The role(s) of hypoxia-inducible factors (HIFs) in the development of renal cysts in mice deficient in *fumarate hydratase (Fh1)*

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Abstract

Fumarate hydratase (FH) encodes a Krebs cycle (also called TCA cycle) enzyme which converts fumarate to malate and also functions as a tumour suppressor gene. Germline mutations in *FH* predispose to hereditary leiomyomatosis and renal cell cancer (HLRCC). Affected individuals develop benign smooth muscle tumours (leiomyomas) of the skin and uterus and renal cysts and cancers; specifically type II papillary and collecting duct carcinomas. Kidney-specific deletion of *Fh1* (the murine orthologue of *FH*) recapitulates HLRCC in part and mice develop renal cysts that are proliferative and express constitutively hypoxia inducible factors -1α and -2α (*Hif-1α* and *Hif-2α*). This phenomenon of pseudo-hypoxia (HIF expression in normal oxygen tension) is also evident in cultured cells deficient in FH and is proposed as a possible mechanism for initiation and/or progression of carcinogenesis. We have utilised our mouse model to investigate the role of *Hif-1α* and *Hif-2α* and *Fh1* in renal cyst development and show remarkably that combined genetic inactivation of *Hif-1α* and *Fh1* increased both cyst size and frequency. However, kidneys deficient for both *Fh1* and *Hif-2α* were comparable to those deficient for *Fh1*. Immunohistochemical analyses show that *Fh1* inactivation leads to increased proliferation, DNA damage and affects the cell cycle in the renal cysts. *Hif-1α* and *Hif-2α* inactivation is not shown to contribute to these. Through genome-wide mRNA expression analysis we have identified that *Ccnd1* is significantly upregulated (or the most significantly upregulated gene) in *Fh1/Hif-1α* knockout kidneys compared to wild type and *Fh1* knockout kidneys. Other upregulated genes in the double knockout kidneys include genes involved in cell cycle, proliferation and structural organization of the cell.

Dedication

I would like to dedicate my thesis to my dear parents Nurcihan and Ali Hatipoglu and my brother Fehim. I would like to thank them for giving me the inspiration and the opportunity to pursue my passion in science and for their unconditional love. This thesis would not have been possible without their support, encouragement and loving care. I also dedicate this thesis to my grandfather whom I miss and love very dearly.

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Abbreviations

HLRCC - Hereditary Leiomyomatosis and Renal Cell Cancer

HPGL - Hereditary pheochromacytomas and paragangliomas

TCAC - Tricarboxylic acid cycle (Krebs cycle)

PGL - Paraganglioma

RCC - Renal Cell Carcinoma

FH - Fumarate hydratase

SDHx - Succinate Dehydrogenase, x = A,B,C or D subunit

BHD - Birt-Hogg-Dube syndrome

VHL - von Hippel-Lindau disease

VEGF - Vascular endothelial cell growth factor

BNIP3 - BCL2/adenovirus E1B 19kDa interacting protein 3

HIF-1α/β- Hypoxia Inducible Factor 1 – alpha subunit / beta subunit

ARNT - Aryl hydrocarbon receptor nuclear translocator

EPAS1 - endothelial PAS domain protein 1 (also called HIF-2α)

