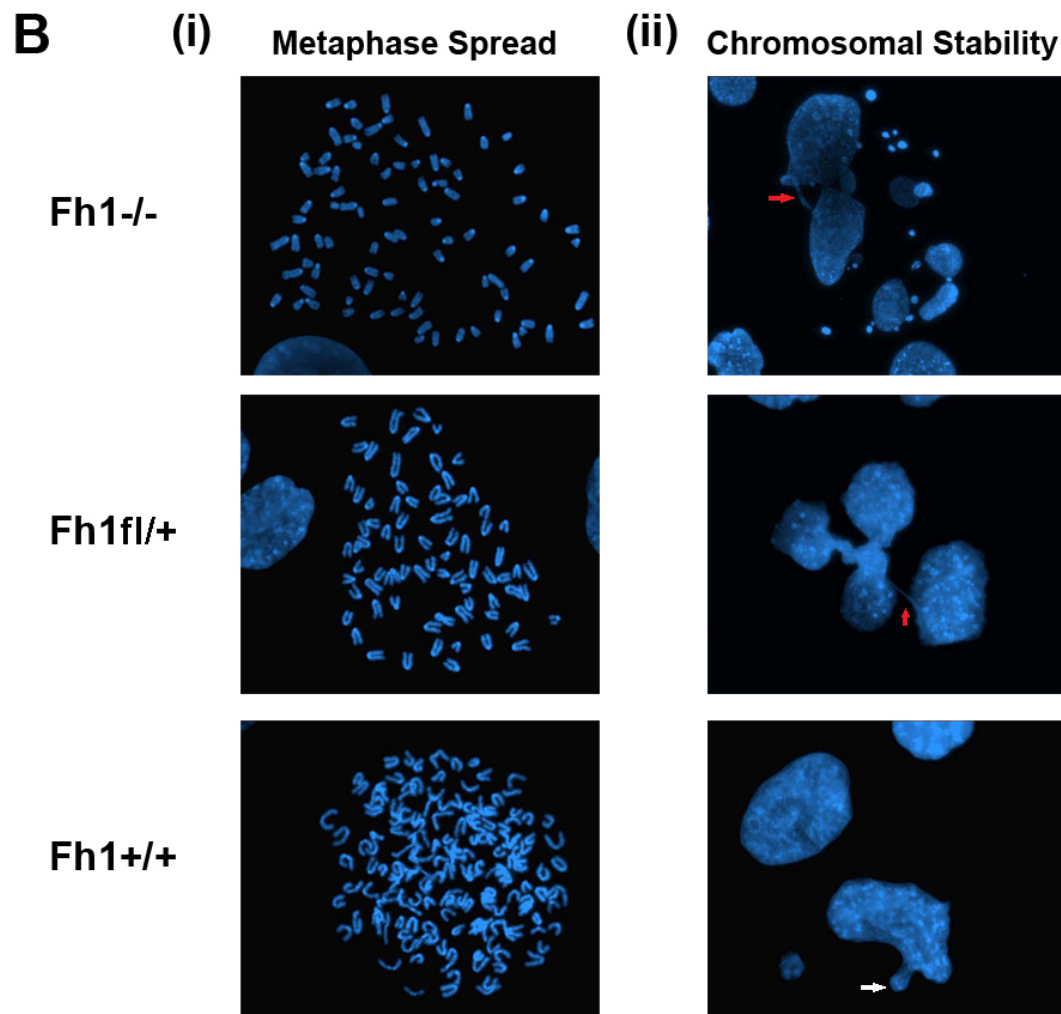
Generation and characterisation of *Fh1*-deficient murine embryonic Fibroblasts**A***Fh1* $-/-$ *Fh1* fl/+*Fh1* +/-

Figure 2.2 mFISH Analysis

*Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts***Figure 2.2 mFISH Analysis**

Chromosomal preparations from $Fh1^{-/-}$, $Fh1^{fl/+}$ and $Fh1^{+/+}$ MEFs were analysed by (A) mFISH analysis (employing 24 colour karyotyping) and (B) DAPI counterstaining and chromosome-specific ‘painting’ of a representative metaphase from each line. Overall, approximately 20 metaphases for each line were analysed. (A) mFISH confirmed that all of the $Fh1$ MEF lines were polyploid. $Fh1^{-/-}$ MEFs are predominantly tetraploid for the majority of chromosomes but hexaploid for five chromosomes and similarly $Fh1^{fl/+}$ MEFs are tetraploid with the exception of three chromosomes. In contrast, chromosomes from $Fh1^{+/+}$ MEFs are mainly in a hexaploid or octoploid state with only a small number present in tetraploidy.

Generation and characterisation of *Fh1*-deficient murine embryonic Fibroblasts**Figure 2.2 Analysis of chromosomal stability**

(B)(i) DAPI staining of *Fh1* MEF chromosome preparations illustrating the condensed and coiled form of the chromosomes during metaphase. The *Fh1^{+/+}* chromosomes are more dense and coiled than the *Fh1^{-/-}* and *Fh1^{fl/+}* due to the presence of the additional chromosome sets described in (A). (B)(ii) Nuclear blebs (indicated by white arrow) are present in *Fh1^{-/-}* and *Fh1^{+/+}* MEFs with anaphase bridges (indicated by red arrows) also visible in *Fh1^{-/-}* and *Fh1^{fl/+}* MEFs. Numerous micronuclei are visible in *Fh1^{-/-}* chromosome preparations, compared to chromosome preparations from either *Fh1^{fl/+}* or *Fh1^{+/+}* MEFs, which is suggestive of increased DNA damage or mutations in *Fh1^{-/-}* MEFs.

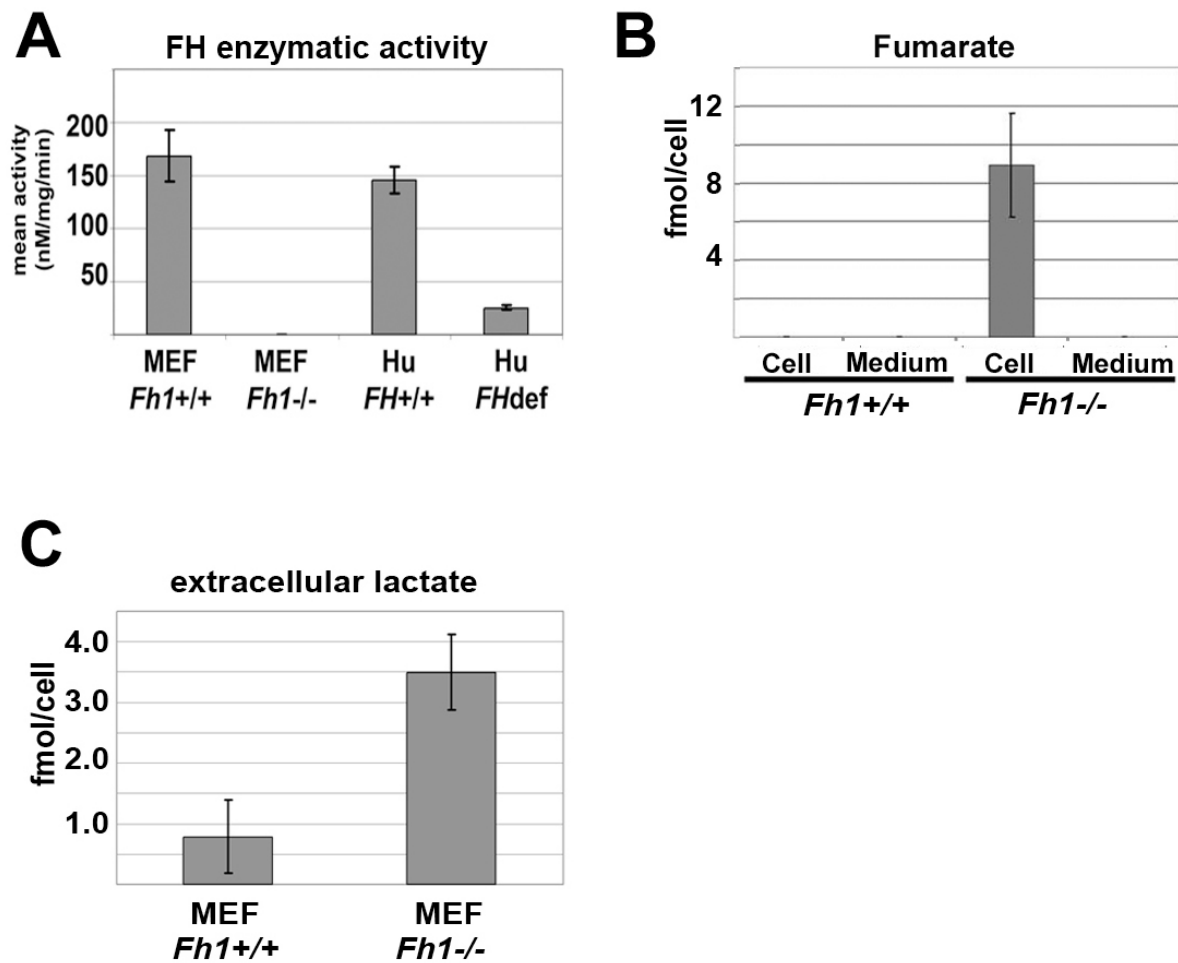
*Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts**2.2.4 Fh1 activity and metabolic alterations in Fh1-deficient MEFs*

To verify that excision of *Fh1* following recombination of the loxP sites led to ablation of cellular Fh1 enzyme function, an FH enzyme activity assay was carried out by Joanne Croft of the Sheffield Children's NHS Trust, UK. Briefly, this assay involved addition of exogenous malic enzyme (from chicken liver) to MEF cellular extracts and measurement of the reduction of NADP^+ to $\text{NADPH}^+ + \text{H}_2$; this assay requires FH/Fh1 activity in order to generate malate as a substrate for malic enzyme (see Chapter 6, section 6.12 for detailed methods). Using skin fibroblast cells from FH-deficient and FH wildtype ($\text{FH}^{+/+}$) patients (Alam et al. 2003) as positive and negative controls respectively, absence of Fh1 activity in $\text{Fh1}^{-/-}$ MEFs was in striking contrast to the fully functional $\text{Fh1}^{+/+}$ MEFs and $\text{FH}^{+/+}$ skin fibroblasts (Figure 2.3A). This provided confirmation that abrogation of Fh1 enzyme activity is complete in $\text{Fh1}^{-/-}$ MEFs. As FH-deficient patients are often carriers of either homozygous or compound heterozygous mutations they retain detectable FH activity, however this is substantially reduced as seen in Figure 2.3A and is sufficient to cause mental retardation and encephalopathies (Coughlin et al. 1998).

It was hypothesised and subsequently confirmed that loss of Fh1 in $\text{Fh1}^{-/-}$ MEFs is responsible for a switch in cellular metabolism from OXPHOS to aerobic glycolysis as a consequence of a defective TCA cycle (O'Flaherty et al. 2010; Zhdanov et al. 2011), as will be demonstrated and discussed in greater detail in Chapter 3. Therefore, it could be predicted that the endogenous levels of metabolites from respiratory and other associated pathways might also be affected. To assess this, a small subset of metabolites was measured by ^1H -MRS. As predicted, accumulation of intracellular

Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts

fumarate was observed in Fh1^{-/-} cells when compared with Fh1^{+/+} MEFs (Figure 2.3B). The inability to detect fumarate in Fh1^{+/+} MEFs could most likely be attributed to the rapid turnover of fumarate to malate in the presence of functional Fh1. To date no specific mechanism for fumarate transport across the plasma membrane has been described and in keeping with this extracellular fumarate was not detected in medium extracts from either Fh1^{-/-} or Fh1^{+/+} MEFs (Figure 2.3B). Increased cellular lactate was also observed in Fh1^{-/-} MEFs (Figure 2.3C) and is suggestive of an increase in glycolytic metabolism. Implications for this phenotype are discussed in further detail in section 2.2.6.

Generation and characterisation of *Fh1*-deficient murine embryonic Fibroblasts**Figure 2.3 Metabolite analyses of *Fh1*^{-/-} MEFs**

Analysis of *Fh1*-deficient (*Fh1*^{-/-}) MEFs. (A) FH activity was compared between *Fh1*^{+/+} and *Fh1*^{-/-} MEFs. Skin fibroblast cells from FH-deficient patients (Alam et al. 2003) and *FH*^{+/+} patients were used as negative and positive controls, respectively. *Fh1*^{-/-} MEFs show no detectable FH activity, whereas the *Fh1*^{+/+} MEFs and normal human skin fibroblasts show equivalent levels of activity. (B) Analysis of intracellular fumarate in cell extracts and extracellular fumarate in medium extracts of *Fh1*^{-/-} and *Fh1*^{+/+} MEFs. (C) Analysis of lactate levels in cellular extracts from *Fh1*^{-/-} and *Fh1*^{+/+} MEFs. *Fh1*^{-/-} MEFs show increased levels of extracellular lactate compared with *Fh1*^{+/+} MEFs.

*Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts**2.2.5 Respiratory defects in Fh1-deficient MEFs and effect on cell growth*

In the absence of FH, fumarate is unable to be catalysed to malate, which in turn is required to supply oxaloacetate as a substrate for citrate synthase in order to commence another round of the TCA cycle. As a consequence of the blockade on the TCA cycle there is no longer a sufficient supply of electrons (in the form of NADH, FADH₂ or NADPH generated by the pentose 5'-phosphate pathway, using TCA cycle intermediates) to feed into the ETC to generate adequate ATP via OXPHOS. In keeping with the lack of FH activity in the Fh1-deficient MEFs, cellular respiration in Fh1^{-/-} cells was found to be markedly suppressed. Using a Clark type oxygen electrode, basal unstimulated mitochondrial respiration was measured in intact cells which was found to be decreased by approximately 80% in the Fh1^{-/-} MEFs compared with Fh1^{+/+} MEFs (Figure 2.4A).

With OXPHOS impaired and the rate of ATP generation compromised as a result (see chapter 3, Table 3.1 and Figure 3.4C) it could be predicted that other signalling pathways and cellular processes, such as anabolic/catabolic reactions and biomass production and transport to list but a few examples, would be considerably affected. In order to ascertain whether the rate of cell growth of Fh1^{-/-} MEFs is altered by their metabolic switch from OXPHOS to glycolysis, compared to their wildtype counterparts, a standard cell count, including trypan blue exclusion as an indicator of cell death, at 12 hour intervals over a 72 hour period was performed. Cell growth was measured under standard 'normoxic' tissue culture conditions (21% oxygen) (Figure 2.4B(i)) and 1% hypoxic conditions to activate the Hif pathway and mimic the hypoxic environment present in solid tumours (Figure 2.4B(ii)).

Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts

The resulting data illustrates an observable difference in the growth rate of the two MEF lines under normoxia with more than a two-fold doubling time observed for Fh1^{-/-} MEFs compared to Fh1^{+/+} MEFs. Fh1^{+/+} cell growth peaked at 24 hours having reached confluency which was followed by a declining growth rate due to increased cell death. Neither the Fh1^{-/-}1A1.8 nor Fh1^{-/-}1A1.19 MEFs reached confluency, however, cell growth decreased for both cell lines after 48 hours. This may be attributed to reduced nutrient and glucose availability as the medium was not changed for the duration of the normoxic and hypoxic proliferation studies. When grown in 1% hypoxia Fh1^{-/-} MEFs showed a subtle increase in cell growth rate (most noticeable at 48 hours) compared to the rate of growth observed when grown in culture under 21% oxygen. These findings may highlight a cellular adaptation that enables Fh1-deficient cells to acquire a selective advantage for proliferating in the hypoxic environments. Adaptation to hypoxia has been observed in numerous neoplastic cell lines capable of sustaining growth within hypoxic regions of solid tumours (Hockel and Vaupel 2001).

In contrast, Fh1^{+/+} MEFs show reduced growth under 1% hypoxia though they do still demonstrate an ability to proliferate under these conditions. This may be attributed to the fact that primary MEF cell lines, compared to human fibroblasts, have extended proliferation at physiological oxygen concentrations (3% O₂) and sustain more cell damage when grown at 20% oxygen (Karatza et al. 1984; Di Micco et al. 2008).

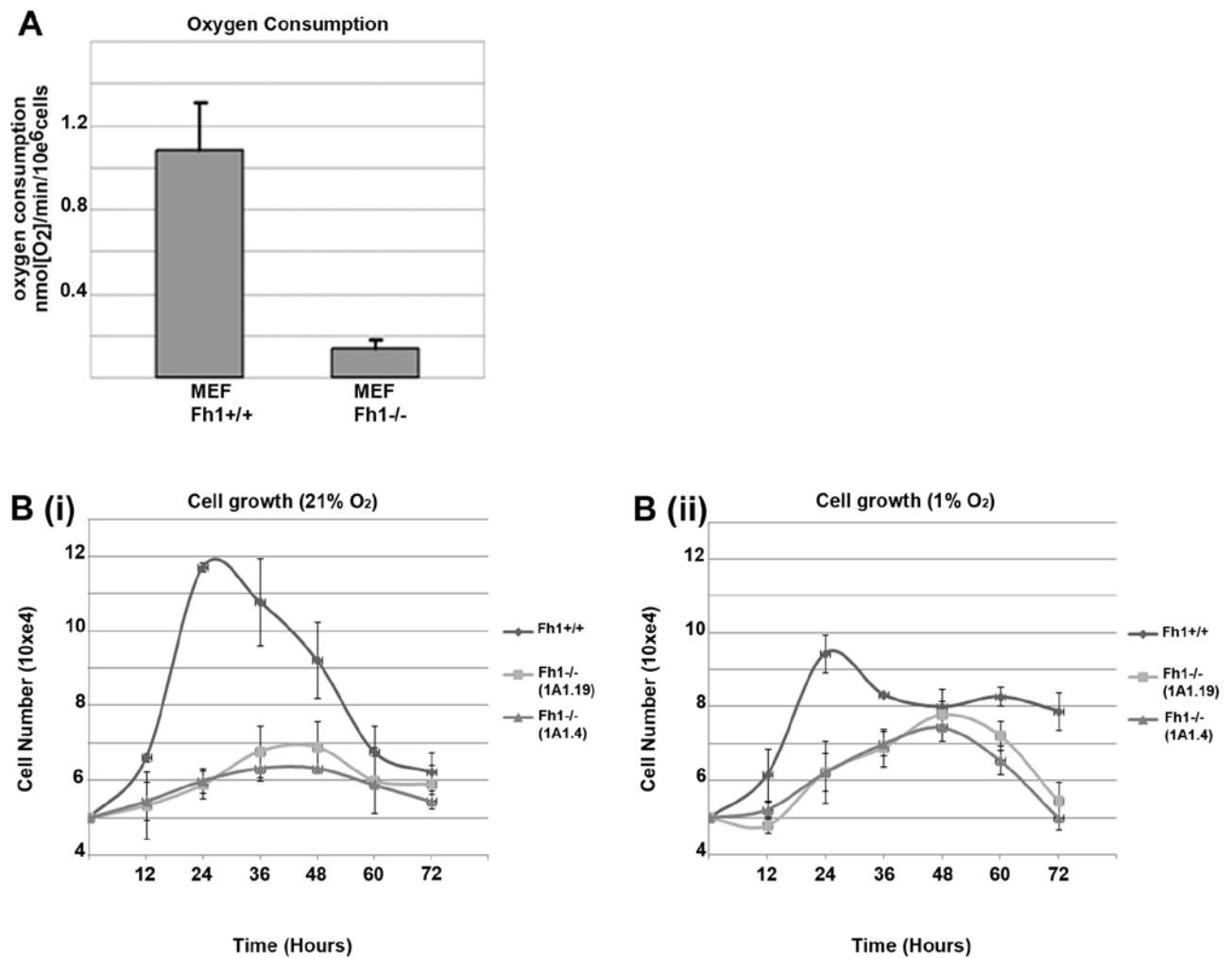
Generation and characterisation of *Fh1*-deficient murine embryonic Fibroblasts

Figure 2.4 Confirmation of respiratory defects in *Fh1*^{-/-} MEFs

(A) A reduction in oxygen consumption was observed in *Fh1*^{-/-} MEFs compared to *Fh1*^{+/+} MEFs. Data shown is mean±S.D. of triplicate assays. (B) Proliferation study conducted under (i) normoxia (21% oxygen) comparing two independent *Fh1*^{-/-} clones (*Fh1*^{-/-} 1A1.19 and *Fh1*^{-/-} 1A1.4) with *Fh1*^{+/+} MEFs illustrates a diminished cell growth rate in *Fh1*-deficient MEFs. (ii) Under 1% hypoxia the two *Fh1*^{-/-} MEF lines and the *Fh1*^{+/+} cells experience a diminished rate of cell growth rate. Data shown is mean±S.D. of biological triplicates.

*Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts**2.2.6 Knockdown of Ldha in Fh1-deficient MEFs results in increased cell death*

Under anaerobic conditions or conditions of limited oxygen supply, pyruvate, the end product of glycolysis, bypasses conversion to acetyl-CoA which is a crucial intermediate for the TCA cycle and is instead reduced by lactate dehydrogenase isoenzyme A (LDH-A) to produce lactate which maintains glycolysis by supplying NAD^+ as a substrate for glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Due to this close association between increased lactate production and glycolysis, lactate is often used as a marker for indicating a shift from OXPHOS to glycolysis-driven metabolism and is furthermore present at high levels in many cancers, as originally observed by Otto Warburg (Warburg et al. 1927; Walenta et al. 2004; Fantin et al. 2006). Increased levels of cellular lactate in cancer can also be linked to the hypoxic conditions found in many tumours as *LDH-A* is a downstream transcriptional target of stabilised HIF- α (as discussed in further detail in chapter 3). For these reasons there is increased interest in the inhibition of *LDH-A* as a potential strategy for therapeutic intervention in the treatment of cancers, including HLRCC (Xie et al. 2009; Granchi et al. 2010; Le et al. 2010).

In 2009 Seth and colleagues (Xie et al. 2009) showed that knockdown of LDH-A in an FH-deficient A549 lung carcinoma cell line increased apoptosis and furthermore that this combined knockdown was sufficient to reduce tumour growth in a xenograft study. Therefore, having detected increased cellular levels of lactate (Figure 2.2C) and expression of *Ldha* (see Chapter 3, Figure 3.1B) in Fh1-deficient MEFs, it remained to be established whether knockdown of *Ldha* in Fh1-null cells would result in increased cell death. In order to assess this, Fh1^{-/-} cells were infected with three

Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts

independent SMARTvector shRNA lentiviral particles containing a GFP reporter (shRNA 1, shRNA 2 and shRNA 3) targeting *Ldha* at either exon 8 (shRNA 2) or two different regions of exon 9 (shRNAs 1 and 3) for inhibition. Fh1^{-/-} uninfected cells and Fh1^{-/-} cells infected with a non-targeting shRNA were used as controls. All analyses were carried out 96 hours post-infection. The *Ldha*-targeted shRNA sequences were designed with a puromycin-resistance selection cassette, however as the immortalized MEF cells were selected using puromycin, this resistance marker could not be used to select for successful cellular integration of these sequences. Therefore infection efficiency was assessed on the basis of GFP expression following integration of the lentiviral particle into the host cells. Infection efficiency ranged from 20% (shRNA 3) - 90% (shRNA 1), as indicated by the level of GFP expression in each cell population measured using an inverted fluorescence microscope (Figure 2.5A). Although seeded at the same density (3×10^4 cells per well), there are substantially fewer cells at 96 hours for Fh1^{-/-} cells treated with shRNA 1 and marginally reduced numbers for shRNA 3 treated cells when compared with the non-targeting control, which is suggestive of increased cell death. No apparent effect on cell density can be seen with shRNA 2 treated Fh1^{-/-} MEFs, however, as for shRNA 3, this may be a consequence of a lower infection efficiency. The uninfected Fh1^{-/-} MEFs prepared for analysis by fluorescence microscopy were subconfluent at 96 hours and therefore cannot be used as a direct comparison of cell density, but merely as a GFP negative control.

Immunoblot analysis of shRNA-treated cells showed complete knockdown of *Ldha* with all three shRNAs, though not in the non-targeting shRNA control and uninfected Fh1^{-/-} MEFs (Figure 2.5B), as expected. However, validation of *Ldha* knockdown at

Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts

an mRNA level by quantitative analysis did not show ablation of *Ldha* mRNA expression (Figure 2.5D). Possible explanations for this might be that 96 hours is not a sufficient duration of infection to see loss of expression on an mRNA level or simply that the Taqman® Q-PCR gene probe for *Ldha* is capable of recognising and amplifying the 3' region of *Ldha* not silenced by the shRNA sequences. To rule out compensation by *Ldh* isoenzyme b, the expression level of this isoenzymes was also measured by Q-PCR (see Appendix Figure 2.2). Briefly, the results indicated that *Ldhb* expression levels were increased approximately two-fold compared to *Fh1*^{-/-} MEFs infected with shRNAs 1 and 2 compared with the uninfected and non-targeting shRNA controls, respectively; there was no change in *Fh1*^{-/-} MEFs infected with shRNA 3. As there is no distinguishing pattern with regard to increased expression of *Ldh* isoenzymes between the control cells and the shRNA-target *Fh1*^{-/-} cells, compensation by these isoenzymes in an *Fh1*-deficient background following silencing of *Ldha* cannot be inferred.

The effect on cell growth was calculated by carrying out a cell count at 96 hours, 120 hours and 144 hours post-infection. this showed a significant decrease in cell growth for cells infected with shRNAs 1, 2 and 3 with marginal effects observed for the non-target shRNA control (n=3, biological replicates) (Figure 2.5D). Of the three shRNAs, shRNA 3 appears to be slightly less effective at silencing *Ldha* the shRNAs 1 and 2; however this could be due to the lower level of lentiviral expression in these cells. Data shown is mean±S.D. of biological triplicates.

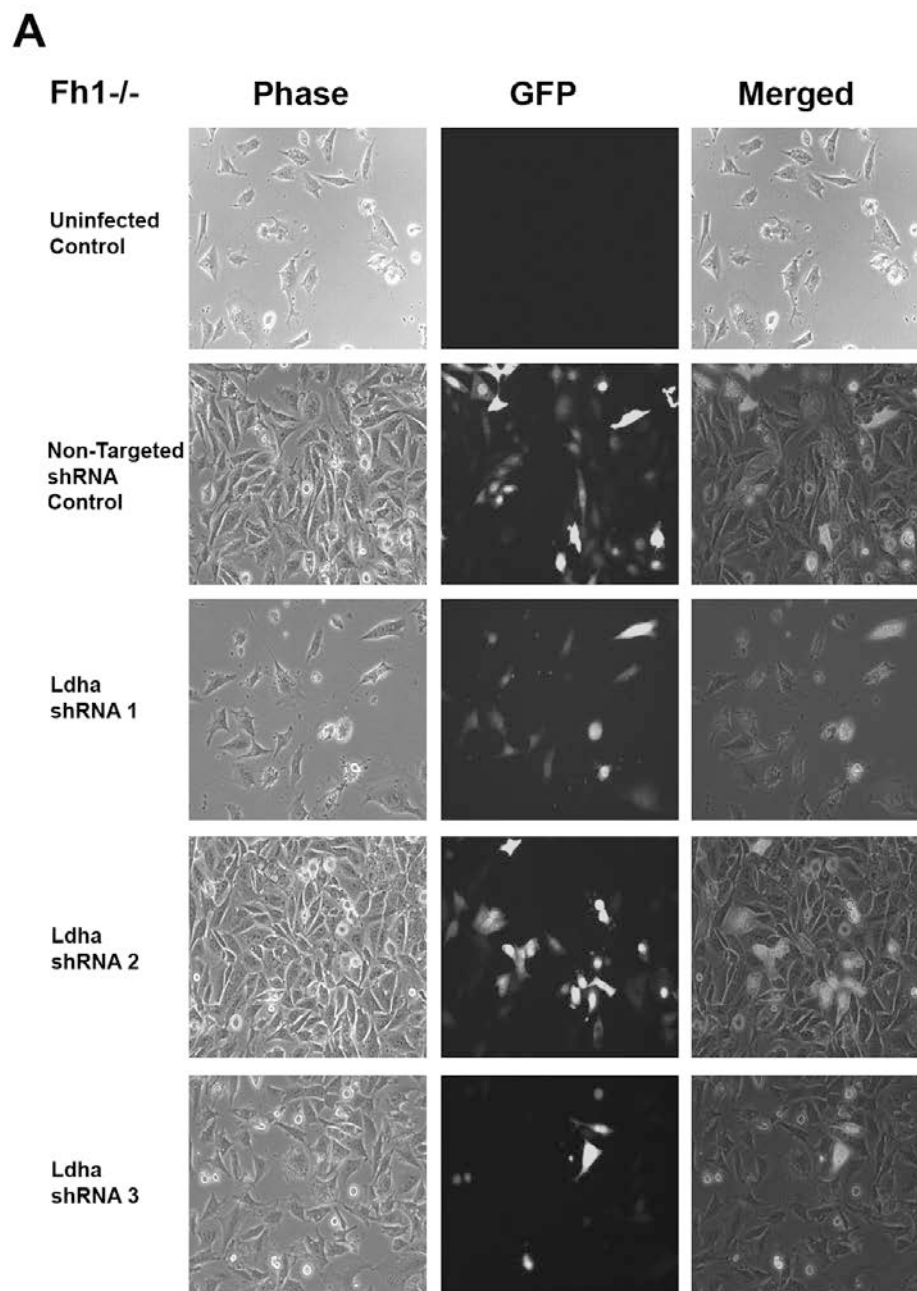
Generation and characterisation of *Fh1*-deficient murine embryonic Fibroblasts

Figure 2.5 shRNA knockdown of *Ldha* in *Fh1*^{-/-} MEFs

(A) Infection efficiency in *Fh1*^{-/-} cells of three independent shRNA sequences targeting *Ldha* for silencing, compared with a non-targeting shRNA control sequence. Infection of the cells was determined by levels of GFP expression measured using an inverted fluorescence microscope (Nikon TE2000U). Magnification shown was set at 60x.

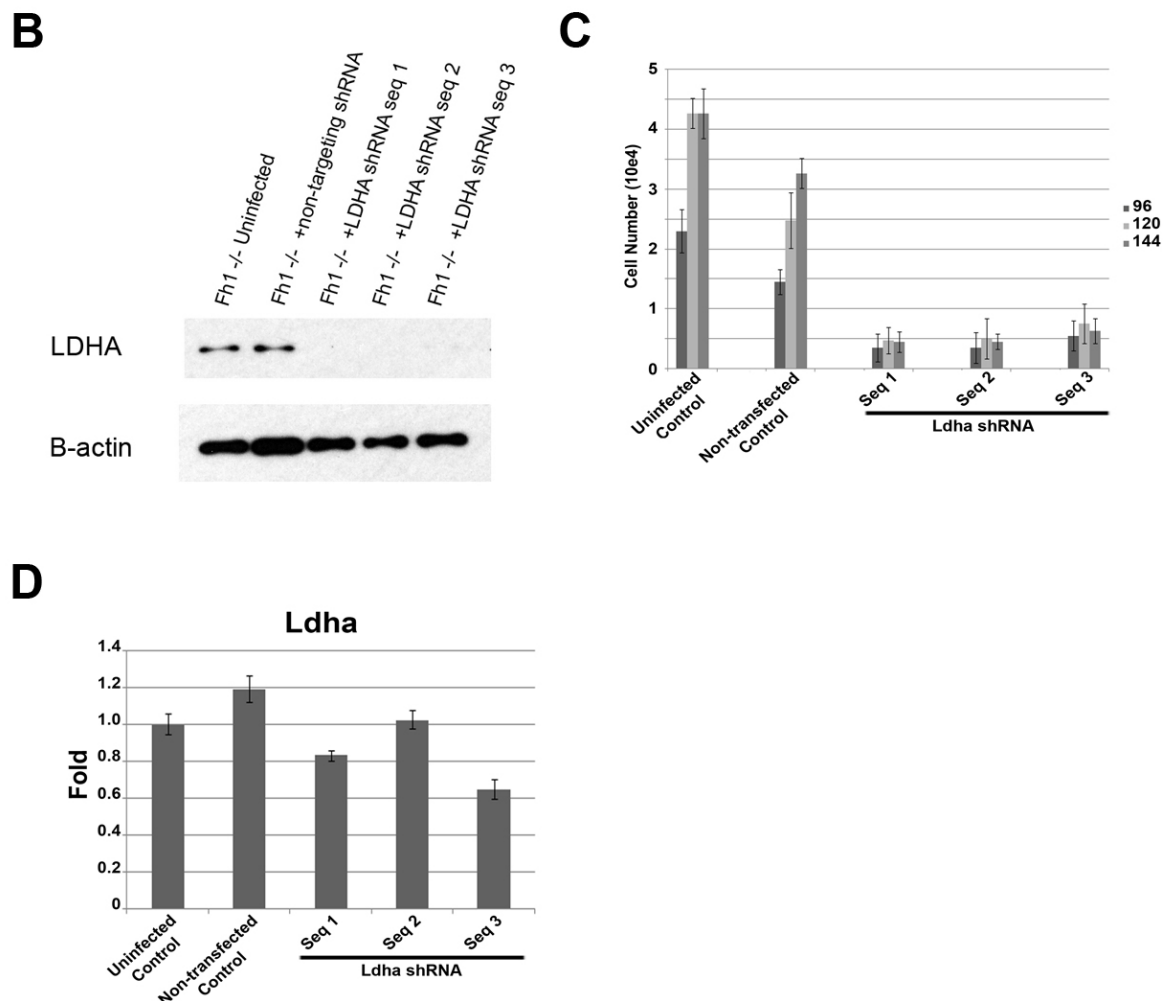
Generation and characterisation of *Fh1*-deficient murine embryonic Fibroblasts

Figure 2.5 shRNA knockdown of *Ldha* in *Fh1*^{-/-} MEFs

(B) Immunoblot confirmation of shRNA-mediated knockdown of *Ldha* in *Fh1*^{-/-} MEFs at 96 hours. (C) *Ldha* shRNA sequences (seq) 1, 2 and 3 significantly decrease cell growth in *Fh1*^{-/-} cells over a 144 hour period compared with uninfected *Fh1*^{-/-} MEFs and non-targeting shRNA controls. Results for (C) are shown as mean $\pm$ SD of biological triplicates. (D) In contrast to results shown in (B), Q-PCR analysis of *Ldha* knockdown at 96 hours shows only a small decrease in *Ldha* mRNA expression levels following infection of *Fh1*^{-/-} cells with shRNA sequences 1 and 3, and no apparent effect following infection with shRNA sequence 2. The disparity between the analysis of *Ldha* shRNA Seq 2 by Q-PCR and the results shown in (B) and (C) may be due to inefficient knockdown of *Ldha* mRNA levels. Results for (D) are shown relative to the mRNA levels in *Fh1*^{-/-} uninfected control cells; mean $\pm$ SD of triplicate assays.

*Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts***2.3 Discussion**

This chapter describes how Fh1-deficient MEFs were generated from Fh1 conditional knockout mice and the subsequent validation and characterisation of these lines with respect to the following: loss of Fh1 activity and expression, cellular oxygen consumption and alteration of cell growth under normoxic and hypoxic conditions. The results and implications for these experiments are discussed here briefly followed by a more detailed appraisal in Chapter 5 of the overall contribution of these findings to the field of FH and FH-associated HLRCC.

The justification for generating an Fh1-deficient cellular model in a MEF background arose from the necessity to address the lack of available model systems at the onset of this work that were capable of facilitating the study of a broad spectrum of primary events and adaptations that occur during early tumourigenesis. Only recently have other cell lines become available for the study of this condition (UOK262 and UOK268 human tumour cell lines and murine renal cell lines, as described previously). These models corroborate the findings of fumarate accumulation, Hif pathway activation and metabolic shift from OXPHOS to aerobic glycolysis that are outlined in the results of this chapter and those to be detailed in chapter 3, therefore strengthening the argument that the Fh1 MEF cell lines, while not of the same histological origin as those cells affected by HLRCC, namely epithelial, renal tubular cells and smooth muscle, are a reliable and robust model for *in vitro* studies in an Fh1-null background. Importantly, the Fh1^{-/-} MEFs have been shown to recapitulate many of the phenotypes observed in Fh1-deficient kidney tissues and also human

Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts

HLRCC-associated tumour tissues (Bardella et al. 2011). These Fh1 MEF models could therefore be described as models for studying the biochemical consequences of loss of Fh1, distinct from the pathological features associated with loss of Fh1. A further advantage for the use of a MEF system is that cell lines derived from established tumours, such as the UOK262 cells, have inevitably acquired secondary ‘hits’ and/or mutations and under some circumstances where patients may have undergone treatment with chemotherapeutics, have acquired resistance to certain compounds. Therefore, such cell lines could be argued to be redundant for analysing primary events *in vitro*.

Following characterisation of the MEFs it can be concluded that excision of *Fh1* by homologous recombination, driven by addition of exogenous AD-cre-GFP, is effective and while there are chromosomal instabilities in both the $Fh1^{-/-}$ and $Fh1^{+/+}$ MEFs following transformation, the cells produce consistent, robust data in relation to their expected phenotype. $Fh1^{-/-}$ MEFs showed a stable loss of Fh1 protein and activity (Figure 2.1C and Figure 2.2A) as well as a predicted increase in cellular fumarate (Figure 2.2B) compared to their $Fh1^{+/+}$ counterparts. Of interest, the data generated in this chapter suggest a reliance of $Fh1^{-/-}$ MEFs on glycolytic metabolism in that lactate production is increased (Figure 2.2C) in the presence of a reduced capacity to consume oxygen (Figure 2.4A) and reduced levels of ATP (see Figure 3.4C Chapter 3), indicative of a mitochondrial defect (O’Flaherty et al. 2010; Zhdanov et al. 2011).

The hypothesis that Fh1-deficient MEFs are dependent on glycolysis could, as with cancerous cells, be classified as exhibiting characteristics of the ‘Warburg effect’. As

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the TCA cycle is the cell's most efficient means of generating ATP and supplying electrons for the electron transport chain (ETC), deletion of one of its required enzymes would be sufficient to severely impair normal TCA cycle function and presumably necessitate a switch from OXPHOS to aerobic glycolysis in order to sustain cell viability. Otto Warburg postulated that such a shift in cellular metabolism enabled cancerous cells to utilize aerobic glycolysis in order to gain selective advantage (Warburg et al. 1927; Warburg 1956). This hypothesis is now referred to as the 'Warburg effect' and is gaining more support in light of growing evidence for metabolic dysregulation in cancer (Kaelin 2009; Bayley and Devilee 2010). While the tumour suppressor function of FH is well established it remained to be established whether deletion of FH in an *in vitro* system would result in a metabolic shift from oxidative phosphorylation (OXPHOS) to aerobic glycolysis, thus corroborating the 'Warburg effect' and potentially highlighting an important process involved in the early stages of tumourigenesis in FH-associated HLRCC. The findings of elevated expression of the glucose transporter, *Glut 1*, and glucose uptake in Fh1-deficient cells (Sudarshan et al. 2009; Ashrafian et al. 2010; O'Flaherty et al. 2010; Frezza et al. 2011) further support the presence of the 'Warburg effect' hypothesis in Fh1-deficient cells. Moreover, Warburg's hypothesis can be utilized in a clinical setting where the increased uptake of glucose in tumours of HLRCC patients can be visualised using 2-deoxy-2-(^{18}F)fluoro-D-glucose (FDG) positron emission tomography (PET) (Yamasaki et al. 2011).

Unsurprisingly, Warburg also observed elevated levels of both intracellular and extracellular lactate as a consequence of glycolytic metabolism. LDH, responsible for catalyzing the reaction for conversion of pyruvate to lactate, is not only a common

Generation and characterisation of Fh1-deficient murine embryonic Fibroblasts

diagnostic marker for tissue damage and haemolysis but is also a downstream target of active HIF-1 α and therefore it is often found to be upregulated in many cancers, including HLRCC (Sudarshan et al. 2009; Semenza 2010). Furthermore, a correlation between the loss of *Fh1* and a corresponding increase in *Ldha* expression has been reported in both *Fh1*^{-/-} MEFs (See Chapter 3, Figure 3.2B) and A549 cells with siRNA knockdown of FH (Xie et al. 2009; Ashrafian et al. 2010; O'Flaherty et al. 2010). Xie et al, also demonstrated that combined siRNA knockdown of FH and LDHA in A549 cells increased cell death *via* apoptosis and was capable of significantly reducing tumour growth in a xenograft model. While the investigation into the effect of shRNA-mediated knockdown of *Ldha* on cell growth in *Fh1*^{-/-} MEFs showed a negative effect on cell growth (Figure 2.5C), these findings were not consistent with the GFP and Q-PCR results. Moreover, the amount of cell death cannot be fully quantified using the method described for this experiment as it does not take into consideration the number of dead cells found in the cell medium. As expression of GFP, *Ldha* mRNA and *Ldha* protein were all measured at 96 hours, the discrepancies between these results could not have been addressed at an earlier point. Due to the limited volume available for each shRNA sequence the experimental design could not be improved upon or repeated for the purpose of this thesis. Given that this experiment was not successful, future efforts to repeat this experiment could be done using inducible shRNA constructs, fluorescent dyes (e.g. Hoechst 33342, acridine orange or propidium iodide) to measure cell culture viability with more accuracy and inclusion of wildtype MEF controls. The use of independent TaqMan® probes to prevent probe cross reactivity or a 3'UTR probe that would amplify all *Ldh* isoforms might also improve the Q-PCR data.

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To develop more effective therapeutic interventions for patients of HLRCC, and indeed all cancer types, and to reduce high mortality rates associated with late presentation, identification of specific and robust biomarkers for early stage tumourigenesis is urgently needed. In order to meet this demand and improve treatments suitable *in vivo* and *in vitro* biological models are required to decipher the mechanisms involved primary changes to cell molecular physiology and biochemistry which can then be extrapolation to understanding the impact on whole body physiology. The Fh1 MEF model described in this thesis has the potential to be utilized for such a purpose as it mimics the phenotype found in FH-deficient tumours and crucially has not been subjected to secondary hits associated with cell lines derived from metastatic tumours.

3 **Chapter 3 The effect of Fh1-deficiency on the Hif-signalling pathway**

*The effect of Fh1-deficiency on the Hif signalling pathway***3.1 Introduction**

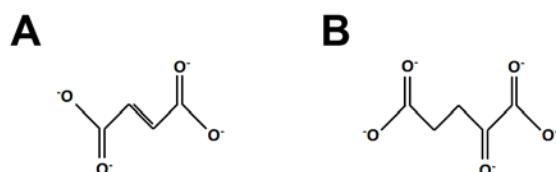
As discussed briefly in Chapter 1, stabilisation of HIF-1 α facilitates survival and metastasis of tumour cells by promoting proliferation and angiogenesis and through transcriptional activation of its downstream target genes. Increased expression of HIF has been reported in numerous cancers (Harris 2002; Semenza 2003; Rankin and Giaccia 2008; Lin et al. 2011), including HLRCC and clear cell renal cell carcinomas (CCRCC) (reviewed by Linehan, 2010 #1399).

Cellular expression of HIF is controlled at a post-translational level by the pVHL-dependent E3 ubiquitin ligase complex which recognises and marks hydroxylated prolyl residues in the HIF oxygen-dependent degradation domain (ODDD) for proteasomal degradation (Maxwell et al. 1999; Ohh et al. 2000; Epstein et al. 2001; Masson et al. 2001; Hon et al. 2002). When cellular oxygen is in ample supply, hydroxylation of proline residues (Pro402 and Pro577 in mice) of HIF occurs *via* the action of the 2-OG-dependent dioxygenase enzymes, the PHDs, thus enabling HIF to be ubiquitinated and undergo proteasomal degradation (Ivan et al. 2001). Factor inhibiting HIF (FIH) is another 2-OG-dependent dioxygenase enzyme which differs from HIF in that it facilitates hydroxylation of a specific asparagine residue (Asn 819 in mice and Asn 803 in humans) at the C-terminal transactivation domain (CAD) of HIF- α thus preventing the recruitment of the transcriptional activators p300/CBP (reviewed by (Pouyssegur et al. 2006; Kaelin and Ratcliffe 2008)). In the event of sub-optimal oxygen levels occurring within the cell, the PHDs are unable to utilise divalent molecules of O₂ as a crucial substrate to sustain enzymatic function and

The effect of Fh1-deficiency on the Hif signalling pathway

therefore HIF escapes degradation while activating transcription of its target genes (Bruick 2003; Coleman and Ratcliffe 2007).

The working hypothesis for stable expression of normoxic HIF- α in an FH-deficient background is that loss of Fh1 leads to increasing levels of cellular fumarate thus exceeding the levels of 2-OG and reducing the availability of 2-OG as a substrate for HIF-PHDs. Fumarate and 2-OG have a similar chemical structure (Figure 3.1) which enables fumarate to bind to the 2-OG substrate-binding site of the PHD enzymes (Hewitson et al. 2007). However, in contrast to the *cis*-orientation of the carboxyl groups in 2-OG, the carboxyl groups of fumarate are in the *trans*-orientation and rather than an oxygen atom, fumarate harbours a double bond between the central carbons. Therefore, fumarate binding to the 2-OG-binding site renders the PHD enzyme inactive (Hewitson et al. 2007; Koivunen et al. 2007). In support of this, the K_i (dissociation constant of inhibition) values for fumarate inhibition of PHDs 1, 2 and 3 were found to be 80, 60 and 50 μM , respectively, whereas succinate has much higher K_i values for inhibition of the same PHDs (PHD 1=350 μM , PHD 2=460 μM and PHD 3=430 μM) (Koivunen et al. 2007). In a similar study by Hewitson et al, fumarate-mediated inhibition of PHD 2 was reported to have an IC_{50} value of 220 μM compared to the insignificant IC_{50} value of >10mM following addition of succinate (Hewitson et al. 2007). It should be noted that IC_{50} values differ from K_i values in that they measure the concentration of inhibitor needed to halve the reaction rate of an enzyme-catalysed reaction whereas the K_i value denotes the dissociation of the inhibitor-bound enzyme complex (Burlingham 2003).

The effect of Fh1-deficiency on the Hif signalling pathway**Figure 3.1** Chemical structure of Fumarate and 2-OG

Chemical structure of A) fumarate and B) 2-OG.

As a consequence of PHD inhibition hydroxylation of HIF- α is prevented, thus leading to transcriptional activation of HIF- α target genes (Isaacs et al. 2005; Pollard et al. 2006; Koivunen et al. 2007; O'Flaherty et al. 2010). Expression of HIF- α and its downstream target genes under aerobic conditions is referred to as 'pseudohypoxic drive' and has been identified in tissues taken from Fh1-conditional knockout mice as well as HLRCC-related RCCs (Isaacs et al. 2005; Pollard et al. 2005). Similarly, tumours associated with mutations or deletions in both *VHL* and the TCA cycle enzyme *SDH*, also exhibit stabilisation of HIF- α through inhibition of the PHD enzymes, though by distinctly different mechanisms; VHL forms an important component of the E3 ubiquitin ligase complex whereas SDH mutations lead to increased cellular levels of succinate (succinate concentrations range from 350-460 μ M) which inhibits PHD activity in a competitive manner similar to that of fumarate (Dahia et al. 2005; Selak et al. 2005; Pollard et al. 2006; Koivunen et al. 2007). Data corroborating the inhibition of the PHD enzymes by TCA cycle enzymes have been described in previous studies (Hewitson et al. 2007; Koivunen et al. 2007).

The effect of Fh1-deficiency on the Hif signalling pathway

To further elucidate the effect of fumarate accumulation in Fh1-deficient MEFs and to determine the extent of its contribution to the pseudohypoxic drive, the status of Hif-1 α hydroxylation in Fh1-deficient MEFs was assessed. As FH exists in a mitochondrial and cytoplasmic form, both encoded by the same transcript, the enzyme has been proposed to have a function beyond that of its role in the TCA cycle by possibly contributing to such pathways as amino acid metabolism and the urea cycle (Salway 1999 ; Nelson 2004). As the HIF hydroxylases are predominantly cytoplasmic enzymes, the role of cytoplasmic FH in context of pseudo-hypoxic drive is unclear. It remained to be determined whether expression of the cytoplasmic variant of FH would be capable of restoring PHD hydroxylase activity in an FH-deficient background. It must also be kept in consideration that the Hif-signalling pathway is predominantly regulated by cellular oxygen concentrations and that there is also evidence for mitochondrial ROS activation of this pathway (Brunelle et al. 2005; Klimova and Chandel 2008; Chandel 2010). Therefore it was also important to determine whether ‘rescuing’ the HIF phenotype by means of cytoplasmic FH expression occurs independently of a persistent disruption to the TCA cycle in the absence of mitochondrial FH, which one would expect to have a negative impact on both oxygen and ROS levels. In order to investigate this hypothesis, the Fh1^{-/-} MEFs were transfected with pEF vectors expressing either human full length, mitochondrial FH, or human FH restricted to the cytoplasm by deletion of the mitochondria targeting sequence (MTS) (See Appendix Figures 4.1 for sequences). Both FH sequences were designed to carry a C-terminal V5-tag to allow for easier screening and visualisation of the protein within the cell having confirmed that the V5-tag does not affect FH activity.

The effect of Fh1-deficiency on the Hif signalling pathway

Also of interest is the growing evidence for the involvement of alternative pathways, acting independently of the dysregulated HIF signalling, such as the NRF2 antioxidant and AMPK signalling pathways, which may make a greater contribution than previously considered to the onset and maintenance of FH-associated tumourigenesis (Adam et al. 2011; Ooi et al. 2011; Tong et al. 2011). The potential contribution of these pathways to pathogenesis in FH-deficiency will be further discussed in Chapter 5. Therefore, for the purpose of this chapter these pathways will not be addressed here.

*The effect of Fh1-deficiency on the Hif signalling pathway***3.2 Results****3.2.1 Normoxic stabilisation of Hif-1 α in Fh1-deficient MEFs as a result of cellular fumarate accumulation**

To assess whether MEFs lacking *Fh1* demonstrate sustained stable expression of Hif-1 α under normoxic (21% O₂) conditions protein levels of Hif-1 α were compared between Fh1^{-/-} and Fh1^{+/+} MEFs. Consistent upregulation of Hif-1 α protein was prominent in Fh1^{-/-} MEFs (Figure 3.2A), an observation which had also been noted in FH-deficient tumour samples and in Fh1-null kidney tissues (Pollard et al. 2005; Bardella et al. 2011). The working hypothesis was that competitive inhibition of the 2-OG-dependent Hif PHDs by increased cellular fumarate, resulting from loss of *Fh1*, was the primary factor contributing to Hif-1 α stabilisation. To ascertain whether this held true for Fh1^{-/-} MEFs, both Fh1^{-/-} and Fh1^{+/+} cells were treated with a cell-permeable esterified derivative of 2-OG (dimethyl-2-OG) in order to raise endogenous levels of 2-OG above that of fumarate. As illustrated in Figure 3.1A, treatment of Fh1^{-/-} MEFs with dimethyl-2-OG is sufficient to restore degradation of Hif-1 α . A marginal stabilisation of Hif-1 α (<5%) can be observed in Fh1^{+/+} MEFs following exposure to dimethyl-2-OG however this can be attributed to increased exogenous substrate concentration saturating the catalytic activity of the PHD enzymes. The *K_m* values for 2-OG binding to the PHDs are as follows: *K_m*=1-2 μ M for PHD 1 and 2 and *K_m*=12 μ M PHD 3 (Koivunen et al. 2007). For further verification, Q-PCR analyses of Fh1^{-/-} and Fh1^{+/+} MEFs was carried out to compare the expression of the following HIF target genes: BCL2/adenovirus E1B 19kDa protein-interacting protein 3 (*Bnip3*), hexokinase 2 (*Hk2*), lactate dehydrogenase A

The effect of Fh1-deficiency on the Hif signalling pathway

(*Ldha*) and glucose transporter 1, *Glut 1* (also known as *Slc2a1*). The results demonstrate a striking increase in expression of HIF-target genes in *Fh1*^{-/-} MEFs which, following addition of exogenous dimethyl-2-OG, are reduced (Figure 3.2B).

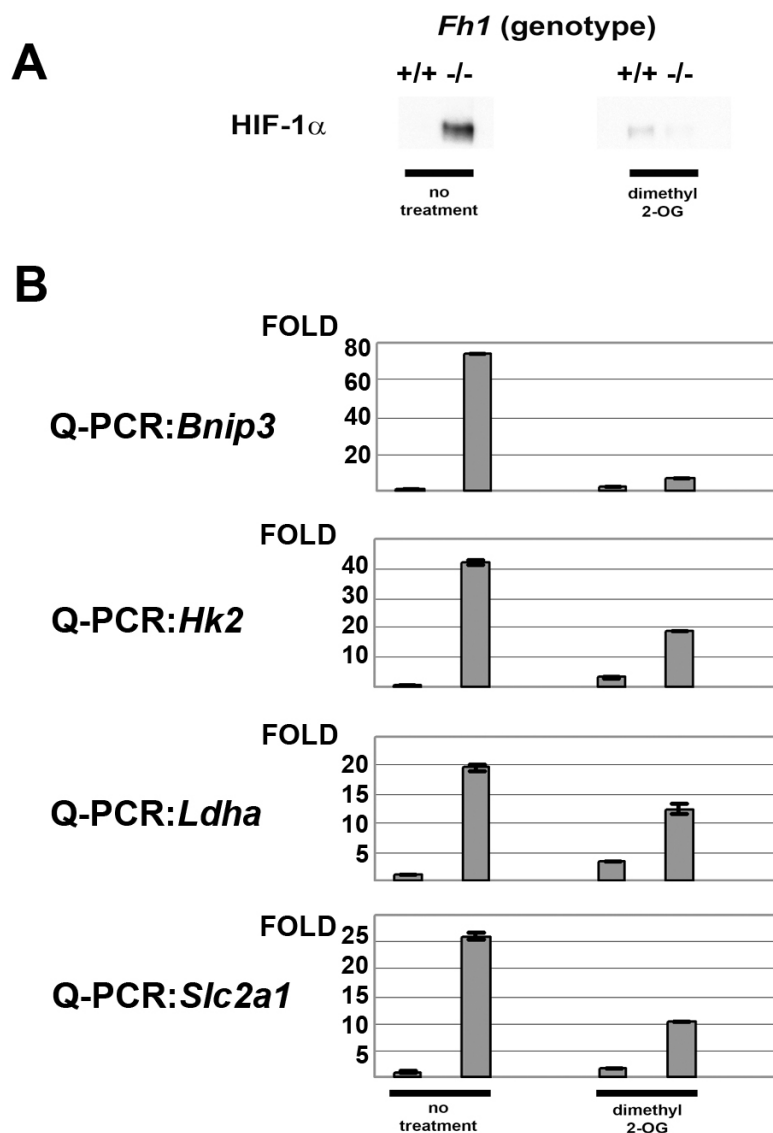
The effect of *Fh1*-deficiency on the *Hif* signalling pathway

Figure 3.2 Confirmation of normoxic Hif-stabilisation and upregulation of Hif target genes

A) Immunoblot analyses of *Fh1*^{+/+} and *Fh1*^{-/-} MEFs demonstrate that exogenous addition of dimethyl-2-OG (2mM) for 8 hours is capable of restoring Hif-1 α protein levels in *Fh1*^{-/-} MEFs to those found in *Fh1*^{+/+} cells under normoxia. B) Comparison of untreated and dimethyl-2-OG treated *Fh1*^{+/+} and *Fh1*^{-/-} MEFs by Q-PCR analyses confirms that increased expression of Hif-target genes in *Fh1*^{-/-} is substantially reduced following treatment with dimethyl-2-OG. Results are shown as mean $\pm$ SD of triplicate assays.

*The effect of Fh1-deficiency on the Hif signalling pathway**3.2.2 Determining the hydroxylation status of Hif-1 α following inactivation of Fh1*

In order for Hif-1 α to be recognised and processed by the E3-ubiquitin ligase pathway for proteasomal degradation, hydroxylation of two proline sites (Pro 402 and Pro577 in mouse Hif-1 α) within the oxygen-dependent degradation domain (ODDD) of *Hif-1 α* must occur (Pugh et al. 1997; Salceda and Caro 1997; Huang et al. 1998; Epstein et al. 2001). Hydroxylation of an asparagine site (Asn819 in mouse Hif-1 α) downstream of Pro402 and Pro507 prevents recruitment of p300/CBP transcriptional co-activators and inhibits transcriptional activation of Hif-1 α (Lando et al. 2002; Schofield and Ratcliffe 2004; Kaelin 2005). To ascertain whether the hydroxylation status of murine Pro402, Pro577 and/or Asn819 was modified in an Fh1-dependent manner, hydroxyl-residue-specific antibodies were employed to verify the hydroxylation status of Fh1^{-/-} MEFs (Figure 3.3A). Due to the fast rate of recognition and degradation by the ubiquitin-proteasome pathway under normoxia, the MEFs were treated with the proteasomal inhibitor *N*-[(phenylmethoxy)carbonyl]-*L*-leucyl-*N*-[(1*S*)-1-formyl-3-methylbutyl]-*L*-leucinamide (MG132) so that the hydroxylation status of Hif-1 α could be determined. Fh1^{-/-} MEFs showed a striking lack of hydroxylation at both Pro402 and Pro577 and moreover hydroxylation was restored following addition of dimethyl-2-OG, which is in keeping with the hypothesis of PHD inhibition in an Fh1-deficient background. In contrast, asparaginyl hydroxylation was not found to be inhibited in Fh1^{-/-} MEFs. From these results it can be inferred that absence of prolyl hydroxylation rather than, asparaginyl hydroxylation in Fh1^{-/-} MEFs, leads to accumulation of Hif-1 α and activation of downstream transcriptional targets.

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To investigate whether these findings of dysregulation of Hif hydroxylation in Fh1^{-/-} MEFs also occur in FH-associated pathogenesis, hyperplastic renal cystic tissue from Fh1^{-/-} mouse kidneys and human FH-deficient papillary renal carcinoma were examined (Figure 3.3B). The results of these experiments corroborate the findings shown in Figure 3.3A, where there is a clear inhibition of prolyl hydroxylation but no observable effect on asparaginyl hydroxylation. However, this is not the case for Von Hippel-Lindau (VHL)-deficient samples, used here as controls, where stabilisation of HIF occurs irrespective of hydroxylation on both proline and asparagine residues. These results corroborate the findings of independent studies (Isaacs et al. 2005; Pollard et al. 2005), specifically that FH inactivation can activate the HIF signalling pathway through accumulation of cellular fumarate, and therefore the Fh1^{-/-} MEFs can be considered to be a robust *in vitro* model for the study of either Hif or non-Hif associated signalling pathways in FH-related tumourigenesis and disease.

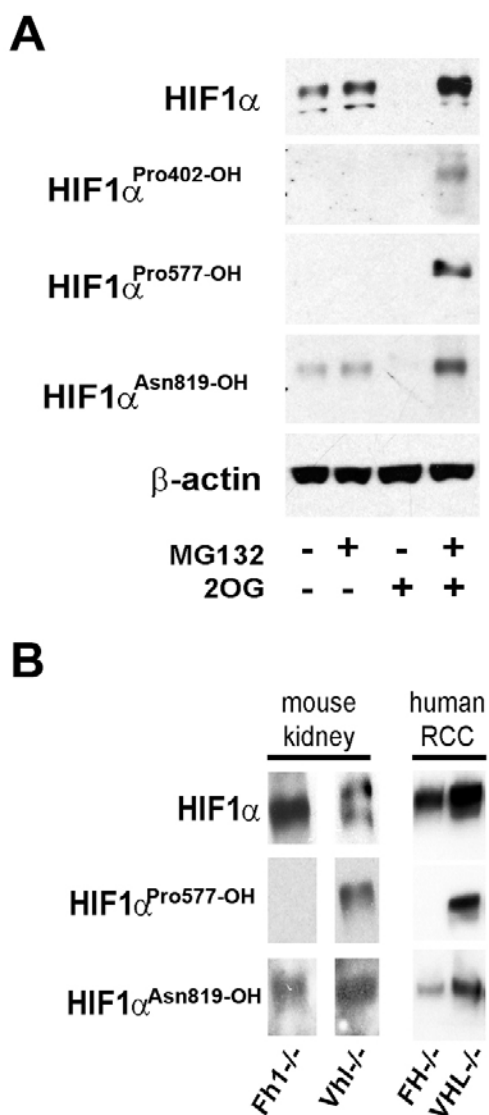
The effect of *Fh1*-deficiency on the *Hif* signalling pathway

Figure 3.3 Hydroxylation status of Hif *in vitro* and *in vivo*

A) Using hydroxyl-residue-specific antibodies, absence of hydroxylation at Pro402 and Pro577 of Hif-1 α is demonstrated in *Fh1*^{-/-} MEFs and following treatment with 2mM dimethyl-2-OG the blockade on hydroxylation is reversed. However, asparaginyl hydroxylation of Asn819 was relatively unaffected in an *Fh1*-deficient background. B) Tissues from *Fh1*^{-/-} or *Vhl*^{-/-} mice kidney and human type II papillary RCC (*FH*^{-/-}) tissues emphasise that suppression of HIF-1 α prolyl hydroxylation is predominant in *FH*-null, but not *VHL*-null tissues, as confirmed by immunoblot analyses.

*The effect of Fh1-deficiency on the Hif signalling pathway***3.2.3 Complementation of *Fh1*^{-/-} MEFs with intra- and extra-mitochondrial FH**

Inevitably the loss of Fh1 activity results in disruption to the TCA cycle by preventing the conversion of fumarate to malate. This has strong repercussions for cellular respiration, most especially the ability of the cell to generate ATP *via* OXPHOS and cells thus affected are described as having a mitochondrial defect. A mitochondrial defect can be described as a diminished supply of electrons from a non-functional TCA cycle reduces the demand for oxygen to act as the terminal electron acceptor for complex IV of the ETC, thus altering cellular oxygen concentrations. Cells with a mitochondrial defect may experience a disruption to and thus cellular oxygen levels have a potential role in the stabilisation and over-expression of Hif-1 α in an Fh1-deficient MEF background. To determine to what extent the mitochondrial defect contributes to the pseudo-hypoxic drive, transient infection of Fh1^{-/-} MEFs with an Adenovirus delivered full-length FLAG-tagged human *FH* gene (Ad-FH) (designed by Violetta Steeples) was carried out and analysed at 24 hour and 48 hour post-infection. The mouse ortholog *Fh1* shares 87.52% sequence homology with *FH* (based on nucleic acid comparison. See Chapter 6, Figure 6.1) and as predicted, the introduction of human *FH* into Fh1^{-/-} cells ablated normoxic over-expression of Hif-1 α protein compared to Fh1^{-/-} cells infected with a Ad-GFP control vector when assessed at both time points (Figure 3.4A). In conjunction with protein measurements, comparison of mRNA expression levels of the egl nine homolog 3 (*Egln3*), *Hk2*, and *Glut1*(*Slc2a1*) was quantified by Q-PCR at 24 and 48 hours post-infection in Fh1^{-/-} MEFs infected with Ad-FH compared to Ad-GFP infected Fh1^{-/-} MEFs. Expression of the Hif-target genes measured was significantly depressed at 48 hours (*Egln3*, 0.09 $\pm$ 0.04-fold; *Hk2*, 0.14 $\pm$ 0.01-fold and *Slc2a1*, 0.19 $\pm$ 0.01-fold) in the Fh1^{-/-} cells

The effect of Fh1-deficiency on the Hif signalling pathway

treated with Ad-FH following normalisation of the signal against β -actin (Figure 3.4B).