IHC - Immunohistochemistry

FIH1 - Factor inhibiting HIF1

ODDD - Oxygen-dependent degradation domain

EPO - Erythropoietin

ROS - Reactive oxygen species

mtDNA - Mitochondrial DNA

ATP - Adenosine tri-phosphate

PHD - Prolyl hydroxylase enzyme

mTOR - mammalian target of rapomycin

PI3K - phosphoatidylinositol 3-kinase

SOD - superoxide dismutase

NTAD- N terminal transactivation domain

CTAD- C-terminal transactivation domain

1. INTRODUCTION

1.1. Renal Development and Physiology

Development of the kidney starts at the intermediate mesoderm in the vertebrates. As the embryo develops, the intermediate mesoderm forms three subparts called pronephros, mesonephros and metanephros. The pronephros stage is when the basic tubule development of the kidney occurs. As embryonic development progresses, the pronephros is replaced by the mesonephros which allows for development of the glomeruli. Also, the tubules gain a more complex structure and start looking more like proximal tubules. Mesonephros then develops into metanephros. The metaphros consists of two main structures called the ureteric bud and metanephric mesenchyme. These structures then form the nephron of the kidney which is the renal functional unit (Rumballe et al., 2010). The nephron has two units: the renal corpuscle and the renal tubule. The renal corpuscle consists of Bowman's capsule and the glomerulus. These two structures are responsible for plasma filtration. The renal tubule is the main site for reabsorption of ions, water and various other molecules. The proximal convoluted tubule, loop of Henle, distal convoluted tubule and the collecting duct are the main substructures of the renal tubule. The proximal convoluted tubule consists of simple cuboidal epithelium and a brush border with microvilli. It plays a vital role in renal function and maintaining body physiology by reabsorbing 65% of the glomerular filtrate. Na+/K^+-ATPase (the sodium-potassium pump) allows the proximal convoluted tubule to reabsorb Na^+, glucose and amino acids. The distal convoluted tubule is another important part of the kidneys. Its main function is in maintaining the acid- base balance through H^+ and K^+ secretion. This balance is regulated by aldosterone that is secreted from the adrenal cortex. The kidney is also critical in regulation of the renin-angiotensin system in the body (Young, 2003).

1.2. Renal cancers and cysts

Cysts and malignant tumours are two important and commonly seen pathologies of the kidney. Both of these diseases can be subdivided into different categories based on the presentation and molecular mechanism involved in the pathology (Wilson, 2004, Bisceglia et al., 2006).

Although presentation of cysts can be evidence of different clinical forms of renal cystic disease, the biological events which lead to the formation of cysts are similar. It has been suggested that the sequence of events leading to cyst formation are as follows: proliferation in the renal tubular epithelium, fluid accumulation in the sac which is formed as a result of increased proliferation of the epithelium and rearrangement of the extracellular matrix surrounding the cyst. In addition to increased proliferation, epithelial cells in the kidney also show general morphological changes. In the proximal tubule, the epithelial cells lose their apical brush border (Martinez and Grantham, 1995). It has also been shown that the epithelial lining of the renal cysts shows increased expression levels of apical Na^+/K^+-ATPase, which is also known to be a characteristic of undifferentiated renal epithelial cells. An increased number of Na^+/K^+-ATPase pumps allow more chloride (Cl^-) into the cysts. The interstitial cells of the cysts also become less sensitive to vasopressin and the epithelial cells start showing secretory characteristics. Therefore, this change in the apical Na^+/K^+-ATPase pump and other changes described above are important mechanisms leading to fluid accumulation in the cysts (Martinez and Grantham, 1995, Avner et al., 1992).

One of the most common causes of cysts in the kidney is polycystic kidney disease (PKD). PKD can be inherited or acquired at later stages of life. The inherited form of the disease can present in two ways: autosomal dominant PKD (ADPKD) and autosomal recessive PKD (ARPKD) (Martinez and Grantham, 1995). ADPKD has a prevalence of 1 in 1000. Patients develop multiple cysts in both kidneys. Some of ADPKD patients develop cysts in other organs such as the liver, pancreas and spleen.

The disease follows a progressive pattern and causes complications such as hypertension, aneurysms and mitral valve prolapse at later stages of life. ADPKD is caused by mutations in *PDK1* and *PDK2* genes which are located at 16p13.3 and 4q21-23 and code for the polycystin 1 and polycystin 2 proteins respectively (Wu and Somlo, 2000). ARPKD is seen less frequently and has a prevalence of 1 in 20,000. The gene most commonly involved in ARPKD is different from the gene involved in ADPKD and is called polycystic kidney and hepatic disease gene *(PKHD1). PKHD1* is located on chromosome 6 and codes for the polyductin protein which is also called fibrocystin. In addition to developing renal cysts, ARPKD patients also develop cysts in the liver. ARPKD presents at an earlier age (i.e. infancy and childhood) compared to ADPKD and complications of the disease include hypertension, lung hypoplasia and liver disease. Examples of other inherited cystic kidney diseases include micro cystic kidney disease and tuberous sclerosis complex. There are also a number of sporadic cystic kidney diseases such as unilateral renal cystic disease, simple renal cysts, multicystic dysplastic kidney and glomerulocystic kidney disease (Bisceglia et al., 2006).