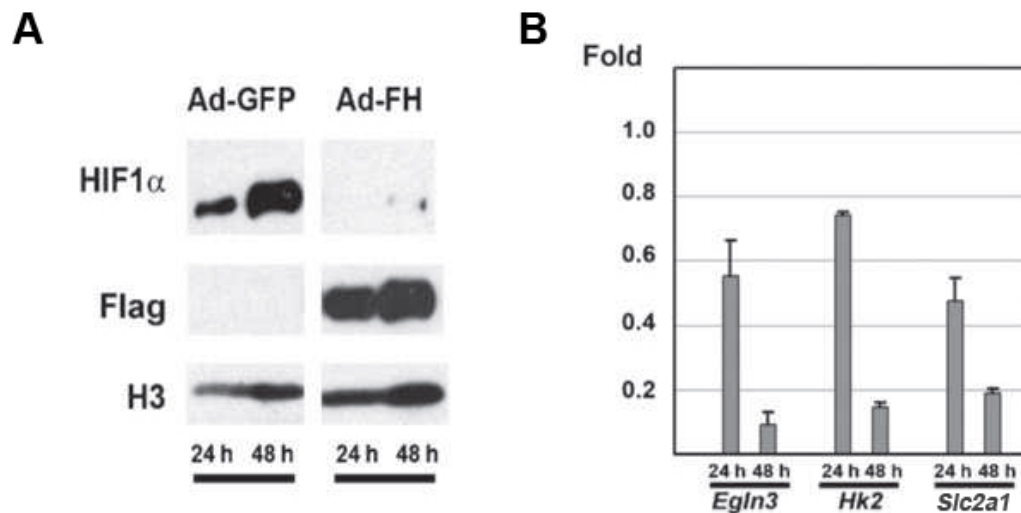
The effect of *Fh1*-deficiency on the *Hif* signalling pathway

Figure 3.4 *Fh1*^{-/-} MEFs re-expressing human FH

A) Re-introduction of a Flag-tagged human FH (Ad-FH) reverses normoxic accumulation of Hif-1 α in *Fh1*^{-/-} MEFs and expression of the Flag-tag was confirmed only in Ad-FH treated MEFs. Cells treated with an Ad-GFP control showed no amelioration of HIF-1 α protein expression. Both untreated and treated *Fh1*-null MEFs were assessed by immunoblot at 24 hours and 48 hours with levels of Histone H3 indicative of protein loading. B) Q-PCR analysis of Ad-FH treated *Fh1*^{-/-} MEFs demonstrates that expression of HIF-target genes is significantly reduced at 48 hours. mRNA levels of each target gene were normalized against β -actin expression. Error bars indicate ± 1 SD from two biological replicates, both in triplicate, with $p < 0.01$.

The effect of Fh1-deficiency on the Hif signalling pathway

Initially Fh1^{-/-} MEFs were treated with an Ad-GFP vector expressing FLAG-tagged human FH to investigate the effects on Hif expression. While the FLAG-tag was capable of abolishing Hif-1 α accumulation (Figure 3.4A) this expression vector did not lend itself to stable, long-term expression in MEFs. For studying the prolonged effects of FH re-expression, in particular its expression in either intra- or extra-mitochondrial compartments, transfection of Fh1-null MEFs using a pEF vector expressing either a full-length C-terminal V5-tagged human FH gene (FH-V5), or a cytoplasmic-restricted variant of the same gene lacking the N-terminal mitochondrial targeting sequence (FH-V5 Δ MTS) (Appendix Figures 3.1) was undertaken, as described in the materials and methods, Chapter 6, section 6.2. To verify that the C-terminus V5-tag was not having a confounding effect on the function of the re-expressed FH, Fh1^{-/-} MEFs were transfected with un-tagged FH and FH- Δ MTS constructs as controls.

Localization of the FH-V5 gene to the mitochondria and the FH-V5 Δ MTS to the cytoplasm in individual transfected clones was confirmed by immunofluorescence microscopy (Figure 3.5A(i) and (ii), respectively). An alternative means of demonstrating differential localisation of the FH-V5 and FH-V5 Δ MTS genes within the MEFs would be to carry out a cellular fractionation experiment to analyse the mitochondrial and cytoplasmic fractions in isolation and to immunoblot against FH, Fh1, V5-tag and cytoplasm- and mitochondria-specific protein markers (e.g. α -tubulin and cytochrome c, respectively). Metabolite analysis of the four MEF lines using ¹H magnetic resonance sounding (MRS) showed that transfection of the Fh1^{-/-} MEFs with either FH-V5 or FH Δ MTS-V5 attenuated the surplus levels of fumarate (¹H MRS analysis was carried out by Dr. Simon Olpin). In contrast to the Fh1^{-/-} +FH MEFs,

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which were comparable with Fh1^{+/+} cells, Fh1^{-/-} + FHΔMTS MEFs retained low levels of fumarate accumulation (Figure 3.5B). Fh1^{-/-} +FH MEFs restored oxygen consumption rates to levels comparable with Fh1^{+/+} cells under conditions of both substrate-stimulation (using glutamate) and uncoupling of mitochondrial respiration, thus enabling respiration independent of ATP synthesis, using carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP). However FH-V5ΔMTS transfected MEFs (Fh1^{-/-} +FHΔMTS) were unable to rescue the respiratory defect in Fh1^{-/-} cells, as demonstrated by a lack of significant difference between the two lines (Table 3.1) and by oxygen being consumed at a rate associated with non-mitochondrial respiration, an observation that was made in all the cells following treatment with the ATP synthase inhibitor Oligomycin (Table 3.1 and Appendix Figure 3.3). In keeping with the mitochondrial defective phenotype of the Fh1^{-/-}+FH-V5ΔMTS MEFs these cells showed a significantly reduced capacity to consume oxygen following mitochondrial uncoupling with FCCP when compared to the Fh1^{-/-}+FH MEFs. The presence of a V5-tag did not have any apparent effect on oxygen consumption and coincided with the data generated from the non-V5-tagged constructs (Appendix Figure 3.2). A minimum number of three clones per FH variant were selected for comparative analyses of each construct.

A metabolic shift from OXPHOS towards glycolytic-dependency has been hypothesised to occur in the TCA cycle impaired Fh1^{-/-} MEFs. To further test this hypothesis ATP levels were measured and compared between the following MEF lines: Fh1^{+/+}, Fh1^{-/-}, Fh1^{-/-} +FH and Fh1^{-/-} +FHΔMTS. In order to distinguish between the levels of ATP generated *via* OXPHOS from that generated by glycolysis the MEFs were grown in medium supplemented with either 25mM galactose or 25mM

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glucose. Galactose is utilised by glycolysis but in order for ATP levels to be maintained within the cell galactose must be fully oxidised in the presence of a functional TCA cycle. Therefore, galactose-supplemented medium is considered to mimic conditions of OXPHOS respiration. By comparing the amount of ATP generated by the cells under each condition and expressing this as a percentage of galactose (OXPHOS)/glucose (OXPHOS and glycolysis) $\times 100$ the capacity of each cell line to generate ATP can be determined. The results confirm that $Fh1^{-/-}$ MEFs were unable to maintain cellular ATP levels when grown under conditions simulating OXPHOS respiration (i.e. galactose as opposed to glucose containing medium) which was also the case for $Fh1^{-/-}$ +FH Δ MTS cells (Figure 3.5 C). Conversely, ATP levels were 'rescued' following re-expression of mitochondrial *FH* as was evident in $Fh1^{-/-}$ +FH MEFs. Overall these findings demonstrate that OXPHOS respiration can be only re-established in $Fh1^{-/-}$ cells after reintroduction of full-length, mitochondrial FH but not FH Δ MTS. However, re-expression of cytoplasmically restricted FH remains adequate for diminishing fumarate accumulation within the cell.

*The effect of Fh1-deficiency on the Hif signalling pathway***Table 3.1 Oxygen consumption measurements in Fh1 MEFs**

	Oxygen Consumption (nmol O ₂ /min/10 ⁶ cells)											
	Fh1 ^{+/+} vs. Fh1 ^{-/-}		Fh1 ^{+/+} vs. Fh1 ^{-/-} +FH		Fh1 ^{+/+} vs. Fh1 ^{-/-} +FHΔMTS		Fh1 ^{-/-} vs. Fh1 ^{-/-} +FH		Fh1 ^{-/-} vs. Fh1 ^{-/-} +FHΔMTS		Fh1 ^{-/-} +FH vs. Fh1 ^{-/-} +FHΔMTS	
	Fh1 ^{+/+}	Fh1 ^{-/-}	Fh1 ^{+/+}	Fh1 ^{-/-} +FH	Fh1 ^{+/+}	Fh1 ^{-/-} +FHΔMTS	Fh1 ^{-/-}	Fh1 ^{-/-} +FH	Fh1 ^{-/-}	Fh1 ^{-/-} +FHΔMTS	Fh1 ^{-/-} +FH	Fh1 ^{-/-} +FHΔMTS
Basal	1.03	0.187 [#]	1.03	0.667	1.03	0.39**	0.187	0.667	0.187	0.39	0.667	0.39
Glutamate	1.08	0.143 [#]	1.08	0.717	1.08	0.297***	0.143	0.717*	0.143	0.297	0.717	0.297
Oligomycin	0.24	0.18	0.24	0.343	0.24	0.247	0.18	0.343	0.18	0.247	0.343	0.247
FCCP	1.5	0.583 [#]	1.5	1.33	1.5	0.76***	0.583	1.33 ^{##}	0.583	0.76	1.33	0.76****

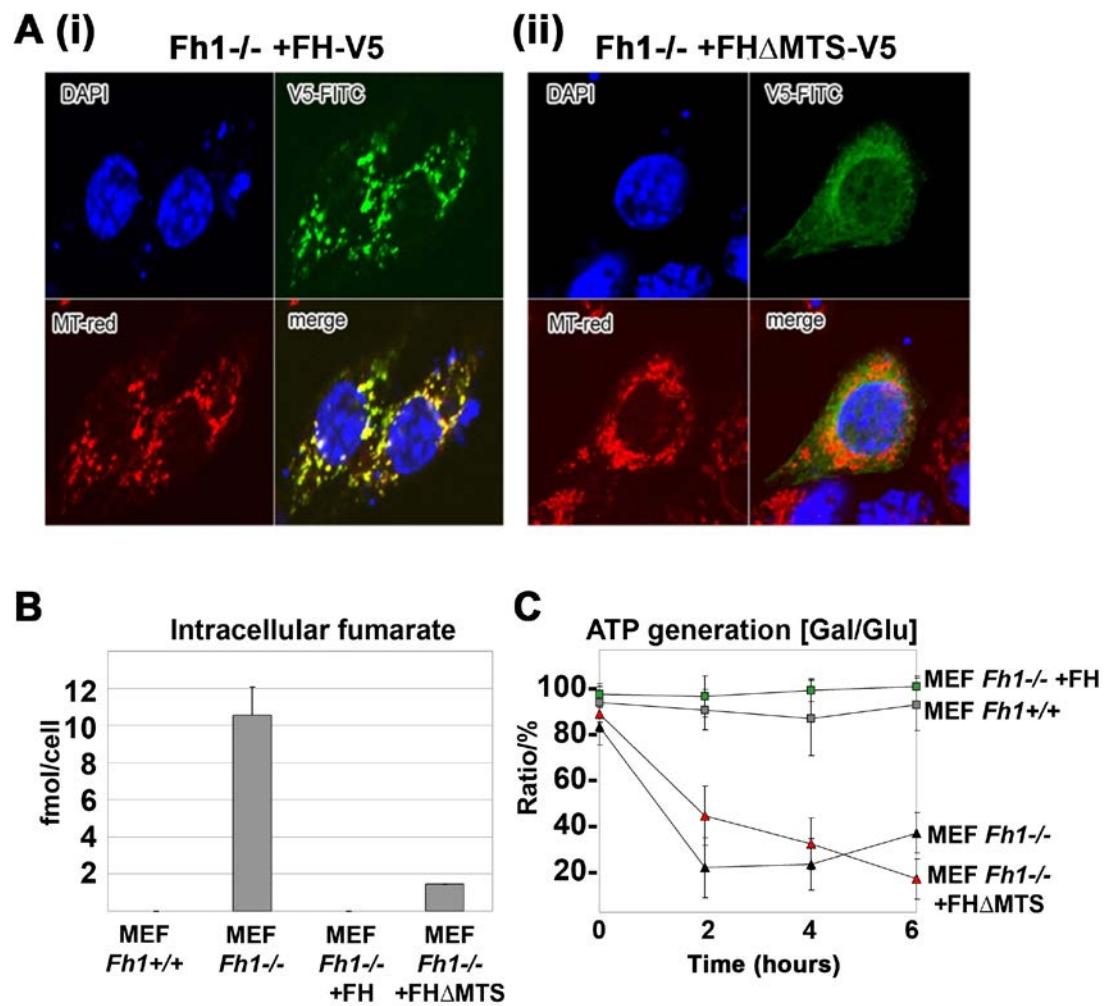
A Clarke-type oxygen electrode was employed to measure the oxygen consumption rate in Fh1^{+/+}, Fh1^{-/-}, Fh1^{-/-} +FH and Fh1^{-/-} +FHΔMTS MEFs following addition of glutamate, oligomycin and FCCP. Statistical analysis was carried out using two-way ANOVA.

[#]p<0.0001, Fh1^{+/+} vs. Fh1^{-/-}

p<0.01, Fh1^{+/+} vs. Fh1^{-/-}+FHΔMTS; *p<0.001, Fh1^{+/+} vs. Fh1^{-/-}+FHΔMTS

*p<0.05, Fh1^{-/-} vs. Fh1^{-/-}+FH; ^{##}p<0.001, Fh1^{-/-} vs. Fh1^{-/-}+FH

****p<0.05 Fh1^{-/-}+FH vs. Fh1^{-/-}+FHΔMTS

The effect of *Fh1*-deficiency on the *Hif* signalling pathwayFigure 3.5 Analyses of re-expression of FH-V5 or FH Δ MTS-V5

*The effect of Fh1-deficiency on the Hif signalling pathway***Figure 3.5 Analyses of re-expression of FH-V5 or FH Δ MTS-V5**

A) Localisation of (i) the FH-V5 construct predominantly in the mitochondrial compartments and (ii) the FH-V5 Δ MTS-V5 construct to the cytoplasm, but not the mitochondria, was confirmed by merging of immunofluorescent signals from MitoTracker (red) and V5-FITC-tagged FH protein (green). B) Increased levels of intracellular fumarate are restored to wild-type levels following re-expression of mitochondrial FH (Fh1^{-/-} +FH). In contrast, re-introduction of cytoplasmic FH (Fh1^{-/-} +FH Δ MTS) does not completely abolish the phenotype of accumulated fumarate but does greatly reduce the levels compared to the Fh1^{-/-} MEFs. Fumarate concentrations were measured by Prof. John Griffiths. C) ATP levels compared across the four cell lines at 0, 2, 4 and 6 hours post-supplementation with either glucose (permissive for OXPHOS and glycolysis), or galactose (permissive for OXPHOS only), indicate that, in contrast to Fh1^{+/+} and Fh1^{-/-} +FH MEFs, both Fh1^{-/-} and Fh1^{-/-} +FH Δ MTS MEFs are unable to maintain ATP levels in galactose-supplemented medium. The ability to sustain generation of [ATP] in galactose medium is calculated as a percentage of that generated in a parallel culture in glucose medium using the following equation: [ATP]Gal/[ATP]Gluc100. Results are shown as mean $\pm$ SD of triplicate assays.

The effect of Fh1-deficiency on the Hif signalling pathway

3.2.4 *Expression of either the FH-V5 or FHΔMTS-V5 transfectant in Fh1^{-/-} MEFs has different effects on cell growth*

As was demonstrated in Chapter 2, section 2.2.5, Fh1^{-/-} MEFs grew at a considerably slower rate compared to their Fh1^{+/+} counterparts under normoxic oxygen tension (21% O₂ in standard tissue culture conditions) (Figure 2.4B(i)). Under 1% hypoxia the growth difference between the two lines became less apparent (Figure 2.4B(ii)). To assess whether the growth rate in Fh1^{-/-} +FH and Fh1^{-/-} +FHΔMTS MEFs was comparable to Fh1^{+/+} cells, proliferation of all cell lines (Fh1^{+/+}, Fh1^{-/-}, Fh1^{-/-} +FH and Fh1^{-/-} +FHΔMTS) was measured at 12 hour intervals over 72 hours. As with the previous study described in Chapter 2, section 2.2.5, normoxic growth measurements were taken in parallel with measurements under 1% hypoxia. The normoxic results showed that Fh1^{-/-} +FH MEFs grew at a rate similar to the Fh1^{+/+} MEFs, whereas the growth of Fh1^{-/-} +FHΔMTS MEFs accelerated beyond the rate of the Fh1-deficient cells after 24 hours (Figure 3.6A). However, the overall growth rate of the Fh1^{-/-} +FHΔMTS MEFs did not increase and could be considered comparable to the Fh1^{-/-} +FH MEFs. These findings are in keeping with the results showing that Fh1^{-/-} +FHΔMTS cells still harbour reduced oxygen consumption (Table 3.1), persistent fumarate accumulation (Figure 3.5B) and reduced levels of ATP generation (Figure 3.5C). It is therefore unsurprising that the growth of these cells would be hindered due to the continued presence of a mitochondrial defect, which on the other hand is restored in Fh1^{-/-} +FH MEFs. A similar growth pattern is observed when the cells are grown under hypoxic conditions, although the difference between the different cell lines becomes less distinct (Figure 3.6B). The Fh1^{-/-} +FHΔMTS cells grew slightly faster than the Fh1^{-/-} MEFs but at a reduced rate when compared to the Fh1^{-/-} +FH

The effect of Fh1-deficiency on the Hif signalling pathway

cells. Unlike either of the FH transfectant cell lines, Fh1^{+/+} MEFs reached a growth plateau at 36 hours which suggests that the wildtype lines are more sensitive to hypoxia.

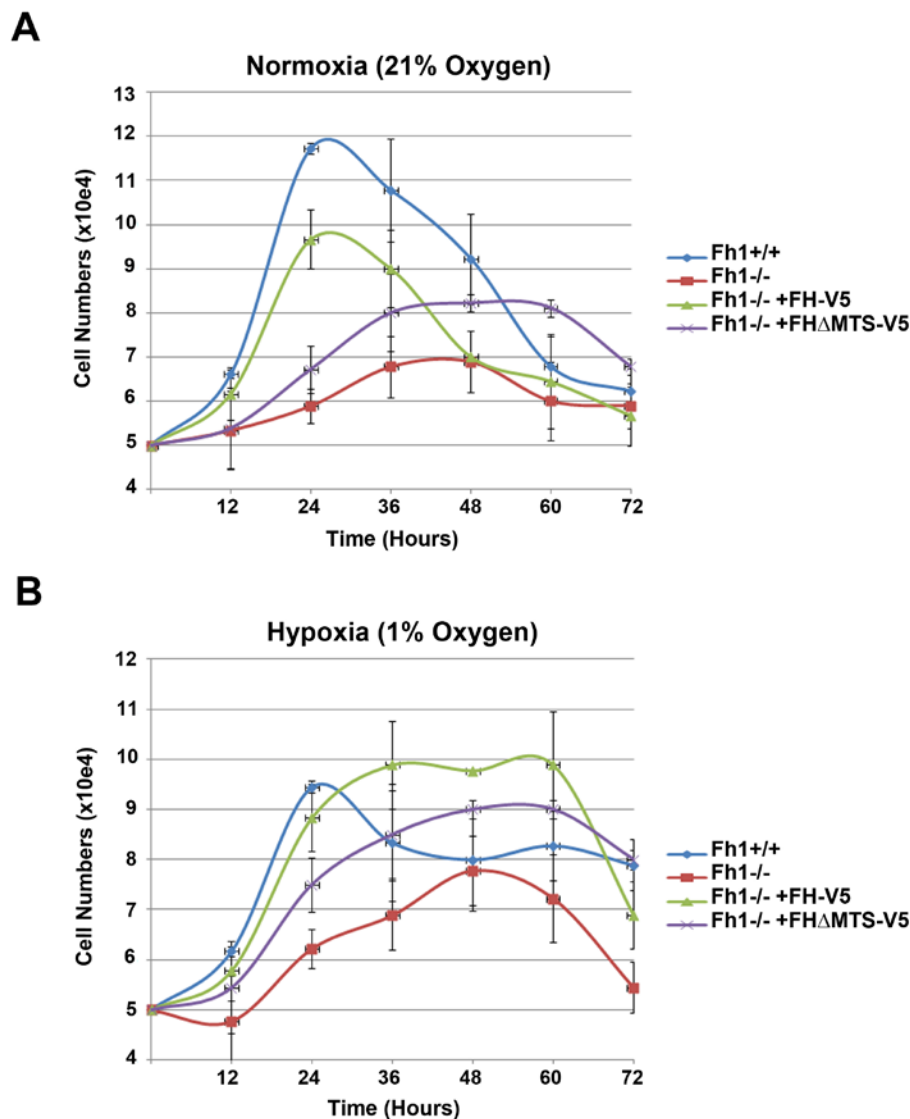
The effect of *Fh1*-deficiency on the *Hif* signalling pathway

Figure 3.6 Effects of re-expression of FH or FH Δ MTS on cell growth

A) Cell growth under normoxic conditions shows that Fh1^{-/-} +FH MEF growth is similar to that of Fh1^{+/+} MEFs. While the Fh1^{-/-} +FH Δ MTS cells proliferate at a greater rate than the Fh1^{-/-} it is not comparable to the rate of proliferation observed with the Fh1^{-/-} +FH MEFs. B) Under 1% hypoxia the Fh1^{-/-} cells show increased growth and the difference between the Fh1^{-/-} +FH and Fh1^{-/-} +FH Δ MTS is less distinct than under normoxic conditions. Conversely the growth of Fh1^{+/+} MEFs peaks at 24 hours and then reaches a plateau for the remainder of the time course. Data shown is mean $\pm$ S.D. of biological triplicates.

*The effect of Fh1-deficiency on the Hif signalling pathway**3.2.5 Complementation with intra- and extra-mitochondrial FH ablates Hif-1 α levels in Fh1^{-/-} MEFs*

Having successfully demonstrated that Fh1-deficient MEFs can be ‘rescued’ with respect to fumarate accumulation following re-expression of either mitochondrial or cytoplasmic FH (and in the case of the former transfectant, also restore oxygen consumption and ATP synthesis), it remained to be confirmed whether the FH-V5 and/or FH Δ MTS-V5 transfectants could abrogate the normoxic Hif-1 α expression observed in untransfected Fh1^{-/-} MEFs. To determine the hydroxylation status of Hif-1 α hydroxylation in Fh1^{-/-} +FH and Fh1^{-/-} +FH Δ MTS MEFs, protein samples from both cell lines were compared by immunoblot analyses using the same hydroxyl-residue specific antibodies and proteasomal inhibitor, MG132, as outlined previously in results section 3.2.2. From the resulting data it was firmly established that FH-V5, and most strikingly, FH-V5 Δ MTS transfectants both promote degradation of Hif-1 α *via* restoration of HIF-1 α prolyl hydroxylation (Figure 3.7A). Corroborating the findings illustrated in Figure 3.2A, no modification of asparaginyl hydroxylation was detected in either Fh1^{-/-} +FH or Fh1^{-/-} +FH Δ MTS MEFs. Further validation of these findings was determined/made by measuring expression of a panel of downstream HIF-target genes by Q-PCR: *Bnip3*, *Hk2*, *Slc2a1* and pyruvate dehydrogenase kinase isoenzyme 1(*Pdk1*). In contrast to the increased expression levels of target genes in Fh1^{-/-} MEFs, expression levels in both Fh1^{-/-} +FH and Fh1^{-/-} +FH Δ MTS MEFs were comparable with Fh1^{+/+} cells (Figure 3.7B). Taken together the findings of this chapter show that ablation of the pseudohypoxic phenotype associated with Fh1-deficiency can be successfully achieved by re-expressing either full length, mitochondrial FH or, interestingly, also extra-mitochondrial FH, irrespective of the

The effect of Fh1-deficiency on the Hif signalling pathway

persistent defect in mitochondrial respiration in the latter transfectant. This suggests that activation of the Hif pathway occurs as a direct result of fumarate accumulation following loss of Fh1 and this activation is independent of the impaired oxygen consumption associated with the mitochondrial defect.

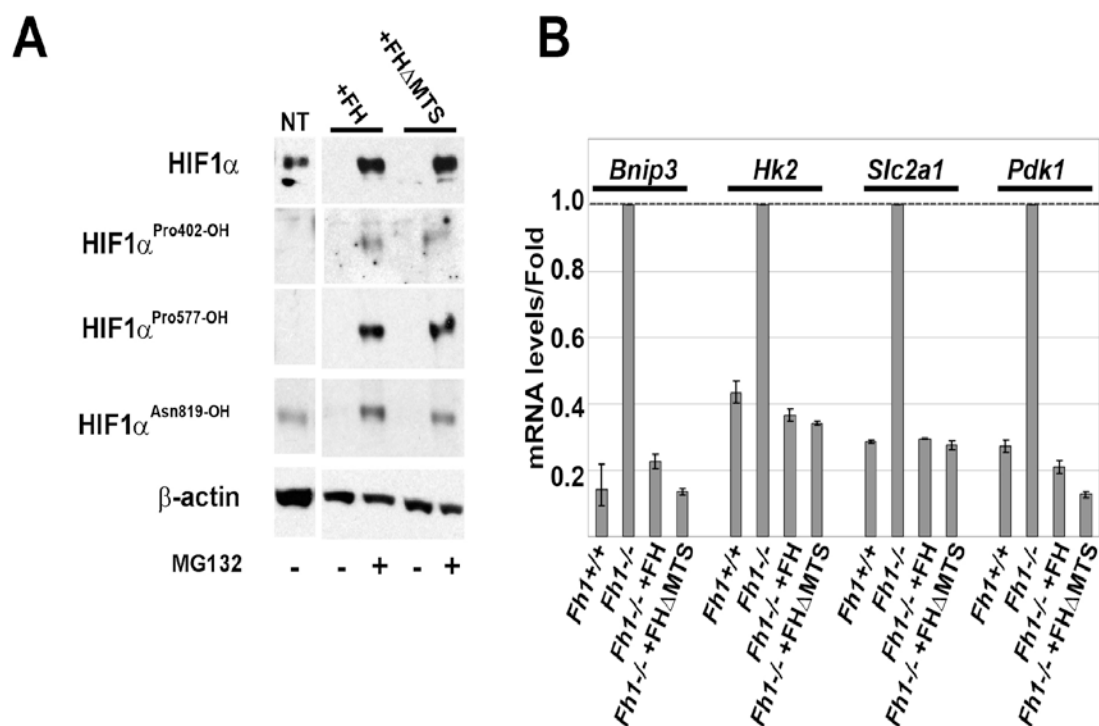
The effect of *Fh1*-deficiency on the *Hif* signalling pathway

Figure 3.7 Restoration of Hif hydroxylation following re-expression of FH-V5 or FHΔMTS-V5

A) *Fh1*^{-/-} MEFs transfected with either FH-V5 or FHΔMTS-V5 show restored hydroxylation at Pro402 and Pro577 when compared against non-transfected (NT) *Fh1*^{-/-} MEFs, thus promoting degradation of Hif under normoxic conditions. There was no notable effect on Asn813 hydroxylation in either cell line. B) These findings were corroborated by Q-PCR data from analysis of a small panel of Hif-1 α target genes. mRNA expression of these target genes are highly expressed in *Fh1*^{-/-} MEFs, however, upon transfection with either FH-V5 or FHΔMTS-V5 mRNA levels are restored to levels comparable to *Fh1*^{+/+} cells. Results are shown relative to the mRNA levels in *Fh1*^{-/-} cells; mean $\pm$ SD of triplicate assays.

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3.2.6 Re-expression of either FH-V5 or FHΔMTS-V5 transfectants in Fh1^{-/-} MEFs restores ROS levels to those detected in Fh1^{+/+} MEFs

It has been reported that the diminished capacity of cells to produce ATP under hypoxic conditions results in the production of reactive oxygen species (ROS) (Brunelle et al. 2005; Klimova and Chandel 2008; Chandel 2010), in particular in renal cancer (Hervouet et al. 2007). To determine whether this hypothesis holds true for the glycolytic-dependent Fh1^{-/-} MEFs, the percentage of ROS generated under basal conditions and treatment with 1mM hydrogen peroxide (H₂O₂) for 90 minutes was measured (n=3) in a single clone from each of the following MEF lines: Fh1^{+/+}, Fh1^{-/-}, Fh1^{-/-} +FH and Fh1^{-/-} +FHΔMTS (Figure 3.8). A titration of H₂O₂ concentrations (ranging from 0.5 mM-2.5mM) was carried out to determine the most effective concentration to induce a ROS response without affecting cell viability. Considerable cell toxicity was observed with 2 mM H₂O₂. Mitochondrial ROS was measured using a fluorogenic dye, MitoSOXTM Red mitochondrial superoxide indicator which enabled positive cells to be sorted by FACS (fluorescence-activated cell sorting) (FACS sorting was performed with the assistance of Dr. Chiara Bardella). There was no significant difference in ROS levels between Fh1^{-/-} MEFs and Fh1^{+/+} MEFs following induction with 1 mM H₂O₂ (p=0.15). These findings indicate that ROS does not directly contribute to the stabilisation of Hif in Fh1^{-/-} MEFs, which is in contrast to the current paradigm that ROS production is significantly increased in FH-deficiency (Sudarshan et al. 2009; Xie et al. 2009). Further investigations are thus required in order to better understand the role of ROS in FH-deficiency and whether it has a significant impact on metabolic regulation or the pathophysiology associated with loss of FH. Re-expression of either mitochondrial or, surprisingly, cytoplasmic

The effect of Fh1-deficiency on the Hif signalling pathway

FH in Fh1^{-/-} MEFS was sufficient to significantly reduce basal ROS levels (for Fh1^{-/-} +FH p=0.023 vs. Fh1^{-/-} and for Fh1^{-/-} +FHΔMTS p=0.033 vs. Fh1^{-/-}) to a range comparable to that detected in wildtype MEFs.

One hypothesis for the FHΔMTS-V5 transfectant reducing ROS to the same extent as the FH-V5 transfectant may be that the presence of cytoplasmic FH facilitates conversion of cytoplasmic fumarate to malate which can then be shuttled across the mitochondrial membrane, either *via* the malate/aspartate shuttle or a carboxylate transporter (Salway 1999 ; Lodish 2000; Nelson 2004). The shuttling of malate into the mitochondria could feed into the TCA cycle beyond the Fh1 blockade to restore the TCA cycle in the Fh1-deficient mitochondria, thus reducing mitochondrial ROS generation. However, this scenario is unlikely to occur in Fh1^{-/-} +FHΔMTS MEFs as it has been clearly demonstrated in Figure 3.5C and Table 3.1 that these cells still harbour a mitochondrial defect. An alternative explanation for this result may be that cytoplasmic FH is capable of diminishing cytoplasmic ROS, generated *via* the unfolded protein response (UPR), endoplasmic reticulum (ER) oxidoreductases or NADH oxidase responses (Kultz 2005; Santos et al. 2009) and that a non-specific signal from the MitoSOXTM probe may have resulted in detection of reduced cytoplasmic ROS.

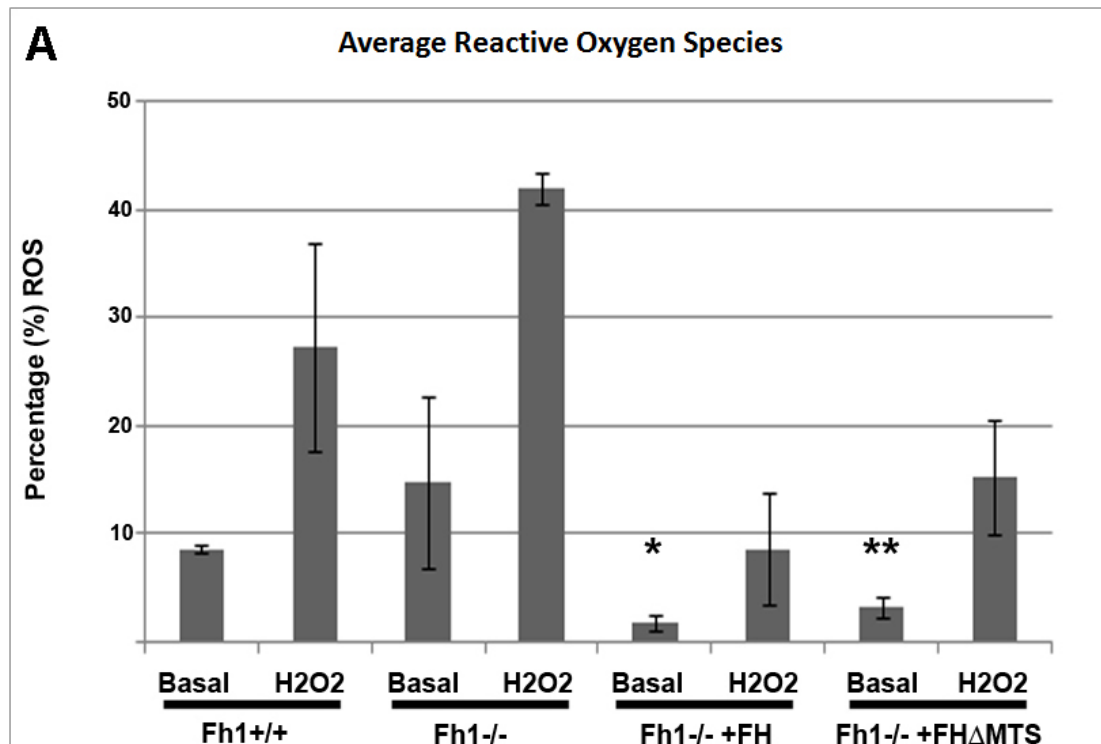
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Figure 3.8 Assessment of mitochondrial ROS levels in *Fh1*^{-/-} MEFs and *Fh1*^{-/-} MEFs complemented with either FH-V5 or FHΔMTS-V5

A) Measurement of mitochondrial ROS with MitoSOX[™] Red fluorogenic dye in *Fh1*^{+/+}, *Fh1*^{-/-}, *Fh1*^{-/-} +FH and *Fh1*^{-/-} +FHΔMTS MEFs in the presence or absence of 1mM H₂O₂ for 90 min. Results are shown as a mean ± S.E of triplicate assays.

p*<0.05 vs. Basal *Fh1*^{-/-}, *p*<0.05 vs. Basal *Fh1*^{-/-}.

*The effect of Fh1-deficiency on the Hif signalling pathway***3.3 Discussion**

This chapter describes the mechanism of Hif activation and signalling in Fh1-deficient MEFs. Also detailed in this chapter is the generation of genetically ‘rescued’ Fh1^{-/-} MEFs which have been transfected with either a wildtype, mitochondrial V5-tagged human FH or a truncated, cytoplasmic V5-tagged human FH in order to address the question of whether accumulation of Hif-1 α in Fh1-null cells is a direct result of a persistent defect in oxidative metabolism. The results and implications for these experiments are discussed here briefly followed by a more detailed appraisal in the final discussion (Chapter 5).

From the results generated above, it is evident that the accumulation of intracellular fumarate is directly involved in the inhibition of HIF-1 α prolyl hydroxylation, but not asparaginyl hydroxylation, thus leading to upregulation of HIF target genes in an Fh1-null background under conditions of environmental oxygen tensions (21% O₂) (O’Flaherty et al. 2010). Furthermore the comparative analysis of Hif-1 α hydroxylation in Fh1^{-/-} cells, tissues and tumours showed an apparent lack of effect on Asn819 hydroxylation in Fh1-deficiency which supports published data demonstrating that fumarate is more efficient at inhibiting PHD enzymes *in vitro* than FIH (Hewitson et al. 2007; Koivunen et al. 2007). Most striking is the finding that activation of the HIF pathway occurs independently of the mitochondrial defect associated with Fh1^{-/-} MEFs. This was confirmed by analyses of Fh1^{-/-} MEFs expressing the cytoplasm-restricted FH-V5 (Fh1^{-/-} +FH Δ MTS) as these cells retain the same mitochondrial defect and dependence on glycolytic metabolism as their parental Fh1-deficient cells (Figures 3.5 and 3.7).

The effect of Fh1-deficiency on the Hif signalling pathway

In comparison to the Fh1 wild-type MEFs, Fh1^{-/-} MEFs had elevated levels of mitochondrial ROS which were restored upon re-expression of mitochondrial FH but also cytoplasmic FH (Figure 3.8). This was surprising given that cells expressing cytoplasmic FH retain a persistent mitochondrial defect and would not be predicted to ablate mitochondrial ROS. Transport of cytoplasmic metabolites across the mitochondrial membrane of Fh1^{-/-} +FHΔMTS MEFs may potentially account for the ablation of mitochondrial ROS in these cells. However, given that the levels of ATP generation and oxygen consumption in Fh1^{-/-} +FHΔMTS MEFs are not restored to wildtype levels (Figure 3.5C and Table 3.1), it may be assumed that metabolite transport (namely malate and/or pyruvate) does not attenuate mitochondrial ROS levels by restoring function to the defective TCA cycle. The role of cytoplasmic-restricted FH in the ROS dynamic may possibly be linked to the function of cytoplasmic oxidases, or regulation by other antioxidant pathways (such as the Nrf2 pathway) in Fh1-deficient MEFs. In support of this, it has recently been published that the Nrf2 pathway is activated in Fh1-deficient cells (Adam et al. 2011; Ooi et al. 2011) and this activation was shown to occur independently of the mitochondrial defect (Adam et al. 2011).

Further investigations are required in order to better understand the role of cytoplasmic FH in conferring protection against cellular ROS and to identify the pathways that may be involved in this proposed function. This phenotype could be further examined by using shRNA-mediated inhibition of cytoplasmic malate dehydrogenase, responsible for converting malate to pyruvate, or targeting of the Nrf2 antioxidant pathway. This may help to determine whether redistribution of metabolites or regulation of antioxidant pathways are altered following cytoplasmic

The effect of Fh1-deficiency on the Hif signalling pathway

expression of FH in an Fh1-deficient background, thus attenuating cellular ROS levels in these cells. As there is conflicting evidence for mitochondrial ROS-dependent stabilisation of HIF-1 stabilisation (Jung et al. 2008) and HIF-1 expression attenuating mitochondrial ROS under hypoxia (Kirito et al. 2009), this complicates the possible interaction between mitochondrial ROS and the HIF pathway and its role in reducing ROS levels in Fh1^{-/-} +FHΔMTS MEFs. One possible approach would be to use siRNA or shRNA constructs to target HIF-1α for knockdown in these cells and compare it to Fh1^{-/-} +FHΔMTS MEFs over-expressing HIF-1α in order to determine whether the reduced ROS levels in these MEFs is dependent on the HIF pathway. Investigations into the effects of Fh1-deficiency on the unfolded protein response (UPR), endoplasmic reticulum (ER) oxidoreductases and NADH oxidase responses may also prove to be informative, especially in relation to the role of cytoplasmic FH in these ROS producing pathways. Finally, the use of whole cell ROS detection probes (such as reduced and acetylated forms of 2',7'-dichlorofluorescein (DCF) by Invitrogen) in combination with mitochondrial ROS detectors, such as MitoSOX™, may identify with more accuracy which ROS sources are responsive to re-expression of cytoplasmic FH in Fh1-deficient MEFs.

Although activation of the HIF pathway is a crucial contributing factor to cancer metastasis there is growing evidence to challenge the assumption that it is the sole primary event responsible for the onset of neoplasia in HLRCC (Adam et al. 2011; Bardella et al. 2011; Ooi et al. 2011; Tong et al. 2011) as it cannot account exclusively for the different biochemical processes and pathways affected in either sporadic or hereditary forms of associated PRCC2 renal cancers. For this reason comparison of the mitochondrial and cytoplasmic variants of FH may allow for a

The effect of Fh1-deficiency on the Hif signalling pathway

clearer understanding of the roles of specific pathways, in specific cellular compartments, that are important to the tumour suppressor function of FH.

As mutations of the MTS domain of FH have yet to be described in HLRCC, the possibility that the tumour suppressor function of FH is subject to MTS function cannot be completely discounted. Indeed, it has recently been reported that cytoplasmic FH forms a component of the DNA damage response (Yogev et al. 2010). While the $Fh1^{-/-}$ +FH and $Fh1^{-/-}$ +FH Δ MTS MEFs have been demonstrated to be informative cellular models it remained to be determined whether or not the results summarized in this chapter could be recapitulated in an *in vivo* mouse model. In light of the dysregulation of the Hif signalling pathway in $Fh1^{-/-}$ MEFs being fully restored following re-expression of cytoplasmic-restricted FH, the development of an *in vivo* model, using the Fh1 conditional knockout mice described previously (Pollard et al. 2007), may provide a powerful system for addressing whether a similar ‘rescue’ can be effected for the cystic phenotype associated with Fh1-deficiency. A detailed assessment of the generation and preliminary analysis of both mitochondrial and cytoplasmic ‘rescue’ transgenic mouse models will be discussed in Chapter 4.

**4 Chapter 4 Re-expression of either mitochondrial or cytoplasmic
FH in Fh1^{-/-} mice**

*Re-expression of either mitochondrial or cytoplasmic FH in Fh1^{-/-} mice***4.1 Introduction**

The aim of the work outlined in this chapter was to design an *in vivo* model ubiquitously expressing either full-length, mitochondrial human FH or cytoplasmic human FH (FH Δ MTS) in mice whose kidneys were specifically targeted for excision of *Fh1* using a Cre/loxP gene targeting system (Pollard et al. 2007). To enable comparisons to be made between the Fh1 MEF model and the *in vivo* model, the same V5-tagged human FH and FH Δ MTS sequences were used when designing the latter model system. The two primary objectives of this work were to determine 1) whether re-expression of FH in either mitochondrial or cytoplasmic compartments would recapitulate the findings of the *in vitro* Fh1 rescue MEFs, thus supporting the use of such a model, and 2) whether re-expression of either FH or more interestingly, FH Δ MTS, alone would be sufficient to diminish the aggressive cystic phenotype associated with Fh1-loss in the kidney. As the Fh1^{-/-} + FH Δ MTS MEFs successfully demonstrated that activation of the Hif pathway in Fh1^{-/-} MEFs occurs independently of the mitochondrial defect, it was hoped that cytoplasmic expression of FH Δ MTS in Fh1-deficient kidneys would elucidate a mechanism by which cystic development occurs, especially as this has also been shown to occur independently of the Hif pathway (Adam et al. 2011). Generating an *in vivo* model which can be used in parallel with a comparable cellular model provides a powerful analytical tool for investigating the regulation of pathways in Fh1-associated pathogenesis, such as HLRCC. When used in combination, these models could allow for high-throughput investigations to be carried out in a low-cost *in vitro* setting prior to extrapolation into an *in vivo* environment, as proof-of-concept for the physiological relevance of a proposed hypothesis.

Re-expression of either mitochondrial or cytoplasmic FH in Fh1^{-/-} mice

As discussed in greater detail in Chapter 1, section 1.5, there are numerous technologies now available for controlling gene expression *in vivo*. The Fh1 murine model discussed in this chapter utilised a site-specific DNA recombination, Cre/loxP system to target endogenous *Fh1* for inactivation in renal tubular cells. This was achieved by excising loxP-flanked exons 2 and 3 in the presence of a kidney specific Cre (expressed from a Ksp1.3/Cre allele), as was previously described (Pollard et al. 2007). Pollard *et al.* utilised the Cre/LoxP system to circumvent the problem of embryonic lethality associated with deletion of *Fh1*. Fh1-conditional knockout mice were then crossed with chimeras expressing either the FH or FH Δ MTS transgene under the control of a CAG (chicken β -actin promoter/CMV early enhancer) promoter. The rationale for expressing the transgenes under a ubiquitous, rather than tissue specific promoter, was to develop a flexible model that could be readily incorporated into other *in vivo* studies in different tissues for investigating a varied range of phenotypes.

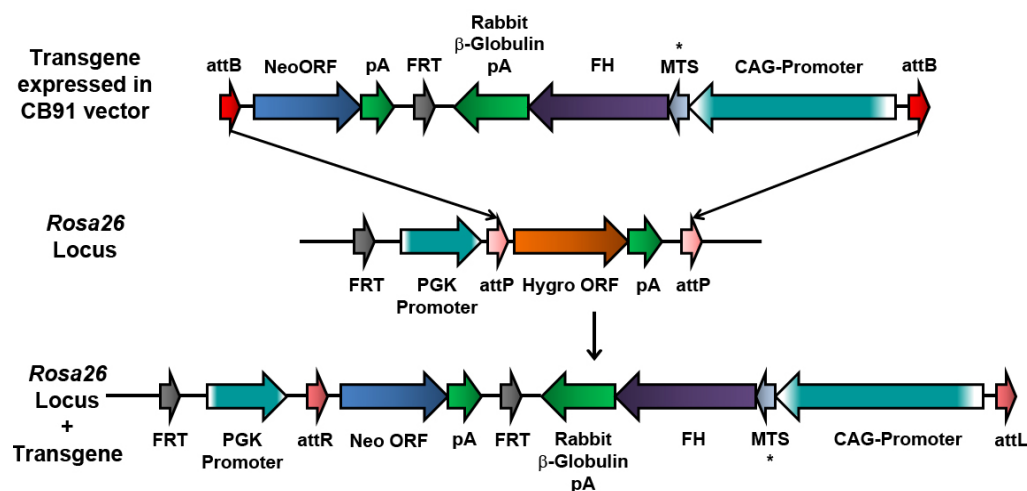
4.2 Results

4.2.1 Generation of CAG-FH transgenic complemented Fh1 conditional knockout mice

The cDNA sequences for V5-tagged full length FH (predominantly targeted to the mitochondria), or V5-tagged cytoplasm-restricted FH (lacking the N-terminal MTS), the CAG promoter and an FRT-flanked Neomycin (neo) selectable marker cassette (which can be removed at a later stage by crossing with a Flp recombinase mouse (Takeuchi et al. 2002)) were cloned into a CB91 exchange vector (carried out by Dr.

Re-expression of either mitochondrial or cytoplasmic FH in Fh1^{-/-} mice

Ben Davies, Transgenic Core, Oxford University). To avoid integration of the transgenes at random sites in the genome, the expression vectors were specifically targeted to the *Rosa26* locus of the acceptor ES cells as this locus is expressed ubiquitously in both embryonic and adult tissues (Zambrowicz et al. 1997; Soriano 1999). This was achieved by co-electroporation of the transgene expression vector with an expression cassette for PhiC31 (bacteriophage) integrase (carried out by Dr. Ben Davies, Transgenic Core, Oxford University). The Phi integrase catalyses the homologous recombination of the attachment site attB of the CB91 vector and the attachment site attP preintegrated into the *Rosa26* locus, thus facilitating integration of the transgene in a site-specific manner (Keravala and Calos 2008) (Chapter 6, Figure 6.4).



Expression vector encoding the FH transgene (Taken from Chapter 6 [Figure 6.4]).

Re-expression of either mitochondrial or cytoplasmic FH in Fh1^{-/-} mice

Recombinant ES cells clones were screened by DNA PCR to confirm correct integration at both the 5' and 3' end of the exchange vector (carried out by Dr. Ben Davies, Transgenic Core, Oxford University). Further confirmation of transgene-positive ES clones was carried out by immunoblot analysis of the V5 protein content and immunofluorescent (IF) localisation of the V5-tagged FH transgenes within ES cell clusters (Figure 4.1A and B). ES cell clones positive for CAG-FH and CAG-FHΔMTS transgenes were then injected into C57BL/6 blastocysts and transferred to pseudopregnant females (Blastocyst injection was performed by Mr. Chris Preece, Transgenic Core, Oxford University). The degree of chimerism within the F1 progeny was assessed on the basis of the percentage agouti coat colour expressed. Mice with >70% chimerism were selected for breeding with Fh1-conditional (C57BL/6) mice (Pollard et al. 2007) followed by a subsequent cross with Ksp1.3/Cre recombinase transgenic mice (Shao et al. 2002; Shao et al. 2002) to knock out LoxP-flanked *Fh1* within the kidneys. The breeding strategy was designed to generate the following littermate phenotypes: Fh1^{fl/fl}, Fh1^{fl/fl}+CAG-FH, Fh1^{fl/fl}+CAG-FHΔMTS, Fh1^{-/-}, Fh1^{-/-}+CAG-FH and Fh1^{-/-}+CAG-FHΔMTS. The FH transgenes, integrated into the genome at chromosome 7, were maintained in heterozygosity to avoid potential compounding factors associated with homozygous expression (consult section 4.3 for a more detailed description of these factors).

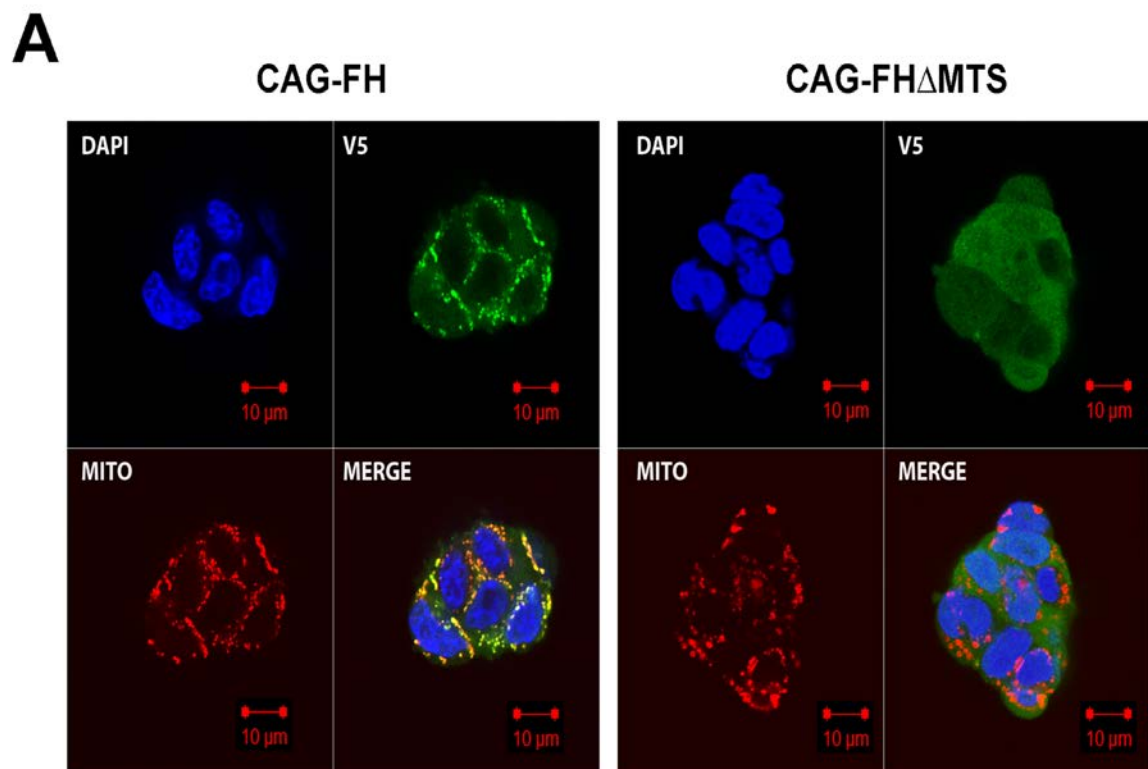
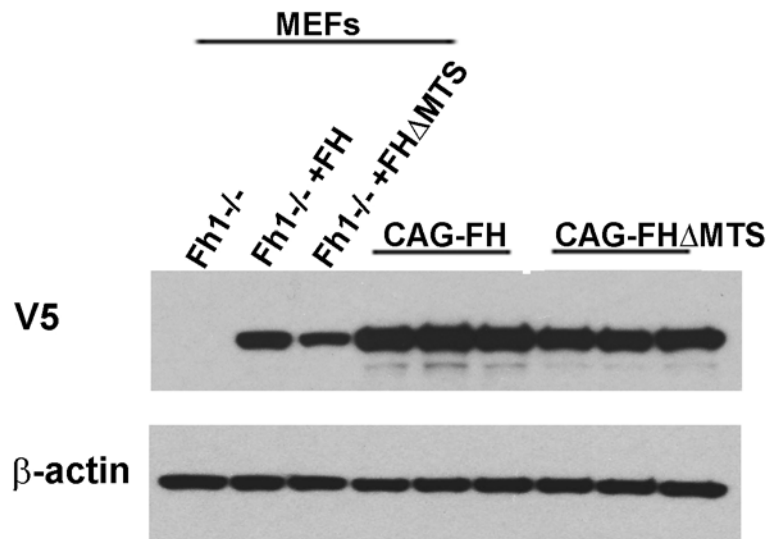
Re-expression of either mitochondrial or cytoplasmic FH in Fh1^{-/-} mice

Figure 4.1 Confirmation of transgene localisation and expression

(A) Localisation of CAG-FH and CAG-FH Δ MTS transgenes in ES cells selected for blastocyst injection, as confirmed by IF staining. The CAG-FH transgene is predominantly mitochondrial whereas the CAG-FH Δ MTS transgene is restricted to the cytoplasmic compartment. Scale bar=10 μ M.

Re-expression of either mitochondrial or cytoplasmic FH in $Fh1^{-/-}$ mice**B****Figure 4.1 Confirmation of transgene localisation and expression**

(B) Immunoblot confirmation of V5-tag protein levels in three independent ES cell lines expressing either CAG-FH or CAG-FH Δ MTS transgenes. $Fh1^{-/-}$ MEFs and $Fh1^{-/-}$ MEFs expressing either FH-V5 or FH Δ MTS-V5 were included as positive controls for V5 expression and an indicator of expression levels.

*Re-expression of either mitochondrial or cytoplasmic FH in Fh1^{-/-} mice**4.2.2 Confirmation of transgene expression in vivo*

CAG-FH and CAG-FH Δ MTS positive and transgene negative (Fh1^{fl/fl}) mice were sacrificed at 8 weeks to confirm ubiquitous expression of the CAG-FH and CAG-FH Δ MTS transgenes *in vivo*. Protein lysates prepared from a selection of tissues (heart, lung, liver, spleen, kidney and uterus) was assessed by immunoblot analysis. All tissues showed strong expression of the V5-tag, located at the C-terminal of the CAG-FH and CAG-FH Δ MTS transgenes, thus confirming ubiquitous expression of both transgenes (Figure 4.2A). Protein loading was controlled for by blotting against β -actin. It should be noted that β -(skeletal) actin is not expressed in cardiac muscle and hence not detected in Figure 4.2A. Faint traces of V5-staining were detected in tissue lysates from CAG-FH Δ MTS negative spleen and uterus; however this is most likely an artefact that resulted from cross contamination during sample preparation.

Primary renal tubular cells were cultured from one of the kidneys harvested from each of the mice (aged 8 weeks) described above. After ~10 days grown under standard cell culture conditions, the cells were harvested and assessed for V5 expression. Whole cell lysates from Fh1^{-/-}, Fh1^{-/-}+FH-V5 and Fh1^{-/-}+FH Δ MTS-V5 MEFs were included to compare the relative levels of transgene expression *in vitro* against the levels expressed *in vivo* (Figure 4.2B).

Microcyst development in Fh1 knockout mice is observed as early as 13 weeks with progression to macrocysts apparent at 24 weeks (Pollard et al. 2007; Adam et al. 2011). In order to clearly distinguish a phenotypic difference in cystic growth between the Fh1^{-/-} +CAG-FH and Fh1^{-/-} +CAG-FH Δ MTS transgenic mice the

Re-expression of either mitochondrial or cytoplasmic FH in Fh1^{-/-} mice

experimental endpoint of this *in vivo* study was set at 19-20 weeks when cysts can be clearly identified.

Mice aged 19-20 weeks were sacrificed and kidneys bisected for paraffin embedding (Histopathox Ltd., London, UK), frozen sectioning (Histopathox Ltd., London, UK), protein and mRNA analysis. Where possible all analyses of the CAG-FH and CAG-FHΔMTS mice were carried out using littermate controls and a minimum number of n=3 animals per cohort studied. Frozen sections of bisected kidneys from each cohort were stained with an anti-V5-FITC conjugated antibody to determine by IF the percentage of either CAG-FH or CAG-FHΔMTS transgene expression, where relevant, within the renal tubules. Although autofluorescence in both the DAPI (405nm) and FITC (488nm) channels was a confounding factor, the variability in V5 expression was apparent both within and between the different transgene variants (n=3 for both Fh1^{-/-}+CAG-FH and Fh1^{-/-}+CAG-FHΔMTS; n=2 for both CAG-FH and FHΔMTS) (Figure 4.3).

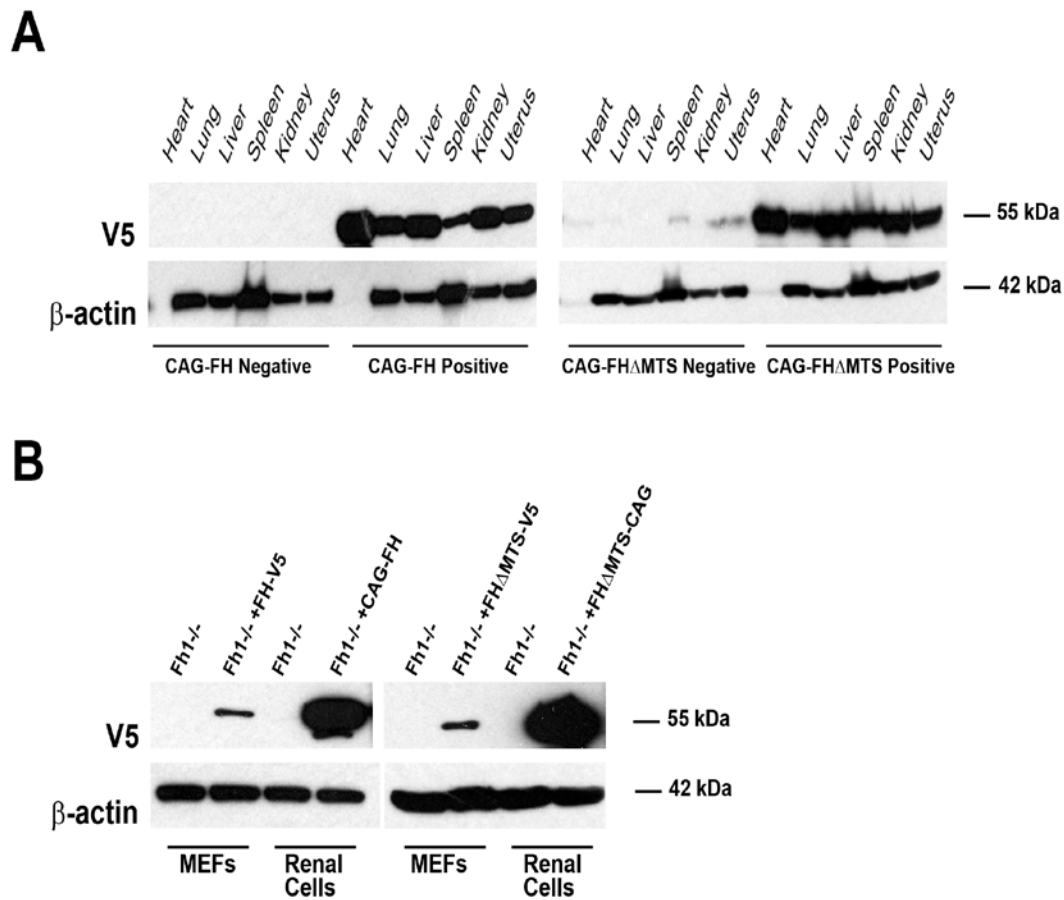
Re-expression of either mitochondrial or cytoplasmic FH in $Fh1^{-/-}$ mice

Figure 4.2 Confirmation of V5-tag expression *in vivo*

(A) Immunoblot analysis of tissue lysates from a selection of CAG-positive and CAG-negative organs confirmed ubiquitous expression of the V5-tag (as a positive indicator for the presence of the CAG-FH and CAG-FH Δ MTS transgenes) in both the CAG-FH and CAG-FH Δ MTS transgenic mice. V5-staining was absent in the CAG-negative littermate control tissues. (B) Primary renal tubular cells harvested from CAG-FH and CAG-FH Δ MTS kidneys have high concentrations of the V5-tagged transgenes. The level of V5-tag expression in the $Fh1^{-/-}$ MEFs complemented with either FH-V5 or FH Δ MTS-V5 is comparable although not as abundant as the V5 levels found in the renal cells. The abundance of V5-tagged FH in the renal cells may be attributed to the ubiquitous expression of the CAG-transgenes in a site-specific manner whereas in the MEFs the transgenes are subject to random integration which may result in reduced expression levels.

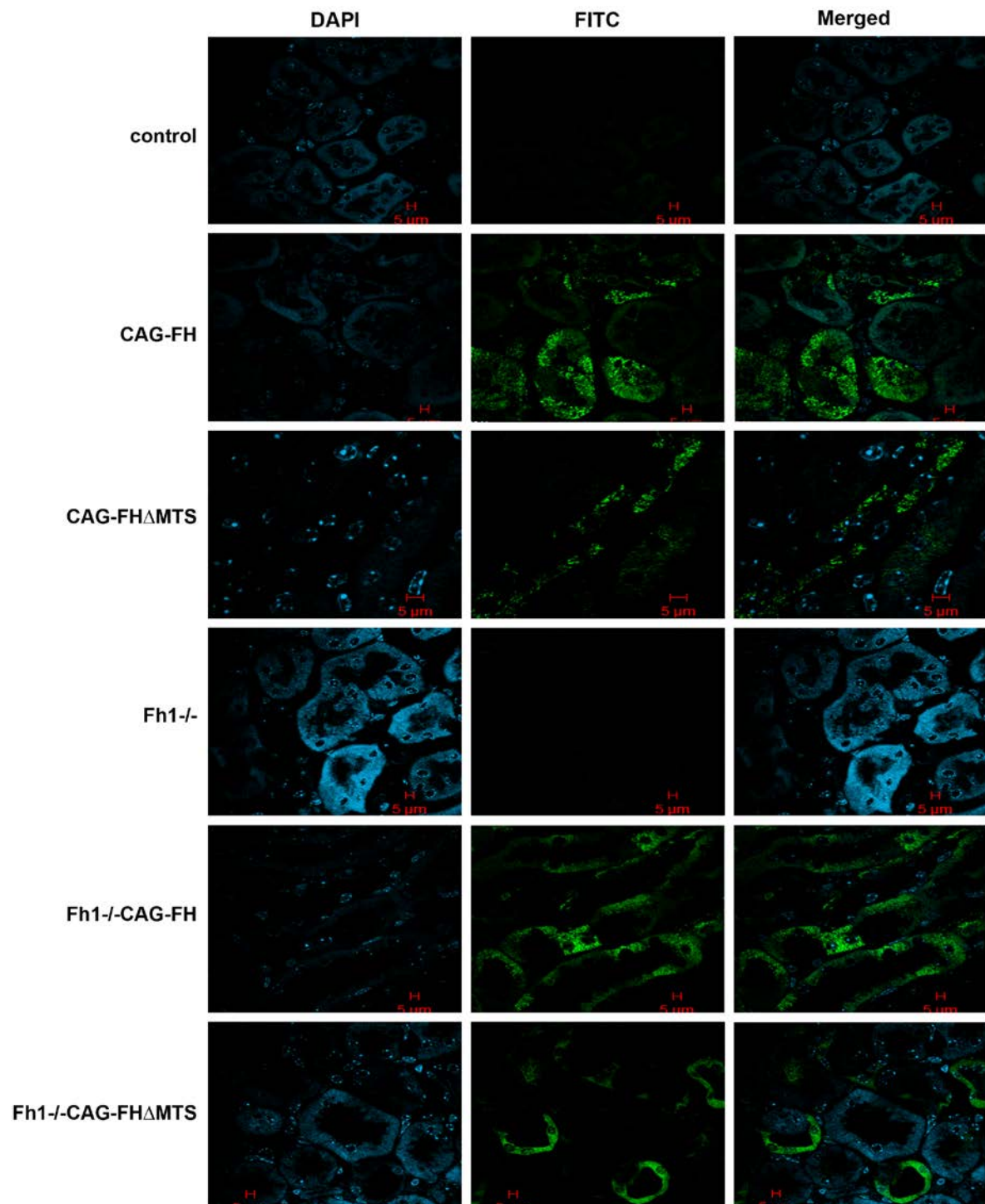
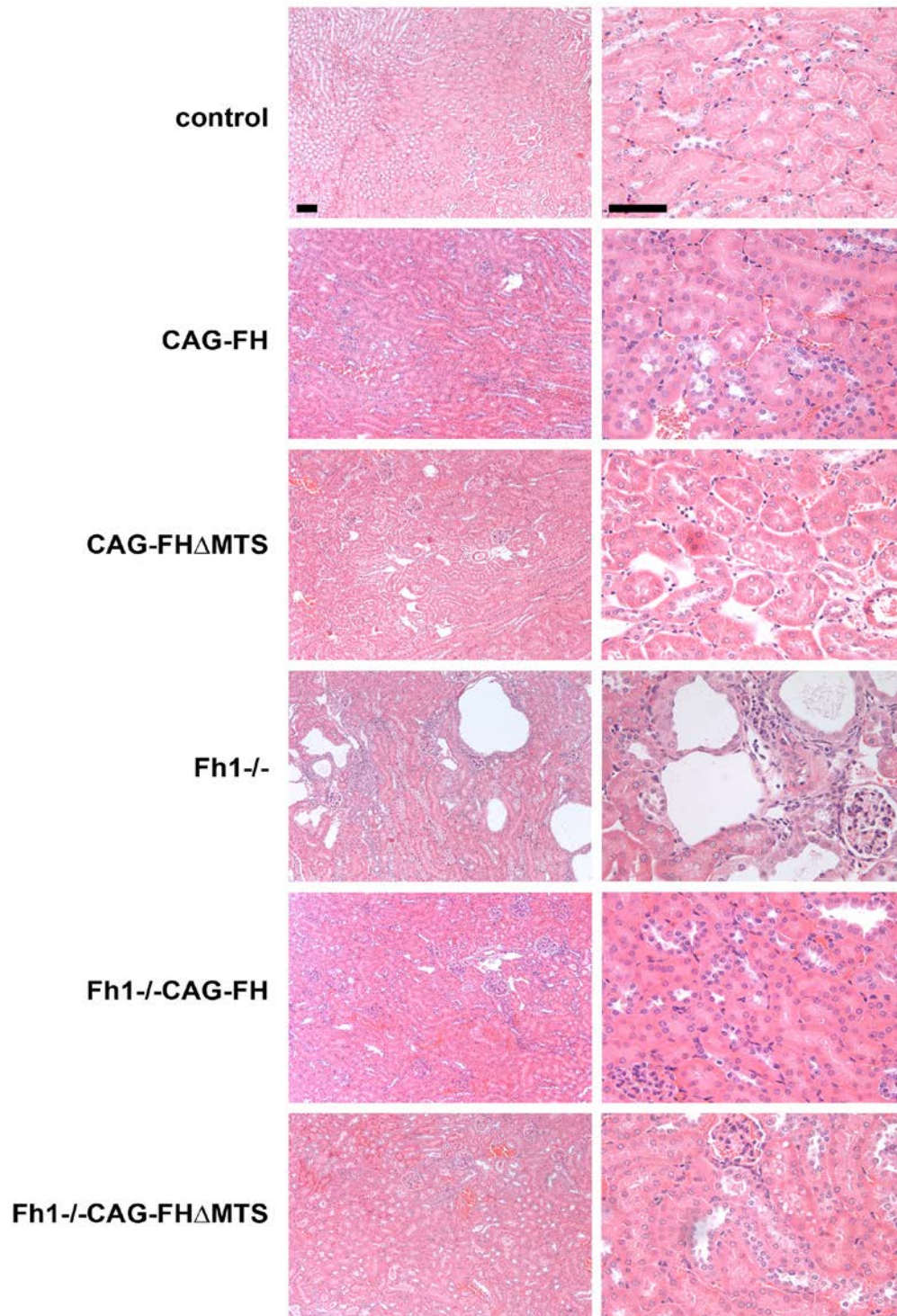
Re-expression of either mitochondrial or cytoplasmic FH in *Fh1*^{-/-} mice

Figure 4.3 Confirmation of V5-tag expression *in vivo*

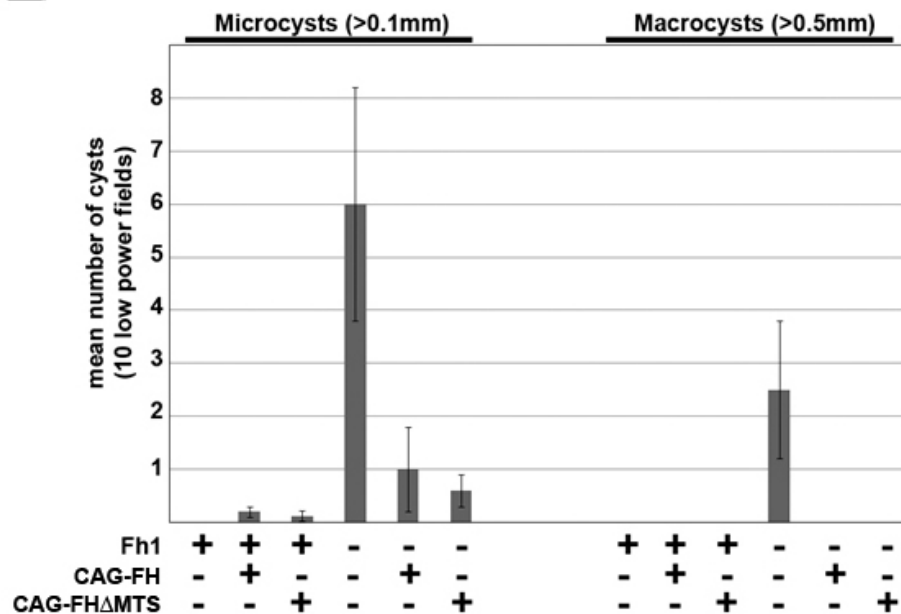
Identification of renal tubular cells (from frozen kidney sections) positive for either the CAG-FH or CAG-FH Δ MTS transgene, as shown by IF using an anti-V5-FITC antibody. Scale bar=5 μ m.