Renal cell cancer (RCC) is a common genitourinary cancer which is more common in men than in women. Studies show that there has been a significant increase in the number of people who have developed RCC in the past years. For instance, since the 1950s the prevalence of the disease has increased 126% in the USA, an increase that has been accompanied by a significant increase in the number of deaths due to RCC (Pantuck et al., 2003). RCC can be divided into four different types based on histological differences: papillary cancer, clear cell cancer (conventional or non-papillary cancer) (ccRCC), collecting duct cancer (Bellini's duct carcinoma) and chromophobe cancer. ccRCC, for which the histogenetic tissue of origin is the distal tubule of the kidney, is the most common type of renal cancer and constitutes 60% of all renal cancers (Kaelin, 2007b, Yan et al., 2009). Papillary renal cell cancer, which is the second most common type of renal cancer, with an incidence of 10-15%, arises

from the proximal tubules of the kidney. There are two different types of papillary renal cell cancer: Type 1 and Type 2. Both clear cell renal cancer and papillary renal cancer can present in sporadic or inherited forms (Yan et al., 2009). Although many different and complex factors are involved in sporadic renal cancers, hereditary types develop due to germline mutations in specific genes. ccRCC is caused by germline mutations in von Hippel Lindau (*VHL*) tumour suppressor gene, located on chromosome 3p25. Somatic mutations in *VHL* are also very common and important in ccRCC. Mutations in the proto-oncogene *c-MET* are associated with type1 papillary cancer whereas germline mutations in *Fumarate hydratase* (*FH*) predispose individuals to Type 2 papillary cancer. *FH* is located on 1q42-44 and codes for the fumarate hydratase enzyme involved in the Krebs cycle where it converts fumarate to malate. Type 2 papillary cancers harbouring *FH* mutations are a component of an inherited syndrome called “hereditary leiomyomatosis and renal cell cancer” (HLRCC). Therefore, inherited type 2 papillary cancer presents as a part of HLRCC. Collecting duct carcinoma and chromophobe cancer are very aggressive cancers but the aetiology of them is not clear (Pantuck et al., 2003, Garcia et al., 2009, Yan et al., 2009, Kaelin, 2007b). It has been suggested that the level of carbonic anhydrase 9 (CA9) and vascular endothelial cell growth factor (VEGF) are important diagnostic and prognostic markers in different types of renal cell cancers. The most important similarity between those two markers is that both of them are hypoxia regulated genes (McGuire and Fitzpatrick, 2009). Birt-Hogg-Dubé *(BDH)* is a syndrome which is inherited in an autosomal dominant manner (Toro et al., 1999). Mutations in the *BDH* gene which is located on chromosome 17p11.2 and codes for folliculin were shown to cause this syndrome. In addition to renal tumours, BDH patients can also present with pulmonary cysts, cutaneous fibrofolliculomas and pneumothoraces (Zbar et al., 2002). Different types of renal tumours are seen in BDH syndrome, most commonly hybrid tumours. Approximately 30% of the tumours are chromophobe renal cancers and the least common type is oncocytomas (Rosner et al., 2009).

Renal cysts are also associated with renal cancer. Cystic presentation in renal malignancies can be in four main ways. The cysts can be multilobular or can present as a single lobule. Renal cancers can also arise from a necrotic cyst or any existing cyst in the kidney. Single lobular cysts are seen mainly in the papillary renal cancers (Bisceglia et al., 2006).