*Re-expression of either mitochondrial or cytoplasmic FH in Fh1^{-/-} mice**4.2.3 Expression of CAG-FHΔMTS and CAG-FH transgenes rescues the cystic phenotype in Fh1-null mice*

Haematoxylin and eosin (H&E) stained sections of kidney tissue from Fh1^{fl/fl}, Fh1^{fl/fl} +CAG-FH, Fh1^{fl/fl} +CAG-FHΔMTS, Fh1^{-/-}, Fh1^{-/-} +CAG-FH and Fh1^{-/-} +CAG-FHΔMTS mice were assessed for the presence and frequency of both micro-(>0.1mm but ≤0.5mm) and macrocyst (>0.5mm) development. Multiple micro- and macrocysts are apparent within the Fh1^{-/-} kidneys (Figure 4.4A). In contrast to this, re-expression of either CAG-FH or, most interestingly, CAG-FHΔMTS diminished cystic growth in Fh1^{-/-} kidneys to such an extent that the kidney tissues from these mice are comparable with those of their wildtype littermates (Figure 4.4A and B). This result is most striking in the kidneys from Fh1^{-/-} +CAG-FHΔMTS mice where macrocyst growth was not only abolished, but surprisingly, the frequency of microcysts was also lower than that observed in Fh1^{-/-} +CAG-FH mice. Expression of the transgenes in wildtype mice did not have any apparent phenotype by 20 weeks of age.

Re-expression of either mitochondrial or cytoplasmic FH in $Fh1^{-/-}$ mice**A****Figure 4.4 Re-expression of CAG-FH or CAG-FH Δ MTS ameliorates cystic development**

(A) H&E stained kidney sections confirming that expression of both CAG-FH and CAG-FH Δ MTS attenuates renal cyst development in $Fh1^{-/-}$ mice. Scale bar=100 μ m.

Re-expression of either mitochondrial or cytoplasmic FH in $Fh1^{-/-}$ mice**B****Figure 4.4 Re-expression of CAG-FH or CAG-FHΔMTS ameliorates cystic development**

(B) Quantification of microcysts (>0.1mm) and macrocysts (>0.5mm) from each cohort demonstrated that microcystic growth in $Fh1^{-/-}$ mice is repressed following complementation with either CAG-FH or CAG-FHΔMTS transgenes. $Fh1^{-/-}$ CAG-FH and $Fh1^{-/-}$ CAG-FHΔMTS mice showed no observable macrocystic growth. Some tubular dilation was noted in $Fh1^{fl/fl}$ mice carrying either the CAG-FH or CAG-FHΔMTS transgene, however these were not significant when compared to kidneys from $Fh1^{fl/fl}$ littermates.

*Re-expression of either mitochondrial or cytoplasmic FH in Fh1^{-/-} mice**4.2.4 Accumulation of 2SC and stabilised Hif-1 α protein is diminished following expression of either the CAG-FH or the CAG-FH Δ MTS transgenes in Fh1^{-/-} mice*

Fumarate accumulation has been shown to correlate directly with an increase in 2SC-modified proteins (as discussed in detail in Chapters 1, section 1.4.1, and 5, section 5.4) and these 2SC proteins can be used as metabolic markers for the detection of Fh1-deficient tissues by IHC (Bardella et al. 2011). Recently, the presence of 2SC protein modifications has also been shown in Fh1-deficient pancreatic β -cells (Dr. Julie Adam, unpublished data). These cells have a diminished capacity for insulin release and mice with Fh1-null β -cells have been found to develop diabetes (Dr. Julie Adam, unpublished data). This is suggestive of a potential role for FH-deficiency in diabetes and obesity. While specific FH-mutations have yet to be identified in human diabetic and obese patients, 2SC remains a marker for FH mutations as demonstrated in the unpublished data by Dr. Julie Adam. As previously described, and demonstrated using the *in vitro* Fh1 MEF model in Chapter 3, there is a close association between Fh1-deficiency and normoxic stabilisation of Hif-1 α (Isaacs et al. 2005; Pollard et al. 2005; Koivunen et al. 2007; O'Flaherty et al. 2010). However, the accumulation of Hif-1 α and 2SC proteins can be restored to wildtype levels by expressing either V5-tagged FH or V5-tagged FH Δ MTS in Fh1^{-/-} MEFs, as shown in Chapter 3. To determine whether expression of either CAG-FH or CAG-FH Δ MTS is sufficient to ablate the accumulation of 2SC-modified proteins and restore normoxic degradation of Hif-1 α *in vivo*, paraffin embedded kidney sections from Fh1^{-/-}, Fh1^{-/-} +CAG-FH and Fh1^{-/-} +CAG-FH Δ MTS mice were stained with 2SC and Hif-1 α specific antibodies.

Re-expression of either mitochondrial or cytoplasmic FH in Fh1^{-/-} mice

Compared to Fh1^{-/-} kidneys, the number of cells that stained positively for 2SC proteins was substantially reduced in the kidneys of CAG-FH and CAG-FHΔMTS transgenic mice (Figure 4.5A). Supporting the observations made on analysis of the H&E sections (section 4.2.3, Figure 4.4A), the phenotypic rescue was more apparent in Fh1^{-/-} +CAG-FHΔMTS rather than Fh1^{-/-} +CAG-FH mice. Where 2SC staining was detected in the Fh1^{-/-} +CAG-FH and Fh1^{-/-} +CAG-FHΔMTS kidney sections it was assumed that the Fh1-knockout phenotype persisted in the absence of transgene expression within these individual cells. This was confirmed by staining the tissues for FH (Figure 4.5A). Similarly, normoxic accumulation of Hif-1α is ablated following expression of either CAG-FH or CAG-FHΔMTS, although this occurs in a mosaic pattern.

Immunoblot analysis of whole tissue lysates from Fh1^{fl/fl}, Fh1^{fl/fl} +CAG-FH, Fh1^{fl/fl} +CAG-FHΔMTS, Fh1^{-/-}, Fh1^{-/-} +CAG-FH and Fh1^{-/-} +CAG-FHΔMTS kidneys corroborated the IHC results by establishing that both transgenes are capable of abolishing the accumulation of 2SC proteins and normoxic Hif-1α that is present in Fh1^{-/-}-tissues (Figure 4.5B), in keeping with the IHC results above. Since Fh1 is specifically targeted for excision in renal tubular cells, Fh1 protein is still detected in Fh1^{-/-} samples due to the presence of non-tubular cells (accounting for most of the kidney tissue) retained within the sample.

As neither the CAG-FH nor the CAG-FHΔMTS transgene is expressed within every renal tubule cell (Figure 4.3A), this may account for the differences between the two transgenic rescue lines with regard to their efficacy in reducing the elevated levels of

Re-expression of either mitochondrial or cytoplasmic FH in Fh1^{-/-} mice

2SC proteins in Fh1^{-/-} mice. Despite the variability in expression, both transgenes clearly rescue the 2SC and Hif-1 α phenotypes associated with Fh1-deficiency, although a small number of macrocysts remained present. The presence of a few 2SC positive tubules suggests that this is due to the transgene(s) not being ubiquitously expressed. These results are in keeping with the findings from the *in vitro* MEF models (Chapter 3); that activation of the Hif pathway and, according to the results of this study, fumarate-dependent 2SC modifications occurs independently of the mitochondrial defect observed in Fh1^{-/-} tissues.

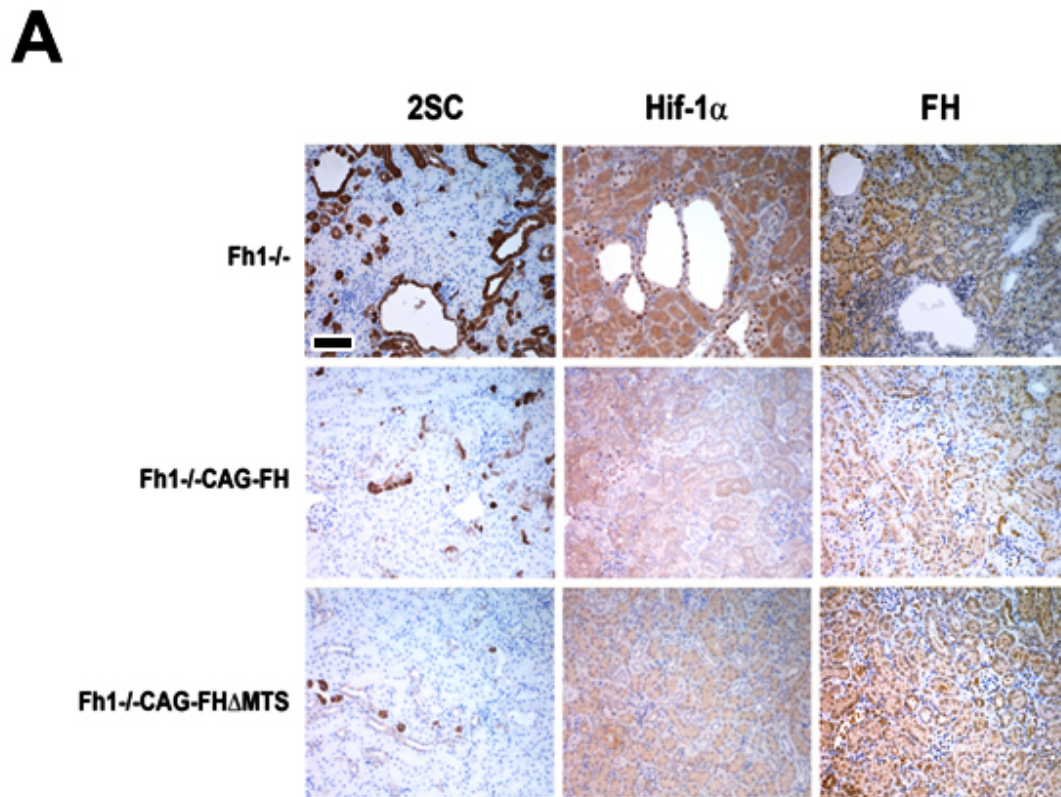
Re-expression of either mitochondrial or cytoplasmic FH in $Fh1^{-/-}$ mice

Figure 4.5 IHC staining for 2SC, HIF-1 α and FH in kidney sections of $Fh1^{-/-}$, $Fh1^{-/-}$

CAG-FH and $Fh1^{-/-}$ CAG-FH Δ MTS mice

(A) IHC staining for 2SC, Hif-1 α and FH in kidney sections from $Fh1^{-/-}$, $Fh1^{-/-}$ CAG-FH and $Fh1^{-/-}$ CAG-FH Δ MTS mice. In comparison to $Fh1^{-/-}$ kidneys, the levels of 2SC are substantially reduced in both $Fh1^{-/-}$ CAG-FH and $Fh1^{-/-}$ CAG-FH Δ MTS kidneys. Furthermore, expression of nuclear Hif-1 α is also ameliorated in $Fh1^{-/-}$ CAG-FH and $Fh1^{-/-}$ CAG-FH Δ MTS kidneys. Scale bar=100 μ m.

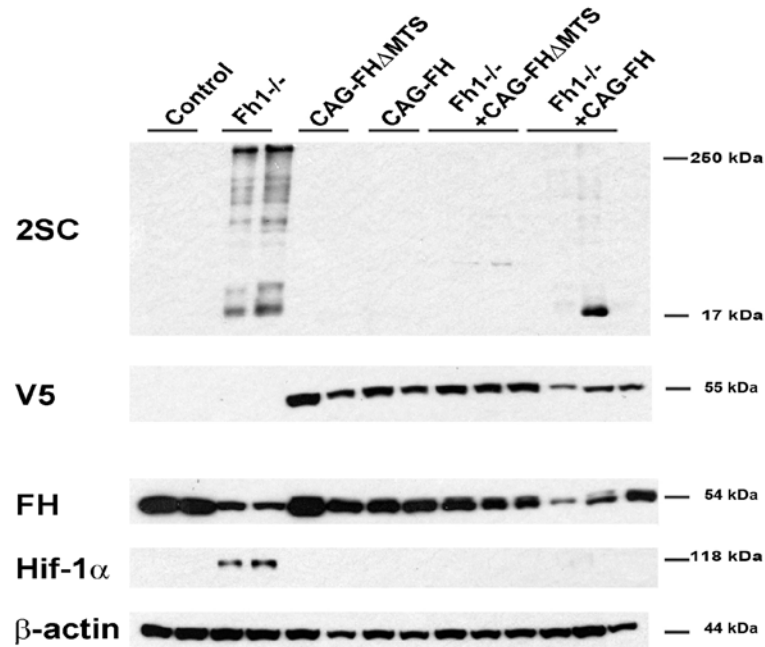
Re-expression of either mitochondrial or cytoplasmic FH in $Fh1^{-/-}$ mice**B**

Figure 4.5 Immunoblot analysis of 2SC, V5, FH and Hif-1α expression in $Fh1^{-/-}$, $Fh1^{-/-}$ CAG-FH and $Fh1^{-/-}$ CAG-FHΔMTS mice

(B) Whole kidney tissue lysates were analysed by immunoblot to confirm the presence of V5-tagged protein in mice positive for the CAG-transgenes and to assess the levels of 2SC, FH and Hif-1α protein in each phenotype. As Ksp1.3/Cre-mediates recombination of the loxP-sites only in renal tubular and collecting duct cells of $Fh1^{fl/fl}$ mice, Fh1 protein is still detected in the cells not targeted by Ksp1.3/Cre for Fh1 excision. β-actin was included as a loading control.

*Re-expression of either mitochondrial or cytoplasmic FH in Fh1^{-/-} mice***4.3 Discussion**

The rationale for developing transgenic Fh1^{-/-} mice complemented with either a mitochondrial or cytoplasmic human FH transgene was to reproduce the *in vitro* findings reported using the Fh1 MEFs in an *in vivo* environment. The primary aim of this *in vivo* model was to determine whether re-expression of FH, in particular cytoplasmic FH (FHΔMTS), would be sufficient to ameliorate the cystic phenotype associated with Fh1-deficiency *in vivo*. The results showed clearly that expression of either the FH or, strikingly, the FHΔMTS transgene can ameliorate cystic growth. These results suggest that the cystic phenotype is independent of the initial mitochondrial defect observed in Fh1-deficiency, although the mechanism through which this is achieved remains to be understood. As observed in the Fh1^{-/-}+FHΔMTS, the Fh1^{-/-}+CAG-FHΔMTS mice are also capable of abolishing the accumulation of normoxic Hif-1α phenotype whilst retaining increased glycolytic activity.

Unexpectedly, the cohort expressing the cytoplasm-restricted (CAG-FHΔMTS) transgene appeared to rescue the cystic phenotype more efficiently than the cohort expressing the full length (CAG-FH). However, it is still unclear how the cytoplasmic FH confers better protection against cyst development than the FH transgene targeted to the mitochondria. It may be that more abundant expression of the CAG-FHΔMTS transgene (see Figure 4.5A and 4.5B) is responsible for diminishing the cystic phenotype. To address the differences between the CAG-FH and CAG-FHΔMTS transgenes, a larger cohort of animals including other time points (e.g. 30 weeks) needs to be assessed in order to determine whether the findings outlined above, with respect to ameliorating the cystic phenotype, remain consistent. Should a difference

Re-expression of either mitochondrial or cytoplasmic FH in $Fh1^{-/-}$ mice

between the efficiency of the CAG-FH and CAG-FH Δ MTS transgenes remain apparent then high resolution metabolomic analysis (e.g. capillary electrophoresis-mass spectrometry) of kidney tissues from CAG-FH and CAG-FH Δ MTS transgenic mice, including wildtype controls, could be employed to identify potential metabolomic pathways whose regulation is altered in a transgene-dependent manner. This may provide an insight into the cellular mechanism involved in FH-associated cystic development and how the cytoplasmic variant of FH is more effective at aborting it. The possibility of the CAG-FH rescue being a mitochondrial-only rescue rather than a full length, wildtype rescue cannot be discounted either and therefore determining the localisation of the FH-transgene rescues and relative expression of FH (using capillary electrophoresis-mass spectrometry) *in vivo* is of great importance. Should this be the case, these findings would suggest that the cytoplasmic isoform of FH playing an important role in cyst development.

Moreover it is apparent from the data that there is some variation in each transgenic line with respect to the number of cells expressing the respective FH transgene and the efficiency of *Fh1* inactivation (as illustrated by IHC, Figure 4.5A, and IF staining, Figure 4.3). In light of this, it is likely that there are in theory four different mosaic patterns ($Fh1^{-/-}$, $Fh1^{-/-}$ +transgene, $Fh1^{fl/fl}$ and $Fh1^{fl/fl}$ +transgene) of expression occurring in each cohort, therefore making it difficult to extrapolate a distinct mechanism through which the CAG-FH and CAG-FH Δ MTS transgenes rescues the cystic phenotype. The *in vivo* model described in this chapter used a CAG-promoter to drive ubiquitous expression of the human FH transgenes at the *Rosa26* locus. However, in the absence of Ksp1.3/Cre, the FH/FH Δ MTS transgene is expressed simultaneously with endogenous *Fh1* and therefore the level of cellular fumarate

Re-expression of either mitochondrial or cytoplasmic FH in $Fh1^{-/-}$ mice

hydratase could be described as over-expressed. The breeding strategy was designed to keep the FH transgene in heterozygosity to reduce the occurrence of potential compounding factors resulting from homozygous expression of the transgenes. For example, homozygous expression of the transgene may disrupt normal transcriptional transactivation of endogenous *Fh1*, or nearby genes, thus reducing mRNA levels and resulting in a hypomorphic allele which can complicate assessment of the transgenic phenotype. It is, however, important to clarify that no effect on viability following expression of either the CAG-FH or CAG-FHMTS transgene was observed in these mice. In light of this it may be an option to adjust the breeding strategy so that the transgene is expressed in both alleles. This may enhance the uniformity of transgene expression.

Another approach to potentially increase the efficiency of the transgene expression would be to flip out the 'neo cassette'. The neo cassette was retained during development of the *in vivo* model however the selectable marker was flanked by an FRT sequence so that it could be flipped out at a later stage by crossing the CAG-transgenic mice with an Flp-recombinase transgenic mouse (Takeuchi et al. 2002) (referred to as the Flp/FRT system). This may prevent confounding effects from occurring in later generations. Inclusion of a selectable marker in the introns or the untranslated (UTR; flanking the coding sequence)/5'- or 3'- flanking regions of a gene of interest remains disputed. There is evidence to suggest that it can compromise either floxed allele or wildtype function by interfering with normal RNA splicing mechanisms, thus resulting in diminished mRNA levels (referred to as a hypomorphic allele) (Meyers et al. 1998; Nagy et al. 1998; Lewandoski 2001; Xu et al. 2001). However, there is also evidence in favour of retaining the neo cassette as it can be

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considerably informative studying the phenotypes generated by hypomorphic alleles in combination with the phenotypes generated by conditional Cre/loxP alleles or null alleles, (Meyers et al. 1998; Nagy et al. 1998; Partanen et al. 1998; Gage et al. 1999).

A more refined method of expressing the transgene *in vivo* would be to use a doxycycline-dependent induction of the transgene in a tissue-specific, as opposed to ubiquitous, manner by combining the Cre/loxP system with the TetR gene expression system. However, this is a more costly approach, complicates animal husbandry and increases the risks of position effects (Lewandoski 2001). Moreover, the *in vivo* model in its current form remains a suitable and robust system for studying Fh1-deficient phenotypes when used in parallel with the *in vitro* Fh1 MEF models, as demonstrated in this chapter.

There was no evidence during validation of the FH-complemented Fh1^{-/-} MEFs to suggest that the presence of a C-terminal V5-tag compromised FH/FHΔMTS-expression *in vitro* when assessed in parallel with the non-V5-tagged transgenes (Appendix Figure 3.3). While the *in vitro* data shows no interference from the V5-tag it may be that expression of a V5-tag in a more physiologically complex *in vivo* environment can interfere with the normal processing and protein folding function of FH. Compromised protein folding function could prevent translocation of the cleaved-population of FH from the mitochondria to the cytoplasm, thus limiting CAG-FH expression solely to the mitochondria compartments. This may suggest that the CAG-FH mice are mitochondrial-restricted rescues rather than wildtype rescues (i.e. with FH expressed in both compartments). This may explain why V5-tag expression in the cytoplasm of Fh1^{-/-} +FH-V5 MEFs could not be visualised by IF staining against the

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V5-tag. One possible explanation for this is that the V5-tag is cleaved from the FH peptide in the process of either a) cleavage of the MTS at the mitochondrial membrane or b) reverse translocation, following processing of FH by MPPs in the mitochondrial matrix, which promotes localisation of the truncated FH peptide to the cytoplasm. Despite the inability to visualise cytoplasmic FH in Fh1^{-/-} +FH-V5 MEFs, these cells have been shown to reduce fumarate and restore the rate of both ATP generation and oxygen consumption to levels comparable with wildtype MEFs (see Chapter 3) (O'Flaherty et al. 2010).

The technical caveats that may be responsible for the disparity between the Fh1^{-/-} +CAG-FH and Fh1^{-/-} +CAG-FH Δ MTS, and their effectiveness at rescuing the Fh1^{-/-} deficient cystic phenotype, have been discussed above. However, in the absence of a 100% efficient model system for *in vivo* gene targeting, limitations are to be expected when using the gene targeting systems currently available. Limitations include random integration and variable copy numbers of the transgene within the genome, mosaicism (multiple phenotypes/patterns of expression within a given cell/tissue) and dysregulation of nearby genes (Bockamp et al. 2002; Tecott 2002; O'Sullivan 2006). Some of these difficulties are unavoidable, but can be minimised by careful design and inclusion of appropriate controls. However, the *in vivo* models described in Chapter 4 circumvent the problems of random integration and variable copy number by specifically targeting the transgene to the *Rosa26* locus. Therefore, the finding that expression of the CAG-FH Δ MTS transgene was more efficient than the CAG-FH transgene at abolishing cystic development should not be dismissed as merely a complication of the model design. To better understand the potential importance of

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this finding we need to examine the role of cytoplasmic FH in a wider physiological context.

Recently it was shown in *S. cerevisiae* that fumarase had a role in the DNA repair response following its translocation to the nucleus. This response was impaired in yeast engineered to be defective in cytosolic, but not mitochondrial fumarase (Yogev et al. 2010). Based on these findings it has been suggested that cytoplasmic fumarase has an important role in regulating this response (Yogev et al. 2011). Furthermore, activation of the Nrf2 anti-oxidant pathway has been shown, by two independent groups, to be markedly increased in Fh1-deficient cells (Adam et al. 2011; Ooi et al. 2011) and Adam *et al.* also demonstrated that this activation occurs independent of the associated mitochondrial defect, thus indicative of this pathway being regulated by cytoplasmic Fh1. Both groups demonstrated that stabilisation of Nrf2 was as a direct result of fumarate-mediated succination of cysteine residues in Keap1. A recent study has also shown that fumarate-mediated activation of the Nrf2 pathway has a role in cardioprotection against ischaemic/reperfusion injury in Fh1^{-/-} cardiac tissues (Ashrafian 2012). It may be that the activity of cytoplasmic FH in Fh1^{-/-} +CAG-FHΔMTS catalyses fumarate, translocated from the Fh1-deficient mitochondria to the cytoplasm, at a rate which prevents these fumarate molecules from initiating 2SC-modification of cytoplasmic-based regulatory proteins. In order to identify a broad range of 2-SC modified proteins NMR, mass spectrometry and chromatography techniques, such as liquid chromatography should be utilised. This is especially relevant for the Fh1^{-/-} +CAG-FH mice as some 2SC modifications are apparent in the kidney tissues when compared to the Fh1^{-/-} +CAG-FHΔMTS kidneys. Identification of these 2SC modified proteins may be important in identifying potential regulatory

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pathways which may be responsible for the cystic phenotype not being as efficiently rescued in the Fh1^{-/-} +CAG-FH mice.

As previously discussed in Chapter 1, fumarate has a structure similar to that of 2-OG and therefore may be responsible for competitively binding to, and inhibiting, a number of the 2-OG-dependent family of enzymes. This could have a significant impact on a broad spectrum of cellular functions such as prolyl and lysyl hydroxylation of collagen, histone lysyl and arginyl demethylation, lysyl hydroxylation of proteins associated with RNA-splicing, different steps in carnitine metabolism, the repair of alkylated bases in DNA, single and double stranded nucleic acid demethylation, and the hydroxylation of 5-methyl-cytosine-(Loenarz and Schofield 2008). Should cytoplasmic FH, the function of which to date remains poorly understood, be shown to have a role in regulating any such pathway independently of its mitochondrial isoform, it would provide a novel insight into and understanding of not only the tumour suppressor function(s) of FH but also altered metabolism in general.

Since the development of the first transgenic mice models, there have always been caveats associated with the use of genetic manipulation *in vivo* and the techniques used to achieve this (Tecott 2002; O'Sullivan 2006). While mosaicism is an undesirable feature of the CAG-FH transgenic mice described in this chapter it is a known limitation of genetic manipulation by means of conditional targeting and homologous recombination (Tecott 2002; O'Sullivan 2006). It may also be that simply extending the endpoint to 30 or 40 weeks, rather than the 20 week endpoint chosen for

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this study, may also allow for phenotypic differences between Fh1^{-/-} +CAG-FH and Fh1^{-/-} +CAG-FHΔMTS lines to be more clearly examined and defined.

In closing, developing a transgenic model, such as the model described in this chapter, enables the activities of cytoplasmic FH to be distinguished from the role of mitochondrial FH and thus is of considerable use in defining the function of cytoplasmic FH, which remains poorly understood to date. This *in vivo* model can be used as a powerful tool for establishing the effects of FH-inactivation (in defined cellular compartments) on a range of biochemical/metabolic processes which may help to identify the oncogenic pathways involved in FH-associated HLRCC, especially when used in combination with the Fh1 MEF model described in Chapter 3. It may also provide a platform for the definition and monitoring of potential therapeutic interventions designed to restrict fumarate accumulation in specific cellular compartments. Finally, by expressing the transgene under the control of a ubiquitous CAG promoter, the transgenic mice described in this chapter provide a versatile model system that can be utilised in a broad spectrum of studies into non-HLRCC disease phenotypes associated with Fh1-deficiency in a variety of tissues and cell systems.

5 Chapter 5 Final Discussion

5.1 Introduction

Individuals bearing germline heterozygous FH mutations are predisposed to a rare cancer syndrome known as HLRCC, which is characterised by uterine and cutaneous leiomyomata and aggressive type II papillary renal cancer, the latter of which has only been reported in 2-16% of the 144 families studied to date (Launonen et al. 2001; Tomlinson et al. 2002; Toro et al. 2003; Chan et al. 2005; Gardie et al. 2011). In order to elucidate better the tumour suppressor function(s) of FH and the early events which lead to tumorigenesis in HLRCC, suitable *in vitro* and *in vivo* model systems for FH-deficiency need to be developed. Such models can be used to address the important questions of how altered metabolism, activation of a pseudo-hypoxic drive, dysregulation of molecular pathways, cystic development and fumarate-mediated 2SC protein modifications contribute to the oncogenic phenotype of HLRCC. *In vitro* models can be used for preliminary investigations to identify candidate pathways, the results of which can then be extrapolated to an *in vivo* setting as proof-of-concept and to test whether the phenotype of interest can be rescued through gene targeting and/or pharmacological interventions.

When work on this thesis began there was only one *in vivo* model available for studying the effects of endogenous knockout of *Fh1* in a kidney-specific manner, using a Ksp1.3/Cre system (Pollard et al. 2007). Therefore the aim of this thesis was to develop versatile *in vitro* MEF models and novel transgenic mouse models for more detailed studies on Fh1-deficiency and to better understand the role of FH, in particular cytoplasmic FH, in context of the phenotypes associated with HLRCC. Chapter 2 describes the generation of immortalised Fh1^{-/-} MEFs and the analyses

conducted to confirm that these cell lines recapitulate the phenotypes associated with Fh1-deficiency. These phenotypes include fumarate accumulation, increased lactate production and secretion as well as normoxic activation of the Hif pathway. Continuing from these findings, Chapter 3 describes the genetic complementation of Fh1^{-/-} MEFs with either V5-tagged human full-length (wildtype) FH or cytoplasmic-restricted FH Δ MTS and details how both variants are capable of reversing the normoxic stabilisation of Hif-1 α . However, only re-expression of the full length FH protein (Fh1^{-/-} +FH) was capable of restoring aerobic respiration and reducing fumarate levels to those levels observed in wildtype, Fh1^{+/+}, MEFs.