1.3. Brief introduction to hypoxia and metabolism

The presence of adequate oxygen levels is vital for the viability of aerobic organisms. The normal oxygen concentration in tissues lies between 10-30 μM. The condition where the oxygen level is suboptimal is called hypoxia. Decreased levels of oxygen can be detected by organisms and the human body through different oxygen detection mechanisms. In mammals, chemoreceptors play a crucial role in the detection of hypoxia. These chemoreceptors are located in the carotid body and neuroepithelial body which detect the levels of oxygen in the respiratory system and blood. Potassium channels are inhibited in response to detection of hypoxia by the chemoreceptors. This activates certain neurological pathways which control the cardiovascular and respiratory system and cause physiological changes in the cardiac and respiratory functions to allow the body to survive under hypoxic conditions. Under acute hypoxic conditions, the cardiac output is increased, causing vasodilation of systemic arteries and the vasoconstriction of the pulmonary arteries to take place. Chronic hypoxia leads to hypertrophy of the heart, angiogenesis and changes in the arterial structure of the pulmonary system (Kaelin and Ratcliffe, 2008, Semenza, 1999, Smith et al., 2008). Experiments conducted on healthy volunteers by exposing them to hypoxia at high altitude show that ninety minutes after staying under hypoxic conditions the erythropoietin (Epo) level in the body is elevated. However, the body adapts to hypoxia within one to two weeks and the levels of Epo go back to the normal values detected at sea level. In contrast to the immediate effect seen with the Epo levels at

high altitude, haemoglobin and heamotocrit levels start to increase only after several days of exposure to hypoxia. Volunteers exposed to hypoxia at an altitude of 4500 m show a significant increase in the red cell count which can increase up to 50% compared to normal levels at sea level. This increase in the red cell count can be maintained for up to several months during exposure to hypoxia. In addition to the haematological adaptive changes, hypoxia also has effects on the respiratory system as mentioned above. Hypoxia causes an immediate elevation in the minute ventilation rate (rate of exhalation of gas from the lungs per minute). This allows the oxygen levels in the blood to be kept at a physiological level. However, the increased ventilation rate then decreases to normal levels a few minutes or hours after staying in hypoxic conditions (Smith et al., 2008).

The systemic changes described above that occur in response to hypoxia are due to the oxygen detection and hypoxic responses occurring at the cellular level. Hypoxia inducible factors (HIF) are the key elements in oxygen sensing and hypoxia signalling at a molecular level (Semenza, 1999). HIFs HIF are transcription factors which regulate gene expression by binding to DNA. HIF is a heterodimer protein which has two different subunits: α and β. The α- subunit is an unstable one whereas the β-subunit is stable. Three types of HIF-α protein are expressed: HIF-1α, HIF-2α and HIF-3α. Expression of HIF-1α is ubiquitous. On the other hand, HIF-2α is expressed in a tissue specific manner. The HIF-1α subunit is 120 kDa in size while the HIF-β subunit is 91-94 kDa (Semenza, 2003). The β subunit of HIF, which is also referred to as Aryl Hydrocarbon Nuclear Translocators (ARNT), has three different forms as well. Both HIF-α and HIF-β have two transactivation domains which are important for transcriptional regulation of HIF target genes (Kaelin, 2005) (this will be discussed in more detail in the following parts of the thesis). These transactivation domains are called N terminal transactivation domain (NTAD) and C terminal transactivation domain. Initiation of transcription by HIF takes place when HIFα-β complex binds to the