While it might be argued that studies of Fh1-deficiency in a fibroblast cell line may not be as relevant as studies conducted in a cancer or renal cell line, such concerns were diminished as both the results and phenotypes observed in the MEFs correlated with the observations made in other *in vitro* models that emerged during the course of this thesis; namely, a cell line derived from a retroperitoneal metastases in a patient with HLRCC kidney cancer (Yang et al. 2010) and two renal cell lines (Frezza et al. 2011; Tong et al. 2011). It could similarly be argued that studies conducted in *Fum* (yeast orthologue of FH)-deficient yeast models (Yogev et al. 2010) are not physiologically comparable with other *in vitro* model systems available and thus not ideal for carrying out investigations into the role of FH in HLRCC tumourigenesis. However, the function of FH is highly conserved between species and, importantly, the purpose of *in vitro* models, such as the Fh1 MEFs and those previously referred to, is to identify biochemical and metabolic pathways adversely affected by the loss of FH function. Upon gaining a better understanding of the dysregulation of such pathways, interrogations into the identified pathways as candidate targets for genetic

or pharmacological interventions that could potentially be carried out. Moreover, these cells have already been implemented in a range of studies on alternative pathways involved in HLRCC tumourigenesis and also in the development of a new, high-throughput *in vitro* analytical method (Adam et al. 2011; Bardella et al. 2011; Thalhammer et al. 2011; Zhdanov et al. 2011). There is also potential for the Fh1 MEF models to be utilised for siRNA synthetic lethality screens.

Chapter 4 describes the development of novel *in vivo* models, which were designed to ubiquitously re-express either a human FH or FH Δ MTS transgene in an Fh1^{-/-} background, under the control of a CAG-promoter. The main questions raised and addressed by the *in vivo* model were: 1) Does normoxic Hif activation occur independently of the mitochondrial defect *in vivo* as well as *in vitro*? 2) Can the accumulation of fumarate-mediated 2SC protein modifications be diminished following expression of either transgene? and 3) Would re-expression of either the full length FH or cytoplasmic FH transgene be sufficient to rescue the renal cystic phenotype, linked to Fh1-deficient kidneys, *in vivo*? In corroboration with the *in vitro* findings, the *in vivo* model successfully demonstrated that degradation of stabilised Hif-1 α , observed under normoxic conditions in Fh1-deficient renal tubular or collecting duct cells, could be restored following expression of either the CAG-FH or the CAG-FH Δ MTS transgene. This confirmed that normoxic activation of the Hif pathway in Fh1-deficiency occurs independently of the mitochondrial defect both *in vivo* and *in vitro*. Furthermore, the CAG-FH and CAG-FH Δ MTS transgenic mice had fewer cells with high levels of fumarate and 2SC protein modifications. However, the most interesting and striking finding observed in these transgenic mice was that expression of the cytoplasmic FH transgene alone could abolish the cystic phenotype,

just as effectively as the full length FH transgene. These results highlight the usefulness of these new *in vivo* models in better understanding the role of cytoplasmic FH and their further potential in determining specific *in vivo* mechanism(s) through which FH carries out its tumour suppressor function in HLRCC.

5.2 *Fh1^{-/-}MEFs complemented with either FH-V5 or FHΔMTS-V5 as an *in vitro* system for the study of Fh1-deficiency*

As no means of looking at early events following the loss of FH activity existed at the outset of the work outlined within this thesis, the MEF models were designed to provide a system which would enable a better understanding of early events following loss of FH activity. These models have been shown to be useful tools for interrogating the affects of Fh1-loss, such as fumarate accumulation, activation of the Hif pathway and altered cell metabolism (Ashrafian et al. 2010; O'Flaherty et al. 2010). Furthermore the Fh1^{-/-} MEFs recapitulated previously reported data (Isaacs et al. 2005; Pollard et al. 2005) by showing that fumarate accumulation *in vitro* directly stabilises Hif-1 α . The four MEF lines (Fh1^{+/+}, Fh1^{-/-}, Fh1^{-/-} +FH and Fh1^{-/-} +FHΔMTS) have proved to be powerful tools, when studied in combination or in isolation, for understanding the phenotypes that are directly linked to loss of Fh1 (or the resulting accumulation of cellular fumarate) and distinguishing between the function of cytoplasmic-restricted FH and mitochondrial-targeted FH, of which the former has to date remained poorly understood. In Fh1-deficient cells there is a decreased capacity to generate ATP and an impaired ability to consume cellular oxygen, which is indicative of a mitochondrial defect resulting from disruption to

normal TCA cycle function. Only Fh1^{-/-} cells expressing the FH-V5 construct rescued the mitochondrial defect by restoring both ATP and oxygen consumption to levels comparable with wildtype Fh1^{+/+} MEFs. Conversely the Fh1^{-/-} +FHΔMTS were unable to rescue this phenotype. Interestingly, Fh1^{-/-} +FHΔMTS MEFs were capable of ablating normoxic accumulation of Hif-1α to the same extent as the Fh1^{-/-} +FH MEFs which suggests that the Hif phenotype occurs independently of an inability to utilize OXPHOS following the loss of Fh1.

The hypothesis of ROS-dependent stabilisation of HIF (Chandel et al. 2000; BelAiba et al. 2004; Goyal et al. 2004) remains highly contested. The literature suggests that disruption to the ETC chain reduces the availability of cellular oxygen required by the PHD enzymes, thus activating the HIF pathway (Hagen et al. 2003). In this capacity, the mitochondria are considered to behave as oxygen sensors in their own right by increasing ROS production which has itself been reported to activate a hypoxic response (Chandel et al. 1998; Agani et al. 2000; Chandel et al. 2000). A study published by Brunelle *et al.* also suggested that mitochondrial ROS is required for oxygen sensing independent of oxidative phosphorylation (Brunelle et al. 2005). This may partially explain the results shown in Chapter 3 (section 3.2.6, Figure 3.8), where OXPHOS remains perturbed in Fh1^{-/-} +FHΔMTS MEFs; but both Hif and mitochondrial ROS levels in these cells are restored to levels comparable with Fh1^{+/+} and Fh1^{-/-} +FH MEFs. Furthermore, the rate of oxygen consumption and ATP generation within Fh1^{-/-} +FHΔMTS MEFs remain comparable to those rates observed in Fh1^{-/-} MEFs (O'Flaherty et al. 2010). Therefore it remains unlikely that the observed rescue of the ROS phenotype in Fh1^{-/-} +FHΔMTS MEFs is due to restoration of OXPHOS function, in accordance with the published literature. It may,

however, be that generation of ROS *via* alternative pathways such as the unfolded protein response (UPR), the action of endoplasmic reticulum (ER) oxidoreductases or cytoplasmic NADPH oxidases (Santos et al. 2009) may be regulated by cytoplasmic FH and thus reduce ROS levels in this setting.

Interestingly, localisation of FH to the nucleus has been observed in *Fh1^{-/-}* MEFs complimented with cytoplasmic-restricted FH (See Figure 3.5A(ii)). Potentially this may provide a function for cytoplasmic FH, whereby FH, upon activation by a stimulus, can translocate to the nucleus and regulate expression of genes associated with the cystic phenotype and tumourigenesis. A study by Yogev et al., reported that cytoplasmic fumarase is capable of protecting cells from double-stranded breaks by recruiting fumarase to the nucleus, where it activates the DNA damage response in *S. cerevisiae* in a fumarase-dependent manner (Yogev et al. 2010). This proposed role for cytoplasmic/nuclear fumarase in the DNA damage response may be one of the underling mechanisms for the tumour suppressor function of this enzyme and is further evidence that Hif-independent pathways may be central to tumour onset and progression in FH-deficient cells. In further support of a Hif-independent hypothesis, the cystic phenotype in *Fh1^{-/-}* mice has been shown to occur independently of the Hif/Phd pathway (Adam et al. 2011). The tumour suppressor function of FH may also be linked to competitive inhibition of other 2-OG-dependent enzymes, in a manner similar to fumarate-mediated inhibition of the Hif Phd hydroxylases (i.e. as a result of elevated fumarate levels). The human genome has been reported to encode as many as 60-70 known or predicted 2-OG-dependent oxygenases with described functions for some of these oxygenases ranging from: prolyl and lysyl hydroxylation of collagen, histone lysyl and arginyl demethylation, lysyl hydroxylation of proteins associated

with RNA-splicing, carnitine metabolism, the repair of alkylated bases in DNA, single and double stranded nucleic acid demethylation, and the hydroxylation of 5-methylcytosine, reviewed in (Loenarz and Schofield 2008). Fumarate-mediated disruption to the function of one or more of these oxygenases could be predicted to be capable of initiating and/or sustaining tumourigenesis. As Hif prolyl and asparaginyl hydroxylations are differentially inhibited in *Fh1*^{-/-} cells (Chapter 3, section 3.3.2, Figure 3.3A), in a manner that is consistent with *in vitro* studies of fumarate as a catalytic inhibitor (Hewitson et al. 2007; Koivunen et al. 2007), studies on the action of fumarate across a range of 2-OG oxygenases should be of interest in defining a variety of possible consequences of *FH* inactivation (Pollard and Ratcliffe 2009), especially within defined cellular compartments.

Pharmacological interventions targeting HIF-1 α target genes are currently undergoing clinical trials (e.g. Bevacizumab, an inhibitor of VEGF-receptor binding reviewed in (Bergsland and Dickler 2004)). The specificity of such pharmacological compounds for targeting cancer cells for programmed cell death is assessed using cell-based synthetic lethality screens (perturbing cell viability by loss of function of two, rather than one, gene targets) (Weidle et al. 2011). To investigate whether the *Fh1* MEF lines have the potential be used for testing novel pharmaceutical compounds and subsequent synthetic lethality screens, *Fh1*^{-/-} MEFs were treated with siRNA-against *Ldha*, a Hif-1 α target gene routinely found upregulated in *Fh1*-deficiency (Xie et al. 2009; Ashrafian et al. 2010; O'Flaherty et al. 2010), which successfully resulted in a reduction in cell growth (Chapter 2, section 2.2.6, Figure 2.5C). Targeting of the Hif-independent, haem oxygenase pathway in *Fh1*-deficient murine renal cells also successfully induced synthetic lethality in these cells (Frezza et al. 2011). By

exploiting the haem oxidation pathway (responsible for haem biosynthesis and degradation), the activity of which can be measured by the rate of bilirubin secretion, Fh1^{-/-} kidney cells were able to utilise the accumulated TCA cycle intermediates for generating NADH in the mitochondria and consequently were able to maintain viability. Thus both Fh1-deficient cell lines, renal (Frezza et al. 2011) and MEFs (O'Flaherty et al. 2010), can be used to identify and subsequently target new pathways in Fh1-deficiency, with the potential to develop targeted and effective treatments for HLRCC patients. Although the Fh1^{-/-} mice described in this thesis develop a cystic phenotype, they did not develop cancer during the time course of experimentation. Therefore these mice would not be suitable for assessing the affects of pharmaceutical interventions on tumour growth.

Glucose concentration used in standard tissue culture conditions range from 5 mM glucose (low glucose) to 25 mM glucose (high glucose), depending on the requirements of the cell line. In general, most cancer cell lines or cell lines with oncogenic potential are grown in medium supplemented with high glucose concentrations as, in most cases, these cells have a tendency to rely on aerobic glycolysis and thus increased glucose uptake (Warburg et al. 1927; Kroemer and Pouyssegur 2008). Yogev and Linehan have previously reported that Fumarase/Fh1-deficient cells are glucose-addicted due to their dependency on glycolytic respiration (Yang et al. 2010; Yogev et al. 2010). The ratio of ATP generated by OXPHOS versus glycolysis (Chapter 3, section 3.2.2, Figure 3.5C) in Fh1^{-/-} and Fh1^{-/-}+FHΔMTS MEFs demonstrated that these cells were unable to utilise galactose for OXPHOS when grown in galactose-supplemented / glucose deficient medium, thus confirming the dependency of these MEF lines on glucose. Concerns that the full

length FH propeptide in Fh1^{-/-} +FH MEFs may not be cleaved, in order to facilitate its translocation from the mitochondria to the cytoplasm, were addressed following cellular fractionation of these cells (Adam et al. 2011). Immunoblot analysis of the subcellular fractions was carried out in a study of fumarate-dependent regulation of the Nrf2 signalling pathway (Adam et al. 2011). Protein levels of NAD(P)H dehydrogenase quinone 1 (Nqo1) protein, an Nrf2-target gene, were shown to be similarly reduced in both the cytoplasmic and mitochondrial fractions in this study. This indicates that FH is at least partially active in the cytoplasm of Fh1^{-/-} +FH MEFs. Furthermore, the Fh1^{-/-} +FH MEFs were fully capable of rescuing the accumulated fumarate and Hif phenotypes associated with Fh1-deficiency (Chapter 3).

5.3 CAG-FH or CAG-FH Δ MTS transgenic mice are capable of abolishing the cystic phenotype observed in Fh1-deficient kidneys

To corroborate the findings obtained using the *in vitro* model (thus providing further evidence for the relevance and robustness of this system) and to determine whether the cystic phenotype in Fh1-deficient kidneys could be reversed by ubiquitous expression of either FH or FH Δ MTS transgenes, an *in vivo* model utilising the same FH sequences as used for the MEF transfectants was developed. By restricting expression of FH to either the cytoplasmic or mitochondrial cellular compartments *in vivo*, it was hoped that the functions of the two FH isoforms *in vivo*, in relation to cyst development, could be better distinguished. Interestingly, expression of cytoplasm-restricted FH (CAG-FH Δ MTS) was found to be more efficient than the full length CAG-FH transgene in reversing the cystic phenotype in Fh1^{-/-} kidneys, despite the

persistence of perturbed TCA cycle function in Fh1-deficient renal tubular cells. This suggests that cystic development is regulated independently of metabolic perturbations resulting from dependency on glycolytic metabolism and accumulation of TCA cycle intermediates. The role of cytoplasmic FH in the overall tumour suppressor function of FH has, to date, not been described. However the results of Chapter 4 demonstrate the potential of the Fh1 transgenic mouse model in facilitating the discovery of mechanism involved in the function of cytoplasmic FH.

As expected with the development of a new transgenic mouse model, there are some limitations that need to be addressed and optimisations made, where appropriate, to ensure that interpretation of the results is not confounded by inconsistencies. As was previously discussed in Chapter 4, the expression pattern of the CAG-FH and CAG-FHMTS transgenes is mosaic rather than uniform among all cells, as illustrated in frozen sections of bisected kidneys from each cohort (Chapter 4, section 4.2.2, Figure 4.3). There may be two possible explanations for this: 1) caveats associated with design of the mouse model (Chapter 4, section 4.3) or 2) that cytoplasmic FH may have a more dominant role than mitochondrial FH in regulating cystic development and that this phenotype occurs independently of the mitochondrial defect apparent in Fh1^{-/-} cells.

With regards to point 1), the transgene expression vectors could have been alternatively designed to be expressed exclusively in the kidneys, driven by a Ksp1.3/Cre-specific promoter. As the CAG-promoter is ubiquitously expressed, it should therefore be no less efficacious in driving expression of the transgene in renal tubular cells (Hong et al. 2007). Moreover, by utilising the CAG-promoter, the Fh1

transgenic models can be readily incorporated into a broader range of *in vivo* studies investigating the effects of FH-deficiency in a range of diseases affecting different cell systems and metabolic pathways. For example, a protective function for Fh1-deficiency in cardiac tissue was recently described (Ashrafian 2012); The Fh1 transgenic mice could potentially be used to investigate whether this new role for FH is specific to cardiac tissues or whether it has a protective function in other tissues. Another option to increase the uniformity of the transgene expression would be to adjust the breeding strategy so that the transgene is expressed on both alleles rather than maintained in heterozygosity. It can also be argued that a mixed cell population is to be expected both in healthy and disease-state tissues.

To address point 2), the design of the CAG-FH mouse model specifically targeted Fh1 for inactivation in renal tubular cells by expression of a Ksp1.3/Cre allele. Therefore, it may be that the persistent mitochondrial defect in Fh1^{-/-} +CAG-FHΔMTS mice is compensated for by the surrounding wildtype non-tubular cells. This might mask the mechanism by which the CAG-FHΔMTS transgene regulates the pathways leading to the onset of Fh1-associated pathogenesis. As the Fh1^{-/-} mice did not develop cancer at the 20 week endpoint specified in the studies of this thesis, it is difficult to fully understand the impact that regulation of the cystic phenotype by cytoplasmic FH has on tumourigenesis. It could, however, be argued that should the Fh1^{-/-} mice be aged for an substantially longer period live that some might go on to develop cancer within this system but given the rapid progression of cystic disease, it is likely that many of these animals succumb to kidney failure and therefore would be unethical. However, for those mice that do not succumb to kidney failure, there is the possibility that a few cells lacking Fh1 might survive long enough to acquire a second hit or undergo a

metabolic modification, leading to these cells becoming cancerous. Publications are emerging that address the role of cytoplasmic FH in newly identified pathways that may be important in promoting and sustaining FH-related tumourigenesis (Adam et al. 2011; Frezza et al. 2011).

5.4 Identification of pathways regulated in an FH-dependent manner and the potential use for the *in vivo* and *in vitro* models in these settings

Validation of the Fh1 MEF lines and the Fh1^{-/-}, Fh1^{-/-} +CAG-FH and Fh1^{-/-} +CAG-FHΔMTS transgenic mice was focused predominantly on the Hif pathway, with some preliminary investigations carried out to study the effects of re-expression of FH on 2SC modifications *in vivo*. The latter studies were conducted to corroborate the findings previously reported in this field (Adam et al. 2011; Bardella et al. 2011). However, both the *in vitro* and *in vivo* models systems have the potential to be utilised as tools for proof-of-concept of proposed hypotheses involving the dysregulation of non-Hif-related molecular and metabolic pathways in Fh1-deficiency.

A study by Bardella et al, recently highlighted the use of 2SC as a highly specific biomarker for loss of FH and its potential to be used for diagnostic screening of patients suspected of being affected by HLRCC (Bardella et al. 2011). By implementing both the FH-V5 and FHΔMTS-V5 complemented MEFs, another study demonstrated that the effects of fumarate accumulation on succination to be assessed in separate cellular compartments where it was confirmed that both FH variants were capable of ablating the aberrant succination of proteins occurring in Fh1-deficient

cells (Adam et al. 2011, supplementary figures). *In vivo*, the situation appears to be more complex, as Fh1^{-/-} mice harbouring the CAG- FHΔMTS transgene were shown in Chapter 4 of this thesis to prevent succination more readily than their Fh1^{-/-} +CAG-FH counterparts.

Of significant interest was the recent finding by Adam *et al.* that cystic development in Fh1^{-/-} mice occurs independently of Hif-1 α and Hif-2 α and more surprisingly, that knockdown of Hif-1 α in an Fh1^{-/-} background exacerbated the cystic phenotype (Adam et al. 2011). These findings further fuelled the search for non-Hif-related pathways that may be responsible for promoting cystic growth in Fh1-deficiency. Gene expression profiles of Fh1^{-/-} and Fh1^{-/-}/Hif-1 α ^{-/-} 15 week old mice (with early stage cystic growth) showed significant upregulation of the Nrf2 antioxidant pathway. A key regulator of this pathway is the Keap1 protein, which binds to, and subsequently targets Nrf2 for degradation by ubiquitination when the cell is not under conditions of oxidative stress. Keap1 was shown to be succinated at Cys38, Cys151, Cys241, Cys288, Cys319, and Cys613 (Adam et al. 2011), of which Cys151 and Cys288 have previously been reported to regulate KEAP1 under oxidative stress (Nguyen et al. 2009). Fh1-mediated succination of Keap1 was independently reported by Ooi *et al.* (Ooi et al. 2011). The Fh1 MEF lines described in this thesis were successfully employed in the study by Adam *et al.* to confirm that Nrf2 activation was in direct response to fumarate accumulation rather than defective oxidative metabolism.

In contrast to the poor prognosis associated with fumarate accumulation in HLRCC-related renal cancer, it has recently been reported that increased fumarate levels in

Fh1^{-/-} mice (Pollard et al. 2007), crossed with mice expressing Cre recombinase under the cardiomyocyte-specific promoter of myosin light chain-(MLC2v)-Cre (Chen et al. 1998) for cardiac tissue-specific knockdown of *Fh1*, also activates the Nrf2 antioxidant pathway to confer cardioprotection against ischemia-reperfusion injury (Ashrafian 2012).

It has been reported that transformation and tumourigenesis following loss of FH is sustained by activation of the *MET* oncogene (Costa et al. 2010). Costa *et al.* showed that knockdown of *Fh1* by shRNA in MEFs lead to activation of the MET receptor and normoxic Hif-1 α stabilisation. This is of interest as the MET oncogene has been shown to be upregulated in and associated with kidney cancer (Natali et al. 1996; Inoue et al. 1998; Yamauchi et al. 2004; Miyata et al. 2006; Giubellino et al. 2009; Linehan et al. 2010). It would be predicted that Met activation would be exacerbated in Fh1 *knockout* MEFs in comparison with Fh1 *knockdown* MEFs (Costa et al. 2010) which retain some Fh1 protein and residual enzymatic activity.

Another pathway implicated in the metabolic alteration of FH-deficient tumourigenic cells is the AMP-activated kinase (AMPK) pathway. A study by Tong *et al.* showed that diminished AMPK levels in FH-deficient UOK262 kidney cells resulted in decreased expression of divalent metal transporter 1 (DMT1) and subsequent activation of the iron regulatory proteins, IRP1 and IRP2, thus leading to stabilisation of HIF-1 α (Tong et al. 2011). This study proposes a link between glycolytic-dependency and altered iron metabolism that conveys a cell growth/survival advantage to FH-deficient cells and thus a mechanism for tumourigenesis. In contrast, a study by Bardella et al, using the Fh1^{-/-} MEFs generated and discussed in this thesis,

showed Hif-1 α -independent activation of AMPK and ERK1/2 following loss of Fh1 function (Bardella *et al.*, paper in review at time of thesis submission). This study proposed that tumourigenesis is upheld following FH-dependent activation of AMPK by promoting cell survival *via* hyperphosphorylation and inactivation of BAD which prevents it from forming a pro-apoptotic signalling complex with either BCL-2 or BCL-XL. These findings were reproduced in FH-depleted HK-2 (human kidney) cells. The different species and tissue origins from which the cell lines were derived and also the different interventions/conditions to which they were exposed to may offer a possible explanation for the profound differences in experimental results between these two studies. The UOK262 cells were derived from a retroperitoneal metastasis in a patient with recurrent HLRCC kidney cancer and are therefore likely to have acquired second ‘hit’ mutations and to have been exposed to chemotherapeutic compounds during clinical treatment of the patient, for which there are no available records. On the other hand, the Fh1 MEFs were immortalized using a pBabe-puro SV40 Large T antigen and the Fh1^{-/-}, Fh1^{-/-} + FH and Fh1^{-/-} + FH Δ MTS lines were further infected with an adenoviral Cre-GFP vector to facilitate homologous recombination of the loxP-flanked exons of *Fh1*. Furthermore, the latter two MEF lines were also transfected with a pEF1/V5-His vector to facilitate expression of either the FH or FH Δ MTS transgene. Further investigations, using both the UOK262 and Fh1 MEF lines, are required in order to establish a convincing and reproducible mechanism for the role of AMPK signalling in FH-related tumourigenesis.

5.5 Future Work

This thesis details the results from the primary stages of validation of a novel *in vivo* Fh1 model, however further work is necessary to optimise this system so that it may be used to its full potential. Firstly, to address the issue of mosaicism in the Fh1^{-/-} +CAG-FH and Fh1^{-/-} +CAG- FHΔMTS transgenic mice, it is essential to study a larger number of animals per cohort (>n=3) to determine whether this occurrence is consistent when assessed on a larger scale. Secondly, the end point will be extended to 30 weeks to better distinguish between the FH and FHΔMTS transgene-related phenotypes. Finally, removal of the Neomycin-resistance cassette ORF from the Fh1 transgenic lines should also be considered. This can be achieved by crossing the Fh1^{-/-} +CAG-FH and Fh1^{-/-} +CAG- FHΔMTS lines with Flp-recombinase transgenic mice (Takeuchi et al. 2002). The presence of Flp-recombinase facilitates the recombination, and excision, of the FLP-FRT sites flanking the neomycin resistance cassette.

There are numerous possibilities for using the Fh1 MEFs as cellular models, either alone or in combination with the Fh1 *in vivo* model. Metabolomic analysis in collaboration with Prof. Tomoyoshi Saga (Institute for Advanced Biosciences, Keio University, Japan) showed significant increases in argininosuccinate and adenylysuccinate in Fh1^{-/-} kidneys, which may be suitable candidates to investigate as these metabolites are broken down to form fumarate in the urea cycle and pyridine nucleotide cycling, respectively (Salway 1999 ; Lodish 2000; Nelson 2004). These pathways may be important in removing the accumulation of fumarate that occurs in Fh1-deficient cells. Regulation of metabolism in cancer is an area of growing interest and employing the Fh1 MEFs to carry out *in vitro* experimentation into metabolic adaptations may prove to be very informative. As previously mentioned, it may also

be possible that fumarate can competitively inhibit other 2-OG-dependent oxygenases; the family of JMJD-histone demethylases would be of particular interest due to the possible implications in epigenetic regulation and control of gene expression in FH-deficiency and potentially HLRCC. Epigenetic regulation has also been shown to be achieved *via* the activities of the Tet family of 2-OG dependent enzymes (reviewed in (Veron and Peters 2011)). Tet enzymes carry out their functions by hydroxylating the DNA methyl group to 5-hydroxymethylcytosine (5hmC) (Kriaucionis and Heintz 2009; Tahiliani et al. 2009; Ito et al. 2010). It would therefore be interesting to investigate whether there is a correlation between the function enzyme function of Tet and FH. Inhibition of a number of 2-OG-dependent enzymes was also found to occur following accumulation of (*R*)-2-hydroxyglutarate (2HG) and (*S*)-2HG, the latter of which is the more potent inhibitor, as a result of IDH1 and IDH2 mutations (Xu et al. 2011; Koivunen et al. 2012). As this is similar to the fumarate-mediated inhibition of the 2-OG-dependent oxygenases, studying the 2HG –inhibition pathway may provide functional and mechanistic insights into the effect FH-inactivation has on the regulation of candidate pathways linked to 2-OG-dependent oxygenases.

5.6 Conclusion

Despite the complexities associated with the design and interpretation of the results from the Fh1^{-/-} mice complemented with either CAG-FH or CAG-FHΔMTS, this *in vivo* model system successfully 1) recapitulated the phenotypic findings of the *in vitro* Fh1^{-/-} and Fh1^{-/-}-complemented MEF model and 2) demonstrated that attenuation of

the cystic phenotype could be achieved by expressing either of the two transgenes. Investigating of such pathways may also shed light on whether cyst development occurs independently of the cancer phenotype. Moreover, the corroboration between the *in vivo* and *in vitro* results further supports the relevance and use of the Fh1 MEF models for investigations into the molecular and cellular pathways involved in initiating and exacerbating Fh1-deficient phenotypes, prior to carrying out more complex and expensive investigation *in vivo*.

While the MEFs may be considered an unorthodox model for the study of a renal phenotype, they have been shown to generate a similar phenotype to the immortalised Fh1^{-/-} renal cells derived from murine kidneys (Frezza et al. 2011). It should also be noted that the mice used to generate the Fh1^{-/-} renal cells were of the same background as the mice used to generate the Fh1 MEFs (Pollard et al. 2007; O'Flaherty et al. 2010). Furthermore, skin and uterine leiomyomata (cancers of the smooth muscle) occur at a higher frequency in HLRCC compared to the occurrence of renal cancer and therefore using a non-renal cell line, such as the Fh1 MEF model, makes them a suitable and relevant *in vitro* model system for studying a broader spectrum of oncogenic pathways and metabolism involved in the early stages of HLRCC.

There are caveats in both the *in vitro* and *in vivo* models but the strength of the work outlined in this thesis, some of which has been published, is that it does not rely on just one model system and that the rationale for these models has not been constrained by preconceptions about Hif and the Warburg phenotypes. During the time frame of this thesis, the field of studies in FH and in cell and cancer metabolism has undergone

a transformation, in part due to the findings published using these models (Ashrafian et al. 2010; O'Flaherty et al. 2010; Adam et al. 2011).

In closing, the results of this thesis demonstrate that the *in vitro* and *in vivo* models, used either alone or in combination, are versatile and robust systems for small or large scale investigations into the complex (dys)regulation of metabolic and molecular signalling pathways in FH-deficiency, some of which may contribute to the early stages of HLRCC tumourigenesis. These models also offer further insights into the subcellular functions of the FH isoforms and their roles in promoting/upholding the pathways which contribute to the phenotypes associated with HLRCC. The *in vitro* and *in vivo* models also have the potential to be used as a platform for testing potential therapeutic interventions (e.g. high-throughput *in vitro* screens combined with pharmacological trials *in vivo*) which may go some ways in assisting with the development of effective therapeutics that have a higher degree of specificity for the treatment of HLRCC patients in the future.

6 Chapter 6 Materials and Methods

6.1 Generation and maintenance of immortalised *Fh1* deficient Mouse

Embryonic Fibroblasts

Fh1 conditional knockout mice (Pollard et al. 2007) were maintained in accordance with Home Office guidelines and licensing regulations. Fumarate hydratase (*Fh1*) heterozygote mice were intercrossed to generate embryos with the following genotypes: *Fh1*^{fl/fl}, *Fh1*^{+/+} or *Fh1*^{fl/+}. Murine embryonic fibroblasts (MEFs) were isolated from littermate embryos and dissected at 14.5 days (E14.5) of gestation. To generate pBabe-puro SV40 Large T antigen a packaging cell line, Phoenix cells, were infected with 25 µg/ml pBabe-puro SV40 Large T antigen construct (10⁸ PFU) (construct was a kind gift from Prof. P. Jat, University College London (Gjoerup et al. 2007)) (Figure 6.1) and 100µl polyethylenimine (PEI) transfection reagent. Retroviral medium was harvested 48 hours post-infection and centrifuged (Allegra™ X-12R, Beckman Coulter) at 1000rpm for 5 min to remove contaminating Phoenix cells. Approximately 90% of the retroviral supernatant was removed and filtered through a 0.45 µm sterile filter to remove remaining cells and debris. Aliquots were made of the retroviral medium and stored at -80°C.

Cells (at passage number 3) were treated with 5 µg/ml pBabe puro SV40 Large T retroviral medium to promote immortalisation of the MEFs via inactivation of p53 (Tevethia 1984; Zhu et al. 1991; Conzen and Cole 1995). Infected cells expressing the puromycin-resistance gene encoded by the pBabe puro SV40 Large T antigen construct were selected for using 2 µg/ml puromycin (Sigma). Excision of the *Fh1* loxP-flanked exon was effected by transfection using an adenovirus-type 5 (dE1/E3) vector, with a CMV promoter-driven Cre recombinase transgene and green

fluorescence protein (GFP) tag (Ad-Cre-GFP) (Vector Biolabs). A similar vector lacking the Cre transgene (Ad-CMV-GFP) was used as a control. Transfection efficiency was approximately 80%, as assessed by GFP expression. More than 50 clones were established and analysed by PCR of genomic DNA to confirm the genotypes (Pollard et al. 2007). All MEFs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) containing 4500mg/L (25mM) glucose supplemented with 10% (v/v) foetal bovine serum (FBS) (Sigma), 2mM glutamine (Sigma), 10,000 units penicillin and 10 mg streptomycin/mL (Sigma), and maintained in a humidified atmosphere of 5% CO₂ and 21% O₂.

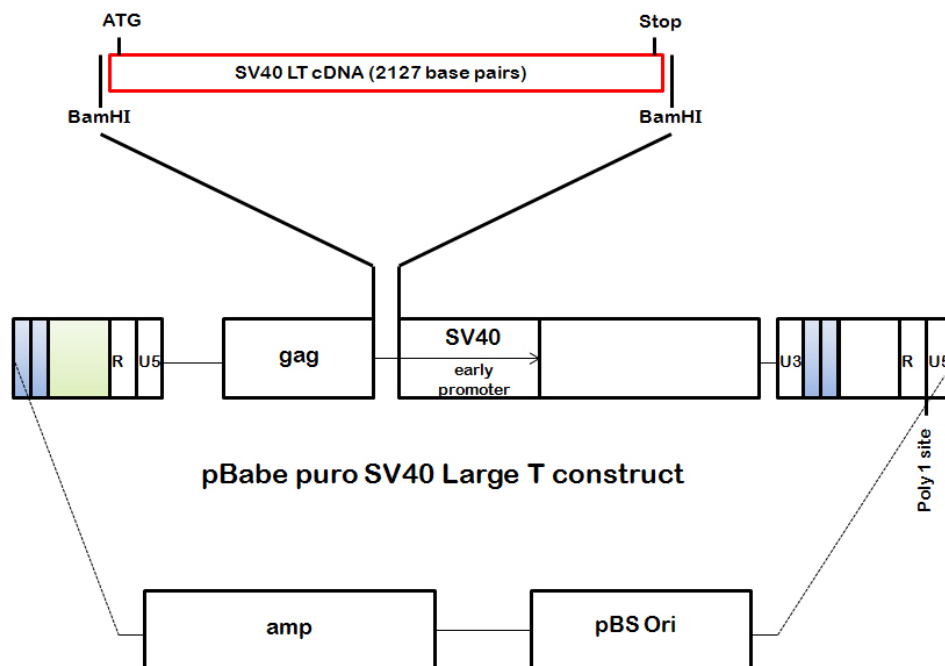


Figure 6.1 pBabe puro SV40 Large T construct

pBabe puro SV40 Large T (LT) construct (Adapted from Prof. P. Jat's schematic).

6.2 Generation of MEFs with stable FH expression

The sequences for human *FH* with or without the mitochondrial targeting sequence (MTS) (sequenced by Violetta Steeples, Oxford University) (Appendix Figure 4.1A and B and Appendix Figure 6.1) were initially inserted into a C-terminal pFLAG-CMV-5.1 adenoviral expression vector (Sigma) (Figure 6.2) for transient expression. However, the transfection efficiency of this construct was very low (~40% of cell population infected). Therefore an alternative approach using a pEF1/V5-His expression vector (Invitrogen) (Figure 6.3) was undertaken to increase the transfection efficiency and stable expression in these MEFs. Insertion of the *FH* sequences was carried out by a Hind III restriction digest. The restriction digest products were run out by gel electrophoresis to confirm the correct diagnostic band (~300 bp for V5-tagged FH). Non-V5-tagged FH sequences were processed in the same manner as the V5-tagged FH with an expected diagnostic band of ~200 bp following a Hind III restriction digest. The V5-tag was designed to target the C-terminus of both FH sequences.

Fh1^{-/-} MEFs were transfected using FuGene[®]6 Transfection Reagent (Roche), according to the manufacturer's instructions. Using 3 µl FuGENE[®]6 reagent (Roche Applied Science) per 1 µg DNA, cells were transfected with the pEF1/V5-His vector (Addgene) carrying either human FH or FHΔMTS DNA sequences (Appendix Figure 3.2). Transfection efficiency was higher using the pEF1/V5-His vector (~70% of cell population transfected). Transfected cells were placed under 800 µg/ml G418 (Sigma) 72 hours post-transfection to select for cells harbouring the neomycin-resistance cassette encoded by the pEF1/V5-His vector. Surviving cells were then analysed by immunofluorescence to confirm appropriate subcellular localisation of V5-tagged FH.

Independent clones (>20) were isolated from these heterogeneous populations of transfected cells, and expression and localisation was re-confirmed by DNA polymerase chain reaction (PCR; see section 6.4) and quantitative reverse-transcription (Q-PCR; see section 6.12).

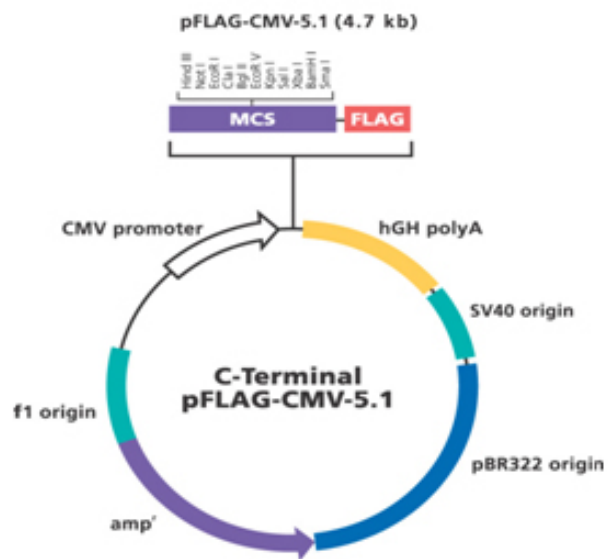


Figure 6.2 pFLAG-CMV-5.1 vector

Schematic for the C-terminal pFLAG-CMV-5.1 adenovirus expression vector (Sigma).

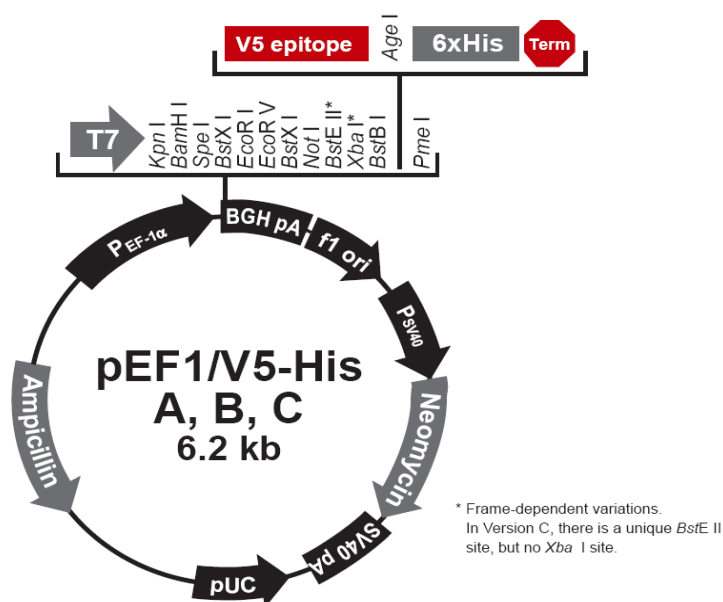


Figure 6.3 pEF1/V5-His vector

Schematic illustrating the pEF1/V5-His vector used for integration of the FH and FHΔMTS sequences into Fh1^{-/-} MEFs (Schematic courtesy of Invitrogen).

6.3 Generation of Primary Renal Cell Lines

Primary renal cell lines were established using standard protocols (Biju et al. 2005). Briefly, dissected kidneys from 8 week old mice were digested in 10 ml of digest buffer at 37°C for 30 minutes. The digest buffer consisted of 100 mg Collagenase IV (Worthington) dissolved in 100 ml Hanks buffered saline solution (HBSS) (Sigma), with 100 mg Soybean Trypsin-Inhibitor (Gibco), and was filter sterilised (using a 0.2 µm filter). The collagenase digest was stopped with 2 ml of FBS and passed through a 70 µm cell strainer (BD Falcon). The supernatant was centrifuged (Allegra™ X-12R, Beckman Coulter) for 5 minutes at 1000 rpm at 4 °C. The cell pellet was washed in Hank's Buffered Saline Solution (Gibco) then grown in DMEM/F12 medium (Gibco) supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH7.0-7.6) (Sigma), 5 ng/ml Amphotericin B (Sigma), 50 nM/ml hydrocortisone (Sigma), 20% (v/v) ITS (Insulin, transferrin and sodium selenite) solution (Sigma). In order to preserve the primary physiology of these cells they were unable to be trypsinised and hence not suitable for long term experimentation.

6.4 DNA extraction

Cell pellets or murine tissues were digested overnight in 5% proteinase K-containing digest buffer (100 mM ethylenediaminetetraacetic acid (EDTA) (pH=8.0), 50 mM TRIS-HCl (pH=8.0), 100 mM NaCl and 1% sodium dodecyl sulphate (SDS)) at 55 °C. DNA extraction was carried out as previously described (Pollard et al. 2007) with the resulting DNA pellet re-suspended in TE buffer (10 mM Tris/HCl and 1 mM EDTA pH 8.0). Briefly, 160µl of concentrated NaCl (5.5M) was added to digested samples, placed on shaker for 2 minutes and centrifuged (MIKRO 200R, Hettich

Zentrifugen) at 14,000 rpm for 10 minutes. The supernatant was transferred to a clean 1.5 ml eppendorf tube and Propan-2-ol (iso-propanol) added. The samples were placed for 2 minutes on a shaker and spun down at 14,000 rpm for a further 10 minutes after which the pellet was washed in 75% ethanol then left to air dry before a final re-suspension in TE buffer.

6.5 DNA polymerase chain reaction

Gene amplification was performed by preparing the PCR reaction mixtures outlined in either Table 6.1 or Table 6.2 with the relevant primer sequences for the gene of interest detailed in Table 6.3. A 200 μ M stock concentration for each primer mixture (made in dH₂O) was made and for each PCR reaction mixture a final concentration of 500 nM per primer oligo. PCR reactions were programmed and ran on a PCR thermo cycler (PTC-225, Peltier Thermal Cycler, MJ Research).

For Fh1 gene amplification the following PCR cycle was run: step 1 (initialisation) at 95 °C for 3 minutes, step 2 (denaturisation) at 95 °C for 30 seconds, step 3 (annealing) at 55 °C for 45 seconds and step 4 at 72 °C for 45 seconds (extension/elongation), with steps 2-4 repeated for 34 cycles and held at 6 °C.

For Ksp1.3/Cre and CAG-FH transgenes (recognises both CAG-FH and CAG-FHAMTS transgenes) the following PCR cycle was run: step 1 at 95 °C for 3 minutes, step 2 at 95 °C for 30 seconds, step 3 at 60 °C for 45 seconds and step 4 at 72 °C for 1 minute, with steps 2-4 repeated for 34 cycles and held at 6 °C.

Table 6.1 Qiagen 1 x PCR reaction mixture

Reagent	Volume
dH ₂ O	31.5 µl
10 x Buffer	5.0 µl
Q Solution	10.0 µl
Mg ²⁺ (25 mM)	0 µl
dNTPs	1.0 µl
Primer Mix	1.0 µl
Taq polymerase	0.5 µl
DNA	1.0 µl
Total Volume	50 µl

Table 6.2 Bioline 1 x PCR reaction mixture

Reagent	Volume
dH ₂ O	38.5 µl
10 x Buffer	5.0 µl
Mg ²⁺ (25 mM)	0 µl
dNTPs	4.0 µl
Primer Mix	1.0 µl
Taq polymerase	0.5 µl
DNA	1.0 µl
Total Volume	50 µl

Table 6.3 PCR primer sequences

Target	Supplier	Oligo Name	Sequence (5'-3')
Fh1	Sigma	FH1	ACCCTGCTAGGTGTCACCAC
		FH2	CCTGGCACTGCAGACTACAA
		FH3	GCTCAGTCACCCATCCAAAT
Ksp1.3/Cre	Sigma	KSPFW	GCAGATCTGGCTCTCCAAAG
		KSPRV	AGGCAAATTTTGGTGTACGG
CAG-FH	Integrated DNA	MLS-F1	AACATGATCGTTGGGATGCAC
transgene	Technologies (IDT)	MLS-R1	GCCTCTGCTAACCATGTTCAT

6.6 Human and murine tissues and cell lines

Access was granted for the use of frozen *FH*-mutant Type II papillary renal cancer, *VHL*-mutant clear cell renal cancer, and *FH*-deficient cells collected and archived by Prof. Ian Tomlinson with full ethical approval [Oxfordshire REC B 05/Q1605/66 “Molecular analyses of archival tumours”]. *Fh1*- and *Vhl*-deficient mice have been previously described (Haase et al. 2001; Pollard et al. 2007).

6.7 Multiplex Fluorescence in situ Hybridization

Metaphase chromosomes were prepared from mouse embryonic fibroblast cultures following standard cytogenetic procedures (Anderson 2010). Karyotyping by Multiplex Fluorescence in situ Hybridization (mFISH) was performed (by Dr. Emanuela Volpi, Molecular Cytogenetics Core, Oxford University) using a

21XMouse Mouse Multicolor FISH (mFISH) Probe Kit according to the manufacturer's instructions (MetaSystems, Catalogue number D-0425-060-DI). Briefly, specimens were prepared on FISH slides and denatured in 20x SSC solution (saline-sodium citrate buffer; 3.0 M NaCl and 0.3 M $C_6H_5Na_3O_7$). Following denaturation of the probe at 75°C for 5 min, the denatured and prehybridised probe was added to the denatured specimens, covered using a cover slip and sealed with rubber cement, then incubated for 1-2 days in a humidified chamber at 37 °C. The cover slip was then removed, specimens washed in SSC solution (prewarmed to 72 °C) and counterstained with 4'6-diamidino-2-phenylindole (DAPI)/antifade (MetaSystems). Image capture and analysis were carried out on a CytoVysion system (Genetix) consisting of an Olympus BX-51 epifluorescence microscope coupled to a Sensys charge-coupled device (CCD) camera (Photometrics). At least 20 metaphases were imaged and analysed for each cell line.

6.8 Immunohistochemistry

Murine and human tissues were fixed in neutral-buffered 10% formalin and processed according to standard methods for paraffin embedding and sectioning. Sections were cut to a thickness of 5-10 µm (Tissues were embedded and cut by Histopathox Ltd., London, UK). Processing of tissue sections and immunohistochemistry was performed using a Dako EnvisionTM+ Kit as per manufacturer's instructions. Briefly, paraffin embedded sections were de-waxed in xylene, a hydrating series of ethanol (EtOH; Sigma Aldrich) and finally water. Using pre-heated 1X Antigen Retrieval (citrate buffer) solution pH 6.0 (DAKO), antigen retrieval was carried out by placing the sections in a pressure cooker (Delicio, Tefal) for 30 minutes. Sections were then

washed in PBS (Sigma) and blocked for endogenous peroxidases for 10 minutes, incubated with primary antibody (diluted to working concentration in DAKO antibody diluent) for 45 minutes and an anti-rabbit horse radish peroxidase (HRP) polymer (DAKO). PBS-Tween (0.1% (v/v) Tween-20®; Sigma) washes were carried out after each antibody incubation step. Each section was exposed using 3, 3'-diaminobenzidine (DAB) solution (one drop of DAB+ substrate-chromogen per 1 ml of DAB+ substrate buffer, both from Dako) was followed by a 2 minute counterstain with haematoxylin. Stained sections were brought through a dehydrating series of alcohol and xylene prior to being mounted in DPX (Fisher Scientific). Primary antibodies used are listed in Table 6.4. Pictures were acquired using a Leica DM 1000 microscope.

Table 6.4 Primary antibodies for immunohistochemistry

Antibody	Supplier	Catalogue #	Concentration
FH	Autogen Bioclear	NE054	1:1000
2SC	Norma Frizzell (Nagai et al. 2007)	N/A	1:20000
V5-HRP conjugated	Invitrogen	R96125	1:5000
HIF-1 α	Caymen Chemical Company	10009269	1:50

6.9 Protein Assay

The protein concentration of a sample was determined using a Bio-Rad Protein Assay kit according to the manufacturer's protocols. Briefly, 20 μ l Reagent S was added to 1ml of Reagent A and vortexed to mix well. Into a round-bottomed disposable 96-well plate (BD Falcon) 5 μ l of each sample (in triplicate) was added to each well including an exogenous control to remove background signal (i.e. sample buffer). To each well 25 μ l of the Reagent A/Reagent S mixture and 180 μ l of Reagent B was added and allowed to develop for 10-15 minutes. The endpoint was measured at 655 nm on a microplate reader (Model 550, Bio-Rad).

6.10 Immunoblotting and antibodies

Whole cell extracts and immunoblot analysis were carried out as described previously (Pollard et al. 2005). Briefly, protein samples were denatured in SDS urea solution (7 M Urea, 8.6 mM Tris/HCl (pH7.5), 8.6% (v/v) glycerol, 0.86% SDS, 1 mM dithiothreitol (DTT) and protease inhibitors [Roche]) and centrifuged at 14,000 rpm (MIKRO 200R, Hettich Zentrifugen) for 5 minutes at 4 °C. The protein concentration of each sample was determined and standardised using the protein assay described above (section 6.8) and 75 μ l of the supernatant transferred to a 1.5 ml eppendorf tube. To the supernatant 25 μ l of 4X Sample Buffer (200 mM Tris/HCl (pH 6.8), 8% SDS, 4% (v/v) β -Mercaptoethanol (Fluka BioChemika), 40% (v/v) glycerol (Sigma) and 0.4% Bromophenol blue [Sigma]) was added and boiled for 10min at 95 °C following a 20 second vortex (TopMix, Fisher Scientific).

SDS-PAGE (polyacrylamide gel electrophoresis) was carried out to separate the sample proteins based on their molecular weight. Samples were loaded onto the gels (see Tables 6.5 and 6.6. Pre-cast, 4-20% gradient Tris-HCl Ready Gels® (Bio-Rad) were also used.) in the presence of a pre-stained protein molecular weight ladder, PageRuler™Plus (Fermentas), and run for approximately 60 minutes in a 1X Running Buffer solution (25mM Tris-HCl, 100mM glycine, 10% methanol and 0.005% SDS) at 20 mAMPs per gel.

Table 6.5 Reagents for running gels

Details of the reagents required to make a 7.5% gel. The volume of 30% Acrylamide used was adjusted to either increase or decrease the gel pore size as required.

Reagent	Volume
Lower Buffer (pH 8.8) (4x Tris/HCl/SDS)	3 ml
30% Acrylamide (Bio-Rad)	3 ml
dH ₂ O	6 ml
10% APS	120 µl
<i>N, N, N', N'</i> -Tetramethylethylenediamine (TEMED) (Sigma)	12.5 µl

Table 6.6 Reagents for stacking gels

Reagent	Volume
Upper Buffer (pH 6.8) (4x Tris/HCl/SDS)	1.25 ml
30% Acrylamide (Bio-Rad)	0.65 ml
dH ₂ O	3.05 ml
10% APS	31 µl
(TEMED) (Sigma)	6 µl

Transfer of protein from gels onto Immobilon®-P (PVDF) membranes (0.45 µm pore size; Millipore) was carried in 1X Transfer Buffer (10 mM Tris-HCl, 100 mM glycine, 10% methanol and 0.005% SDS) at 100 Volts for 45-60 minutes. Membranes were then blocked in 5% skimmed milk powder (Fluka BioChemika) in PBS-Tween for 30-60 minutes. Primary antibodies (made in 5% skimmed milk) were incubated with the membrane-bound proteins for either 90 minutes at room temperature or overnight at 4 °C. Secondary antibodies were made up to working concentrations in 5% milk solution and incubated for a 45-60 minute period at room temperature. After incubation with each antibody the membranes were subjected to three 5 minute washes in PBS-Tween.

Depending on the signal strength of the antibodies used, the membranes were exposed to a suitable substrate prior to being developed by film (X-Omat LS film, Kodak). SuperSignal® West (Thermo Scientific) substrates, consisting of equal volumes of Luminol/Enhancer solution mixed with Stable Peroxide Buffer, were used to develop

the antibody signals. In order of increasing signal intensity the substrate solutions, used were: PICO, DURA or FEMTO (all from Thermo Scientific).

Table 6.7 Antibodies for immunoblotting

Antibody	Supplier	Catalogue #	Concentration
FH	Autogen Bioclear	NE054	1:1000
2SC	Norma Frizzell (Nagai et al. 2007)	N/A	1:20000
V5-HRP conjugated	Invitrogen	R96125	1:5000
HIF-1 α	Novus	100-449	1:500
HIF-1 α	Caymen	#10006421	1:500
Hydroxy-HIF-1 α (Pro564)	Cell Signalling Technology	#3434	1:500
Hydroxy-HIF-1 α (Pro402)	Millipore	#07-1585	1:4000
Hydroxy-HIF-1 α (Asn803)	(Lee et al. 2008)	N/A	1:3000
Anti-FLAG®M2	Sigma	F1804	1:1000
Anti-SV40 T-antigen	Abcam	ab16879	1:200
LDHA	Cell Signalling	#3582S	1:500-1:1000
Histone H3	Abcam	ab1791	1:5000
β -actin-HRP	Abcam	ab49900	1:50000
Swine Anti-Rabbit-HRP	Dako	P0399	1:4000
Goat Anti-Mouse-HRP	Dako	P0447	1:4000

6.11 Immunofluorescence and confocal analysis

Prior to fixation, cells were labelled with 250nM Mitotracker[®] Red CMXRos (Invitrogen) for 15 minutes at 37 °C. Cells were fixed in 10% neutral buffered formalin (Sigma) for 10 minutes, with an additional 10 minute fixation in methanol (Sigma) at -20 °C, and permeabilized with 0.1% Triton-X-100 (Sigma) for a further 10 minutes. Samples were blocked in 3% bovine serum albumin (BSA) in PBS. See Table 6.8 for the list of primary antibodies and secondary AlexaFluor[®] antibodies (Invitrogen) used. Attempts were made to visualise 2SC proteins by IF using a range of antibody concentrations and conditions, however it proved to be unsuccessful. Both primary and secondary antibodies were diluted to working concentrations in 3% BSA. V5-tagged FH protein was visualised with anti-V5-FITC conjugated antibody (Invitrogen). Samples not treated with Mitotracker[®] Red CMXRos and samples treated with secondary antibodies only were included as controls. Samples were mounted in Vectashield[®] (Vector Labs) with DAPI. Pictures were acquired using the Zeiss LSM510 MetaHead confocal Microscope and using LSM 510 software v4.2 (Carl Zeiss GmbH).

Table 6.8 Antibodies for immunofluorescence

Antibody	Supplier	Catalogue #	Concentration
FH	Autogen Bioclear	NE054	1:1000
V5-FITC conjugated	Invitrogen	R96325	1:5000
Donkey Anti-Rabbit AlexaFluor 488	Invitrogen	A21206	1:2000

6.12 Measurement of Fumarate Hydratase enzyme activity

Determination of enzymatic activity of FH was performed as described previously (Hatch 1978; Alam et al. 2003). The principle of this assay is that, in the presence of catalytically active FH, fumarate is converted to malate which can subsequently be converted to pyruvate by malic enzyme. In addition to malate, malic enzyme requires NADP⁺ as a co-substrate which is reduced to NADPH and the reduction of NADP⁺ to NADPH can be measured at 340nm as an indirect measure of FH activity. Briefly, malic enzyme (~26 units) (chicken liver; Sigma) was purified using a 5ml Sephadex G25 Superfine (Pharmacia) column after which 0.95 ml of purified enzyme was mixed with 12 ml 0.43 mM NADP (Sigma) dissolved in assay buffer (20 mM HEPES (Sigma), 1M KOH (pH7.5), 4 mM of KH₂PO₄ and 5 mM of MgCl₂.6H₂O). Fumaric acid (120 mM) (Sigma) was dissolved in 5 ml assay buffer. The final reaction medium consisted of: 10mM Fumaric acid, 25 mM Hepes-KOH Buffer pH 7.5, 0.3 units malic enzyme/ml, 0.27 mM NADP, 4 mM MgCl₂ and 5 mM Potassium phosphate. Cells were gently sonicated, washed in wash buffer (20 mM HEPES (Sigma), 1M KOH (pH7.5) and 2 mM DTT), centrifuged and supernatant removed and protein content determined. Following addition of assay medium to the cell supernatant, kinetic analysis was carried out using a KONELAB chemical analyser (Thermo Scientific) at 37°C at a wavelength of 340 nm. Calculation of FH activity (using 6µl sample)= $\Delta\text{OD (optical density)} \times 3242$ (KONELAB calculation factor for 6µl sample) nmol/mg(protein)/min. Human wildtype skin fibroblasts and FH-deficient skin fibroblasts were included as controls for normal and impaired FH activity, respectively.

6.13 MRS metabolite analysis

Magnetic resonance spectroscopy (MRS) was performed as described previously (Pollard et al. 2005). In brief, metabolites were extracted from cells, using perchloric acid and freeze dried. The samples were resuspended in D₂O and analysed using ¹H MRS. Cell culture media were also collected and analysed by ¹H MRS. Sodium 3-trimethylsilyl-2,2,3,3-tetradeuterpropionate (TSP) was added to the samples for chemical shift calibration and quantification. Results were expressed in fmol/cell.

6.14 RNA extraction

Total RNA was extracted by adding 1 ml Tri®-reagent (Sigma) to cells and left to stand at room temperature for 5 minutes. For RNA extraction from frozen tissue samples, the tissue was first smashed and then homogenised in 1 ml Tri®-reagent using a 21 G needle (BD Falcon) and 1 ml syringe (BD Falcon); remainder of the protocol was carried in the same manner as for cell samples. Following this, 0.2 ml chloroform was added, vortexed for 15 seconds and left to stand at room temperature for a further 3 minutes before centrifugation of the samples at 12,000 rpm for 10-15 minutes. The upper layer was transferred to a new eppendorf tube, 0.5 ml isopropanol added and left to stand at room temperature for ~10 min then centrifuged at 12,000 rpm for 10 minutes. Supernatant was removed and two 1 ml 75% EtOH washes were carried out at 9,500 rpm for 5 minutes. Finally, the resulting pellet was left to air dry for 10 minutes at room temperature prior to re-suspension in 50 µl diethyl pyrocarbonate (DEPC)- treated water. The quality and quantity (ng/µl concentrations) of the RNA was determined using a Nanodrop spectrometer (ND-1000, Nanodrop). All centrifugation steps were carried out at 4°C. Samples were stored at -80°C.

6.15 cDNA amplification

First strand cDNA synthesis was generated from 2µg of total RNA (Applied Biosystems). Total reaction mixture (Table 6.9) was brought to a final volume of 20 µl using nuclease-free H₂O. PCR conditions for cDNA amplification were as follows: 25 °C for 10 minutes, 37 °C for 120 minutes and 85 °C for 1 minute. Completed reactions were held at 4 °C.

Table 6.9 cDNA synthesis 1 x reaction mixture

Reagent	Volume
10 x RT Buffer	2.0 µl
dNTP	0.8 µl
10 x RT Random Primers	2.0 µl
Multiscribe Reverse Transcriptase	1.0 µl
RNase Inhibitor	1.0 µl
RNA (in nuclease free H ₂ O)	13.2 µl
Total Volume	20 µl

6.16 Reverse-transcription Quantitative PCR

Reverse-transcription quantitative PCR (referred to as Q-PCR throughout the text), for quantification of mRNA expression, was carried out by employing Taqman gene expression assays run on a StepOne thermocycler (Applied Biosystems). Total RNA was extracted using Tri®-reagent (Sigma) as detailed above in section 6.10 and cDNA synthesised (Applied Biosystems) from 2µg of the total RNA extracted.

Materials and Methods

Reaction mixtures including the relevant Taqman® probe (Applied Biosystems) against the target gene and an internal control were prepared as detailed in Table 6.10. Normalization was to *β-actin* mRNA and relative gene expression was calculated using the comparative C_T (also known as $\Delta\Delta C_T$) method. Briefly, this method uses the amount of target, normalized to an endogenous reference and relative to a calibrator, as denoted by the formula $2^{-\Delta\Delta C_T}$, to achieve the result for relative quantitation (Applied Biosystems, manufacturer's guidelines). For a valid $\Delta\Delta C_T$ calculation, the efficiency of both the target and reference amplifications must be approximately equal. For each reaction 50ng of cDNA template was used and three biological replicates, each in triplicate, were performed for each experiment. Cycling stages for each Q-PCR run were set at 95 °C for 1 second, 60 °C for 20 seconds for a total of 40 cycles.

Table 6.10 1 x Q-PCR reaction mixture

Reagent	Volume
Taqman® Mastermix	5.0 µl
Taqman® probe (FAM-labelled)	0.5 µl
Exogenous control (VIC-labelled <i>β-actin</i>)	0.5 µl
Nuclease free H ₂ O	2.0 µl
Total Volume	8µl

6.17 Measurement of mitochondrial respiration

MEFs (2×10^6 cells) were harvested and washed in 100mM KCl, 50mM morpholino propanesulfonic acid (MOPS) and 0.5mM Ethylene glycol-bis(2-aminoethylether)-N, N, N', N' tetra acetic acid (EGTA) to remove any cell culture media. Cells were resuspended in respiratory media (100mM KCl, 50mM MOPS, 1mM EGTA, 5mM KH_2PO_4 , and 1 mg/mL BSA, pH 7.4), and transferred to a Clark-type oxygen electrode (Strathkelvin, UK) at 30°C for measurement of mitochondrial respiration (Heather et al. 2010). Respiration was measured under basal unstimulated conditions, following the addition of glutamate (20mM), the ATP synthase inhibitor oligomycin (2 μ M), and the metabolic uncoupler carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (10 μ M).

6.18 ATP measurements

Levels of ATP were determined using a CellTiter-Glo® Assay (Promega, Madison) on white 96-well plate (Greiner Bio One, Frickenhausen, Germany) according to the manufacturer's instructions. Briefly, cells were plated at a density of 5×10^4 cells per well in 96 well plate and grown in DMEM supplemented with either 25mM glucose, or 25mM galactose. Cells grown in each condition were lysed simultaneously at 0h, 2h, 4h and 6h and luminescent signals measured using a plate reader (Victor 2; PerkinElmer Life Science). Maintenance of [ATP] in galactose medium is expressed as a percentage of that in glucose medium at each time point; $[\text{ATP}]^{\text{Gal}}/[\text{ATP}]^{\text{Glu}} \times 100$.

6.19 Measurement of mitochondrial Reactive Oxygen Species Production by FACS

The fluorogenic dye MitoSOXTM Red mitochondrial superoxide indicator (Molecular ProbesTM, Invitrogen) was used to detect reactive oxygen species (ROS) in the mitochondria of live cells, following manufacturer's protocol/ instructions. Briefly, cells were plated in a 6 well plate at 50% confluency and after 24 h either left untreated (controls for basal levels of ROS) or treated with 1mM hydrogen peroxide (H₂O₂) for 2 hours. The optimum concentration for ROS induction, without affecting cell viability, was determined by measuring the effects of a titration of H₂O₂ concentrations (ranging from 0.5 mM-2.5 mM) on cells viability and efficacy in stimulating ROS generation. . Following this the cells were then incubated with 0.5 μ M MitoSOXTM solution for 20 minutes at 37°C protected from light. After PBS washing, cells were harvested with 0.05% trypsin/EDTA, re-suspended in a 500 μ l PBS and analyzed with a flow cytometer (CyAn, Dako). FACS parameters were set accordingly: Excitation of the MitoSOXTM Red mitochondrial superoxide indicator was measured at 400nm and sample acquisition occurred over 2-5 min, with 10,000-30,000 events measured. Cells were gated using forward and side scatter measured using a linear scale ranging from 0-256. Fluorescent signal from the MitoSOXTM Red mitochondrial superoxide indicator was measured on a logarithmic scale, ranging from 10⁰-10⁶, with detection observed between 10⁰ and 10¹.

6.20 Ldha shRNA-targeted knockdown

Three independent SMARTvectorTM 2.0 lentiviral shRNA particles containing a GFP reporter gene (all from Thermo Fisher Dharmacon) targeting against mouse gene LDH1 (Ldha) and a Non-targeting control (2.66 x 10⁸ Titer (TU/ml); Thermo Fisher

Dharmacon) (See Table 6.11). Expression of the shRNA particles was linked to detectable GFP expression. Fh1^{-/-} MEFs were plated at 2×10^4 cells per well of a 6x well plate and infected at a multiplicity of infection (MOI) of 20 in the presence of 5 µg/ml Polybrene in serum-free OptiMem medium (Gibco) for 8 hours after which the medium was replaced with standard DMEM with 10% (v/v) FBS. Cells were infected for a period of 96 hours with cell counts carried out at 24 hour intervals. Trypan blue (Sigma) exclusion was used to distinguish viable cells from dead cells and total cell numbers were counted using disposable haemocytometers (Fast-Read 102®, Immune Systems). Immunoblot and Q-PCR analysis was carried out at 144 hours.

Table 6.11 Ldha shRNA constructs 1-3

Gene	Sequence #	Source Clone ID	Titre (TU/ml)	Gene Target Sequence
Ldha	1	SH-043884-01-10	3.41×10^8	AGTTCCCAGTTAAGTCGTA
	2	SH-043883-02-10	3.53×10^8	AATGAAGAACCTTAGGCGG
	3	SH-043883-03-10	3.65×10^8	CAACTGCAGGCTTCGATTA

6.21 Generation of CAG-FH transgenic complemented Fh1 mice

The cDNA sequences for human full-length FH and FH lacking its N-terminal MTS (FHΔMTS) transgenes, to be expressed under the control of the ubiquitous CAG (chicken-beta actin promoter/CMV immediate early promoter enhancer) promoter, and a neomycin-resistance cassette were cloned into CB91 exchange vectors. The CAG promoter was obtained from a pCX-eGFP plasmid (kindly donated by Prof. Elizabeth Robertons, Oxford University). Sequence analysis prior to integration with the exchange vector confirmed that no mutations were present in either sequence.

These vectors were co-electroporated with the PhiC31 integrase expression cassette into C57BL/6J acceptor ES cells. PhiC31 integrase drives the exchange between the attB sites within the exchange vectors and the attP sites lying within the *Rosa26* locus which have been preintegrated into the acceptor ES cells (Figure 6.4). Correct integration was confirmed at the 5' (ExPGK3 and ExNeo2 binding) and 3' (CMV-IER and Rosa3HR-R binding) ends of the construct by PCR (See table 6.12). For PCR genotyping of the 5' end the following conditions were used: step 1 at 94 °C for 5 minutes, step 2 at 94 °C for 30 seconds, step 3 at 57 °C for 60 seconds, step 4 at 72 °C for 30 seconds and step 5 at 72 °C for 7 minutes, with steps 2-4 repeated for 30 cycles and held at 4°C. PCR genotyping of the 3' end was performed under the following conditions: step 1 at 94 °C for 5 minutes, step 2 at 94 °C for 60 seconds, step 3 at 60 °C for 60 seconds, step 4 at 72 °C for 30 seconds and step 5 at 72 °C for 7 minutes, with steps 2-4 repeated for 35 cycles and held at 4°C. Localisation of both variants of CAG-FH to their relevant cellular compartments in the ES cells was further confirmed by IF staining using an anti-V5 antibody (Invitrogen) to detect the V5-tag present in the C-terminus of the FH transgenes. The above experiments were carried out by Dr. Ben Davies (Transgenic Core, Oxford University).

Two independent ES clones for positive for either the CAG-FH or CAG-FHΔMTS variants, were injected into C57BL/6 blastocysts and the resulting chimeric embryos were transferred to pseudopregnant females (Carried out by Dr. Ben Davies, Transgenic Core, Oxford University). A problem with acceptor ES cell integrity resulted in a second attempt at blastocyst injection of the ES cells expressing the CAG-FH transgene being necessary. Pups were assessed for their degree of chimerism using coat colour markers (the percentage of agouti colouring) to

determine the rate of success with which the injected ES cells colonised the host epiblast. Confirmation of the phenotypes of the three CAG-FH Δ MTS chimeras and two CAG-FH chimeras generated was carried by PCR using the MLS-F1 and MLS-R1 primers described in Table 6.3. In the presence of the full length FH construct an amplification product of 649 bp is obtained whereas in the absence of the MTS (i.e. the FH Δ MTS construct) a truncated amplification product of 510 bp is obtained. All chimeras tested had the correct 5' and 3' recombination. The success rate for generation of the chimeras was variable. A larger number of chimeras with a higher percentage of injected ES cell integration were generated from injection of the ES cells expressing CAG-FH Δ MTS at the *Rosa26* locus. The CAG-FH chimeras had a lower percentage of ES cell integration (ranging from 40-70%) and only generated two chimeras deemed suitable for breeding.

Two CAG-FH and two CAG-FH Δ MTS chimeras with the highest percentage agouti coat colour were intercrossed with *Fh1* mice to initiate the breeding strategy. In order to specifically initiate recombination of the LoxP sites flanking *Fh1* in murine kidney cells, *Fh1* mice were bred with transgenic mice expressing Cre recombinase under the kidney specific-cadherin promoter (Ksp) (Ksp1.3/Cre mice). *Fh1*^{fl/fl} or *Fh1*^{fl/+} mice positive for monoallelic expression of either CAG-FH or CAG-FH Δ MTS were intercrossed with Ksp1.3/Cre mice to generate offspring with the following genotypes: *Fh1*^{fl/fl}, *Fh1*^{fl/fl} +KSP1.3/Cre, *Fh1*^{fl/fl} + CAG-FH (with and without the Ksp1.3/Cre allele), *Fh1*^{fl/fl} + CAG-FH Δ MTS (with and without the Ksp1.3/Cre allele), *Fh1*^{fl/+}, *Fh1*^{fl/+} +KSP1.3/Cre, *Fh1*^{fl/+} + CAG-FH (with and without the Ksp1.3/Cre allele) and *Fh1*^{fl/+} + CAG-FH Δ MTS (with and without the Ksp1.3/Cre allele). This ensured that wildtype, *Fh1*^{-/-}, wildtype +transgene only and *Fh1*^{-/-} +transgene only

controls were available for comparable analysis with the $Fh1^{-/-}$ +CAG-FH/CAG-FHAMTS (assumed throughout text as expressing Ksp1.3/Cre unless otherwise stated) mice. As Ksp1.3/Cre is not expressed in the metanephros and ureter until E15.5 the caveat of early lethality associated with embryonic deletion of *Fh1* *in vivo* is circumvented in $Fh1^{-/-}$ mice who do not express either of the FH transgenes.

The CAG-FH/Ksp1.3/Cre transgenic mice were maintained in accordance with Home Office guidelines and licensing regulations. Mice were weighed weekly until the 20 week end point.

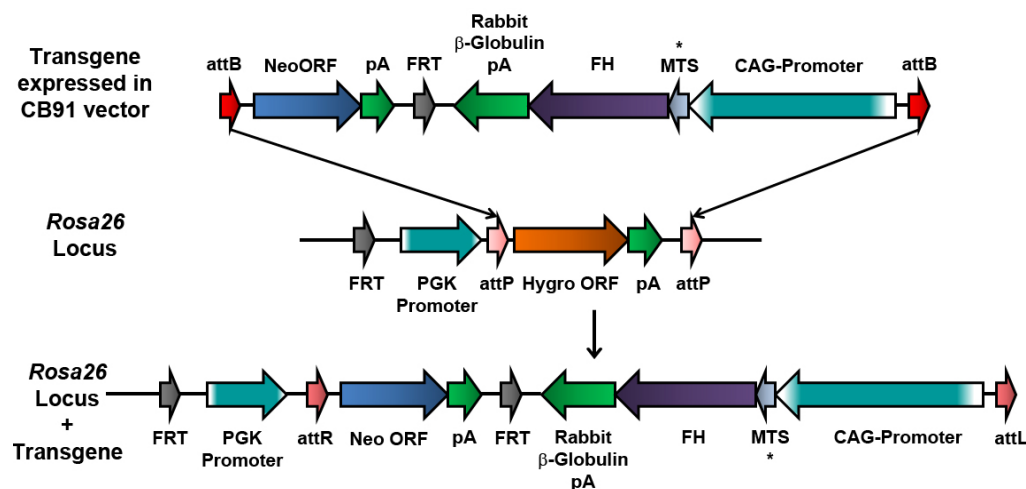


Figure 6.4 FH transgene expression vector targeted to the *Rosa26* locus

The top panel illustrates the CB91 expression vector encoding the FH transgene driven by the CAG-promoter. *The MTS cDNA sequence is absent in the CB91 expression vector encoding the FHAMTS transgene. Exchange between the CB91 attB and *Rosa26* attP sites (pre-integrated into acceptor ES cells) is catalysed by Phi integrase which is expressed *via* an expressing vector co-electroporated into the acceptor ES cells. The bottom shows integration of the transgene at the *Rosa26* locus of the acceptor cells. (Adapted from Dr. Ben Davies' schematic)

Table 6.12 PCR primers used for confirmation of integration of exchange vector

Target	Supplier	Oligo Name	Sequence (5'-3')
PGK promoter	(Hitz et al. 2007)	ExPGK3	CACGCTTCAAAAGCGCACGTCTG
Neomycin Resistance Cassette (ORF)	(Hitz et al. 2007)	ExNeo2	GTTGTGCCCAGTCATAGCCGAATAG
CAG Promoter	(Prof.Elizabeth Robertson)	CMV-IER	GGCGTTACTATGGGAACATACG
Rosa26 Locus	(Hitz et al. 2007)	Rosa3HR-R	CGGGAGAAATGGATATGAAGTACTGGGC

6.22 Cell Proliferation Studies

Biological triplicates of Fh1 MEFs were plated at 5×10^4 cells per well of a 6x well plate and grown in either standard tissue culture conditions (i.e. 21% O₂) or 1% hypoxia. Cells were harvested with 0.05% Trypsin/EDTA solution (Sigma) at 12 hour time points for 72 hours, re-suspended in 1ml standard DMEM medium with 40µl Trypan Blue (Sigma). Cells were counted using a disposable haemocytometers (Fast-Read 102®, Immune Systems) with the number of cells positively stained with trypan blue (i.e. dead cells) deducted from the total cell count to determine the number of viable cells at each time point. The medium was not changed during the time course. Results were plotted using Microsoft Excel.

6.23 Statistical Analysis

Statistical comparisons for Q-PCR were performed using a Student's unpaired *t* test.

Data on mitochondrial respiration were analysed using two-way ANOVA and Tukey's *post hoc* analysis.

7 Chapter 7: Appendix

7.1 Chapter 1

Appendix Table 1.1 Summary of reported *FH* mutations

(Adapted from (Gardie et al. 2011))

Number of families	Nucleotide Mutation	Protein Consequence	Reference of description
1	Whole gene deletion	/	(Tomlinson et al. 2002; Alam et al. 2003; Smit et al. 2011)
1	Whole gene deletion	/	(Tomlinson et al. 2002; Alam et al. 2003; Smit et al. 2011)
1	Whole gene deletion	/	(Tomlinson et al. 2002; Alam et al. 2003; Smit et al. 2011)
1	Whole gene deletion	/	(Smit et al. 2011)
1	DelExon1	/	(Ahvenainen et al. 2008)
1	DupExon7	/	(Campione et al. 2007)
1	IVS3+1delG	Splice Site	(Badeloe et al. 2006)
1	IVS4+1G>A	Splice Site	(Badeloe et al. 2006)
2	c.10C>T	p.Gln4X	(Tomlinson et al. 2002; Chuang et al. 2006)
1	c.28G>T	p.Glu10X	(Smit et al. 2011)
1	c.49delC	p.Leu17fs	(Tomlinson et al. 2002)
1	c.104del	p.Asn35fs	(Smit et al. 2011)
1	c.111insA	p.Lys37X	(Wei et al. 2006)

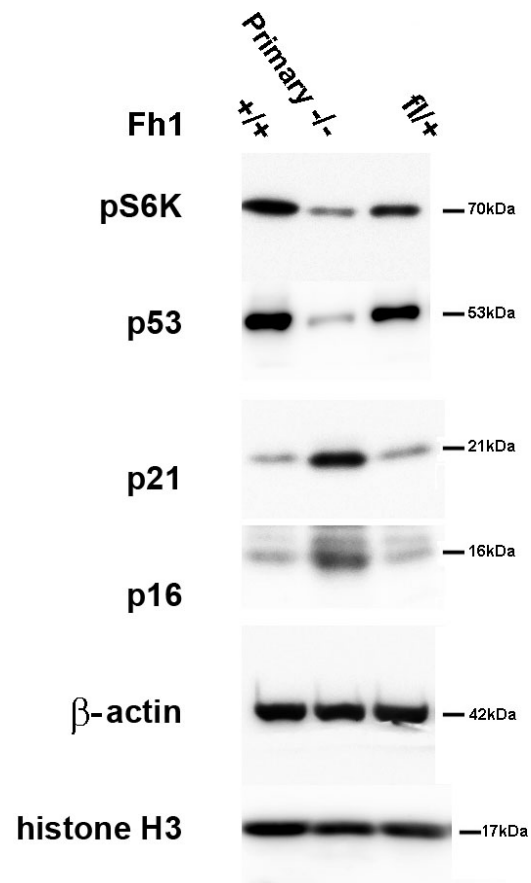
1	c.138+1G>C	Splice Site	(Wei et al. 2006)
8	c.172C>T	p.Arg58X	(Tomlinson et al. 2002; Wei et al. 2006; Vahteristo et al. 2010)
2	c.173G>C	p.Arg58Pro	(Chan et al. 2005)
7	c.191A>C	p.Asn64Thr	(Tomlinson et al. 2002; Wei et al. 2006)
1	c.220G>C	p.Ala74Pro	(Tomlinson et al. 2002)
1	c.266T>C	p.Leu89Ser	(Wei et al. 2006)
1	c.275A>G	p.His92Arg	(Chuang et al. 2005)
1	c.288delG	p.Val97X	(Toro et al. 2003)
1	c.305G>C	p.Ser102X	(Wei et al. 2006)
1	c.346G>T	p.Ser115Ile	(Martinez-Mir et al. 2003)
1	c.349A>G	p.Arg117Gly	(Wei et al. 2006)
1	c.410A>G	p.His137Arg	(Tomlinson et al. 2002)
1	c.424C>T	p.Gln142X	(Martinez-Mir et al. 2003)
1	c.425A>G	p.Gln142Lys	(Badeloe et al. 2006)
1	c.425A>G	p.Gln142Arg	(Tomlinson et al. 2002)
1	c.431C>T	p.Ser144Leu	(Toro et al. 2003)
1	c.434A>G	p.Asn145Ser	(Toro et al. 2003)
1	c.446A>G	p.Pro149Leu	(Chuang et al. 2005)
1	c.455T>C	p.Met152Thr	(Toro et al. 2003)
3	c.458A>G	p.His153Arg	(Lehtonen et al. 2006)
2	c.542_543delAG	p.Glu181fs	(Tomlinson et al. 2002)
1	c.557T>C	p.Ile186Thr	(Alam et al. 2003)
1	c.3bpdel	p.Ile186fs	(Tomlinson et al. 2002)

3	c.560A>G	p.Lys187Arg	(Tomlinson et al. 2002; Alam et al. 2003; Chuang et al. 2005)
1	c.559_561delAAG	p.Lys187fs	(Tomlinson et al. 2002)
1	c.566del	p.Gly189Aspfs	(Parmentier et al. 2010)
2	c.568C>T	p.Arg190Cys	(Chuang et al. 2005; Wei et al. 2006)
1	c.569G>T	p.Arg190Leu	(Toro et al. 2003)
21	c.569G>A	p.Arg190His	(Tomlinson et al. 2002; Alam et al. 2003; Toro et al. 2003; Chuang et al. 2005; Wei et al. 2006; Huter et al. 2008; Smit et al. 2011)
1	c.695G>A	p.Gly232Glu	(Smit et al. 2011)
1	c.716G>T	p.Gly239Val	(Tomlinson et al. 2002)
1	c.780_781delGC	p.Leu260fs	(Toro et al. 2003)
1	c.782_788del7bp	p.Pro261fs	(Toro et al. 2003; Matyakhina et al. 2005)
1	c.799A>T	p.Asn267Lys	(Alam et al. 2005)
2	c.823C>T	p.His275Tyr	(Toro et al. 2003; Smit et al. 2011)
1	c.836T>A	p.Val279Asp	(Toro et al. 2003)
1	c.859A>C	p.Thr287Pro	(Chuang et al. 2006)
1	c.869G>A	p.Cys290Lys	(Makino et al. 2007)
1	c.873T>G	p.Ser291Arg	(Smit et al. 2011)
1	c.875T>C	p.Leu292Pro	(Toro et al. 2003)
2	c.891T>A	p.Asn297Asp	(Toro et al. 2003; Wei et al. 2006)
1	c.898C>T	p.Arg300X	(Tomlinson et al. 2002)

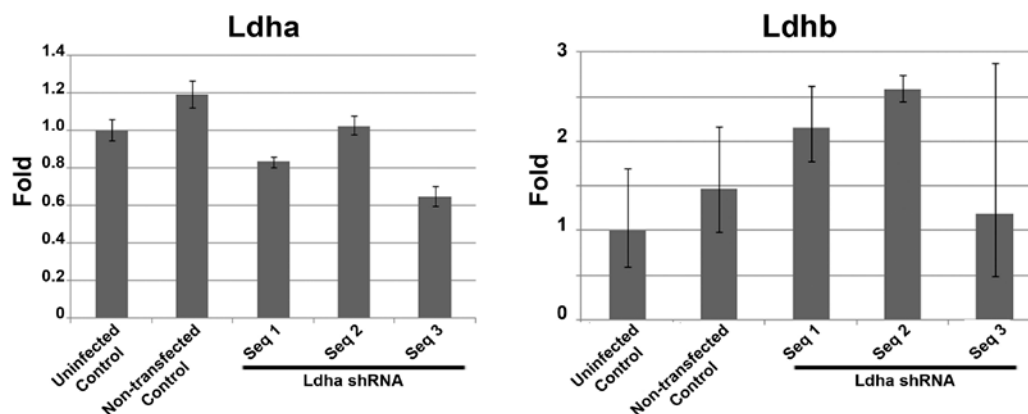
4	c.905-1G>A	Splice Site	(Chuang et al. 2005)
2	c.934G>A	p.Glu312Lys	(Alam et al. 2003)
1	c.1081_1083del4bp	p.Asn318fs	(Martinez-Mir et al. 2003)
1	c.954T>A	p.Asn318Lys	(Alam et al. 2003)
2	c.964A>G	p.Ser322Gly	(Toro et al. 2003; Wei et al. 2006)
2	c.968G>A	p.Ser323Asn	(Alam et al. 2003; Toro et al. 2003)
1	c.989A>G	p.Asn330Ser	(Lehtonen et al. 2007)
1	c.1002T>G	p.Ser334Arg	(Badeloe et al. 2008)
1	c.1004ins2bp	p.Glu335fs	(Toro et al. 2003)
1	c.1015A>G	p.Met339Val	(Smit et al. 2011)
1	c.1025C>A	p.Ala342Asp	(Wei et al. 2006)
1	c.1051G>C	p.Val351Leu	(Martinez-Mir et al. 2003)
8	c.1060G>A	p.Gly354Arg	(Alam et al. 2003; Badeloe et al. 2008; Vahteristo et al. 2010; Smit et al. 2011)
1	c.1081G>T	p.Glu361X	(Smit et al. 2011)
1	c.1103T>C	p.Met368Thr	(Badeloe et al. 2008)
1	c.1105del	p.Met369X	(Smit et al. 2011)
1	c.1126T>A	p.Ser376Pro	(Wei et al. 2006)
1	c.1162delA	p.Thr388Fs	(Toro et al. 2003)
1	c.1187A>C	p.Gln396Pro	(Wei et al. 2006)
1	c.1218delG	p.Ile406fs	(Alam et al. 2005)
1	c.1210G>T	p.Glu404X	(Badeloe et al. 2006)
1	c.1238delA	p.Ile413fs	(Badeloe et al. 2006)
2	c.1265A>G	p.Lys422Cys	(Toro et al. 2003)

1	c.1271A>G	p.Lys424Arg	(Ylisaukko-oja et al. 2006)
1	c.1300- 1307del8bpdup	p.Ser437fs	(Toro et al. 2003)
1	c.1328C>A	p.Ala443Asp	(Onder et al. 2010)
1	c.1339delG	p.Gly447fs	(Toro et al. 2003)
1	c.1340delG	p.Gly447fs	(Pithukpakorn et al. 2006)
1	c.1346-1347delTC	p.Leu449fs	(Pithukpakorn et al. 2006)
1	c.1391T>C	p.Leu464Pro	(Alam et al. 2003)

7.2 Chapter 2

**Appendix Figure 2.1 Confirmation that Fh1^{fl/+} MEFs are similar to Fh1^{+/+} MEFs**

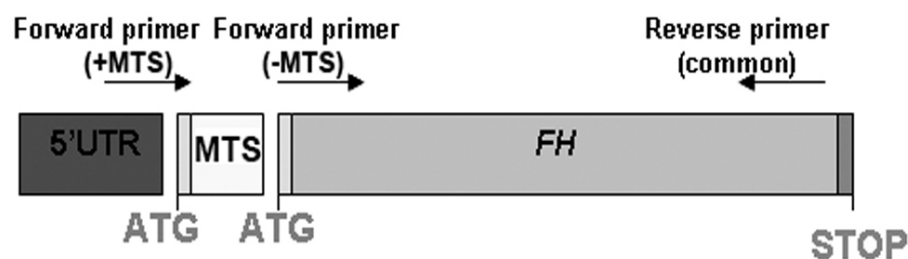
Immunoblot analysis illustrating that Fh1^{fl/+} MEFs are similar to Fh1^{+/+} MEFs with respect to protein levels of phospho-p70-S6 kinase (pS6K), p53, p21 and p16. β -actin and histone H3 were included as loading controls. Late passage (~passage 25) primary Fh1^{-/-} MEFs are shown to be positive for senescence. For this reason immortalised rather than primary MEFs were utilised for long term analysis.



Appendix Figure 2.2 Ldh isozyeme expression of Ldha and Ldhb

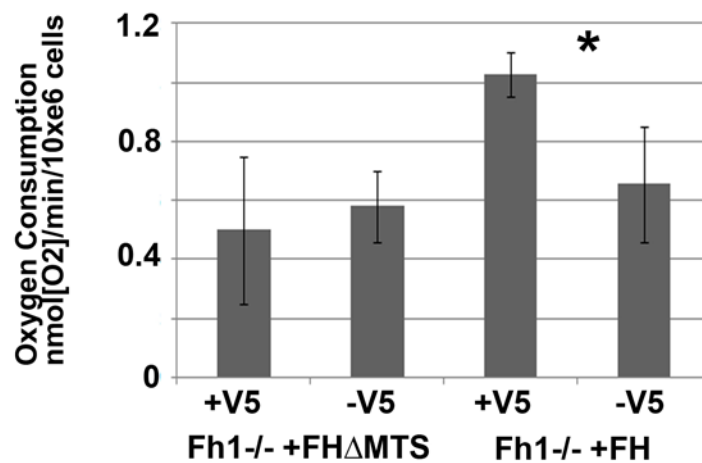
Q-PCR analysis of mRNA expression of Ldh isozyemes Ldha and Ldhb in $Fh1^{-/-}$ MEFs following treatment with Ldha shRNA sequences 1-3 and Non-targeting shRNA. Ldhb mRNA expression is below detection.

7.3 Chapter 3



Appendix Figure 3.1 Schematic of the FH or FHΔMTS transgene

Cartoon illustrating the strategy for generation of the transgenes encoding either a full length or an FH sequence lacking the amino-terminal MTS (FHΔMTS).



Appendix Figure 3.2 Oxygen consumption measured in Fh1^{-/-} +FH and Fh1^{-/-} +FHΔMTS MEFs

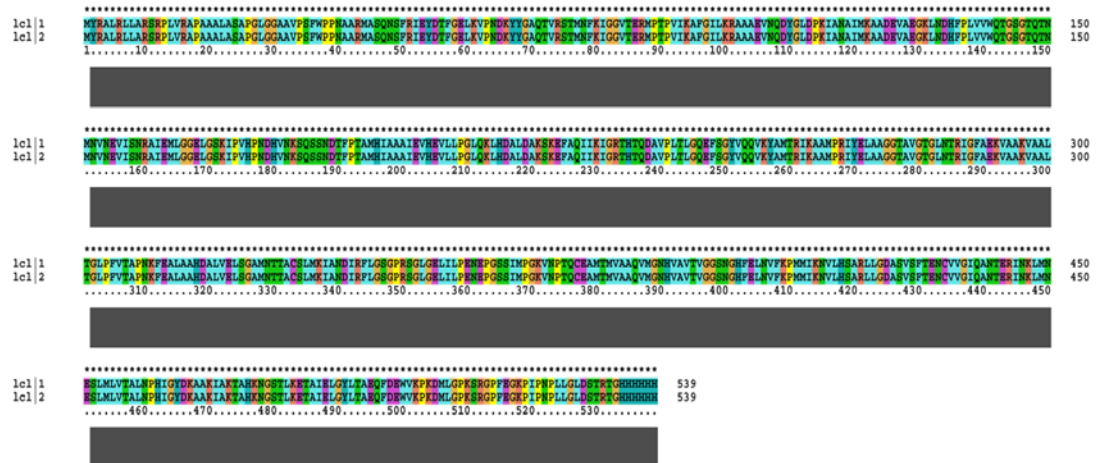
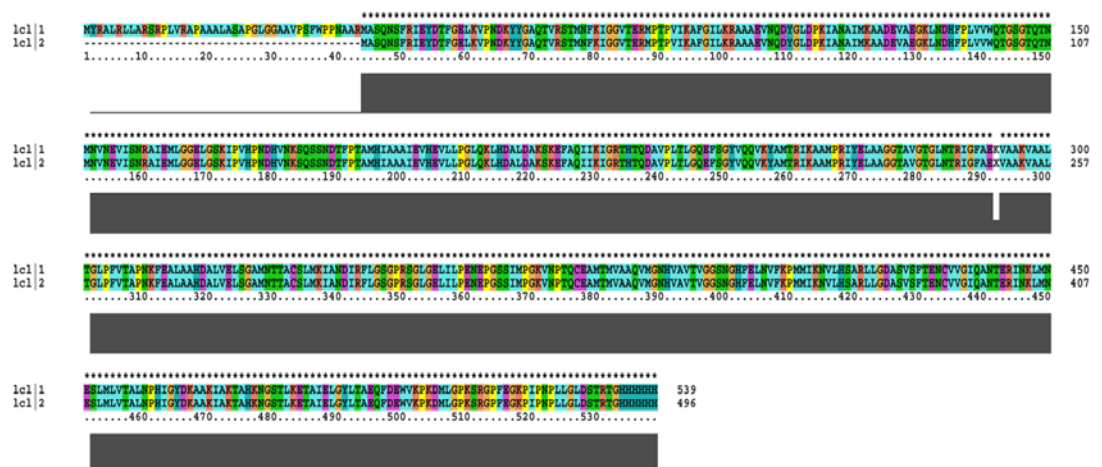
Oxygen consumption measured in Fh1^{-/-} +FH and Fh1^{-/-} +FHΔMTS MEFs with and without the V5-tag showed no significant difference between the presence or absence of V5. Data shown is mean+S.E. of duplicate assays and * p>0.1.

Fh1+/+ vs. Fh1-/-						
Treatment	Fh1+/+	Fh1-/-	Difference	95% CI of diff.	t	P value
Basal	1.03	0.187	-0.847	-1.34 to -0.352	5.74	P < 0.0001
Glutamate	1.08	0.143	-0.94	-1.43 to -0.446	6.37	P < 0.0001
Oligomycin	0.24	0.18	-0.06	-0.554 to 0.434	0.407	P > 0.05
FCCP	1.5	0.583	-0.917	-1.41 to -0.422	6.21	P < 0.0001
Fh1+/+ vs. Fh1-/-+FH						
Treatment	Fh1+/+	Fh1-/-+FH	Difference	95% CI of diff.	t	P value
Basal	1.03	0.667	-0.367	-0.861 to 0.128	2.48	P > 0.05
Glutamate	1.08	0.717	-0.367	-0.861 to 0.128	2.48	P > 0.05
Oligomycin	0.24	0.343	0.103	-0.391 to 0.598	0.7	P > 0.05
FCCP	1.5	1.33	-0.167	-0.661 to 0.328	1.13	P > 0.05
Fh1+/+ vs. Fh1-/-+FHΔMTS						
Treatment	Fh1+/+	Fh1-/-+FHΔMTS	Difference	95% CI of diff.	t	P value
Basal	1.03	0.39	-0.643	-1.14 to -0.149	4.36	P < 0.01
Glutamate	1.08	0.297	-0.787	-1.28 to -0.292	5.33	P < 0.001
Oligomycin	0.24	0.247	0.00667	-0.488 to 0.501	0.0452	P > 0.05
FCCP	1.5	0.76	-0.74	-1.23 to -0.246	5.01	P < 0.001
Fh1-/- vs. Fh1-/-+FH						
Treatment	Fh1-/-	Fh1-/-+FH	Difference	95% CI of diff.	t	P value
Basal	0.187	0.667	0.48	-0.0144 to 0.974	3.25	P > 0.05
Glutamate	0.143	0.717	0.573	0.0789 to 1.07	3.88	P < 0.05
Oligomycin	0.18	0.343	0.163	-0.331 to 0.658	1.11	P > 0.05
FCCP	0.583	1.33	0.75	0.256 to 1.24	5.08	P < 0.001
Fh1-/- vs. Fh1-/-+FHΔMTS						
Treatment	Fh1-/-	Fh1-/-+FHΔMTS	Difference	95% CI of diff.	t	P value
Basal	0.187	0.39	0.203	-0.291 to 0.698	1.38	P > 0.05
Glutamate	0.143	0.297	0.153	-0.341 to 0.648	1.04	P > 0.05
Oligomycin	0.18	0.247	0.0667	-0.428 to 0.561	0.452	P > 0.05
FCCP	0.583	0.76	0.177	-0.318 to 0.671	1.2	P > 0.05
Fh1-/-+FH vs. Fh1-/-+FHΔMTS						
Treatment	Fh1-/-+FH	Fh1-/-+FHΔMTS	Difference	95% CI of diff.	t	P value
Basal	0.667	0.39	-0.277	-0.771 to 0.218	1.87	P > 0.05
Glutamate	0.717	0.297	-0.42	-0.914 to 0.0744	2.85	P > 0.05
Oligomycin	0.343	0.247	-0.0967	-0.591 to 0.398	0.655	P > 0.05
FCCP	1.33	0.76	-0.573	-1.07 to -0.0789	3.88	P < 0.05

Appendix Figure 3.3 Two way ANOVA analysis of oxygen consumption measurements in Fh1 MEFs

Two way ANOVA statistical analysis of the oxygen consumption data generated from the Fh1 MEFs under basal (unstimulated) conditions and following treatment with glutamate, oligomycin and FCCP, as outlined in Table 3.1 and chapter 3, section 3.2.3.

7.4 Chapter 6

A**B****Appendix Figure 6.1 Sequence alignment for FH and FHΔMTS transgenes**

Clustal 2.0.12 Multiple Sequence Alignment for (A) FH sequence and (B) FHΔMTS sequence.

7.5 *List of publications resulting from/associated with the work of this thesis*

O'Flaherty, L., J. Adam, L.C. Heather, A.V. Zhdanov, Y.L. Chung, M.X. Miranda, J. Croft, S. Olpin, K. Clarke, C.W. Pugh, J. Griffiths, D. Papkovsky, H. Ashrafian, P.J. Ratcliffe, and P.J. Pollard. **Hum Mol Genet**, 2010. **19**(19): p. 3844-51.

Ashrafian, H*, **O'Flaherty L***, Adam J., Steeples V., Chung Y.L., East P., Vanharanta S., Lehtonen H., Nye E., Hatipoglu E., Miranda M., Howarth K., Shukla D., Troy H., Griffiths J., Spencer-Dene B., Yusuf M., Volpi E., Maxwell P.H., Stamp G., Poulsom R., Pugh C.W., Costa B., Bardella C., Di Renzo M.F., Kotlikoff M.I., Launonen V., Aaltonen L., El-Bahrawy M., Tomlinson I., and Pollard P.J. **Cancer Res**, 2010. **70**(22): p. 9153-65. ***Both authors contributed equally**

J. Adam, Hatipoglu E*, **O'Flaherty L***, Ternette N, Sahgal N, Lockstone H, Baban D, Nye E, Stamp GW, Wolhuter K, Stevens M, Fischer R, Carmeliet P, Maxwell PH, Pugh CW, Frizzell N, Soga T, Kessler BM, El-Bahrawy M, Ratcliffe PJ, Pollard PJ. **Cancer Cell**, 2011. **20**(4):p524-37. ***Both authors contributed equally**

AV. Zhdanov, Favre C, **O'Flaherty L**, Adam J, O'Connor R, Pollard PJ, Papkovsky DB. **Integr Biol (Camb)**, 2011. **3**(11):p1135-42.

C. Bardella, El-Bahrawy M, Frizzell N, Adam J, Ternette N, Hatipoglu E, Howarth K, **O'Flaherty L**, Roberts I, Turner G, Taylor J, Giaslakitidis K, Macaulay VM, Harris AL, Chandra A, Lehtonen HJ, Launonen V, Aaltonen LA, Pugh CW, Mihai R, Trudgian D, Kessler B, Baynes JW, Ratcliffe PJ, Tomlinson IP, Pollard PJ. **J Pathol**, 2011. **225**(1):p.4-11.

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