part of the DNA reading G/ACGTG which is a part of the hypoxia response elements (HREs) of HIF target genes (Masson and Ratcliffe, 2003). HIF-α has a more important role in detection of oxygen levels in the cell compared to HIF-β which is more important in regulating gene expression. Among the HIF-α proteins, it is known that the HIF-1α and HIF-2α are more sensitive to cellular oxygen levels than HIF-3α is (Schofield and Ratcliffe, 2004). Furthermore, less is known about the function and regulation of HIF-3α. When oxygen is present in the cell, HIF is broken down since expression of HIF is upregulated under hypoxic conditions (Ratcliffe, 2006, Kaelin, 2005, Schofield and Ratcliffe, 2004, Masson and Ratcliffe, 2003, Palmer et al., 2000). Degradation of HIF under normoxia is mediated through hydroxylation of HIF domains by prolyl-4 hydroxylase enzymes (PHD). There are three PHD isoenzymes: PHD1, PHD2 and PHD3; also known as HPH3 or EGLN2 (PHD1), HPH2 or EGLN1 (PHD2); and HPH1 or EGLN3 (PHD3) (Epstein et al., 2001). PHD enzymes hydroxylate the HIF proteins at conserved proline and asparagine residues. The domains where proline hydroxylation takes place are called the N- and C -terminal oxygen dependent degradation domains (NODDD and CODDD, respectively). PHDs use dioxygen to mediate the proline hydroxylation reaction. In this reaction, HIF-1α receives one oxygen atom at the prolyl residue. The other oxygen atom is used to produce succinate from 2-oxoglutarate (2-OG). On the other hand, hydroxylation of asparagine takes place at the C-terminal transactivation domain (CTAD). Factor inhibiting HIF (FIH) is responsible for catalysing this reaction (Mole et al., 2003, Masson et al., 2001, Masson and Ratcliffe, 2003, Dann and Bruick, 2005). Hydroxylation of asparagine residues by FIH, then prevents p300/CBP binding which is important in the initiation of transcription. Therefore, FIH is important in the inhibition of transcription. Similar to the hydroxylation of proline, the asparagine hydroxylation utilizes two oxygen atoms from 2-OG. Both of the reactions, which are 2-OG dependent, use ascorbate and Fe(II) as cofactors (Cockman et al., 2009, Mole et al., 2002, Ratcliffe, 2007, Fedele et al., 2002, Maxwell et al., 1999).

Degradation of HIF-α is mediated through hydroxylation of HIF-α at the NODD and CODD sites. Prolyl hydroxylation of the protein allows the von Hippel-Lindau tumour suppressor E3 ubiquitin ligase (VHLE3) to recognize HIF-α for degradation. VHL protein (pVHL), which is a part of VHLE3, recognizes the hydroxylated proline residue (P564) in the HIFα protein (Masson et al., 2001, Mole et al., 2002). *VHL* is a tumour suppressor gene and mutations in the *VHL* cause VHL disease. Patients with VHL disease are susceptible to developing ccRCC, hemangioblastoma and pheochromocytomas. There are two main forms of ccRCC: Type1 and Type 2B both of which present in the distal tubules of the kidney. The disease can also initially present with renal cysts. VHL mutations in ccRCC cause both HIF stabilization and upregulation of HIF target genes such as vascular endothelial cell growth factor (*VEGF*), platelet derived growth factor (*PDGF*) and transforming growth factor alpha (*TGFα)* (Kaelin, 2007a, Clark, 2009, Cowey and Rathmell, 2009).

As described above, degradation of HIF-α does not take place in hypoxia due to inhibition of prolyl hydroxylation. This leads to stabilization of HIF. However, hypoxia is not the only factor which leads to HIF-α stabilization. HIF can also be stabilised in response to hormones, growth factors and other cellular molecules under normoxic conditions. Nitric oxide (NO) synthesized by nitric oxide synthase has many physiologically important roles such as vasodilatation and cyclic GMP production in the neurons (Sogawa et al., 1998). NO induces HIF-α stabilization under normoxic conditions. It is suggested that NO causes HIF-α stabilization by preventing the recognition of the protein by pVHL. This is believed to happen through the inhibition of hydroxylation of the PHD enzymes by competing with oxygen to bind the hydroxylation sites of the PHD enzyme (Metzen et al., 2003). However, the mechanism by which NO has an effect on HIF-α is still not clear. Some studies suggest that NO causes HIF-α stabilization in a dose dependent manner (Metzen et al., 2003). A faster HIF-α stabilization effect is seen under low NO concentrations compared to higher levels of

NO. Some experiments also imply that inhibition of HIF-α degradation by NO is part of an inflammatory response mediated by NO (Metzen et al., 2003, Brune et al., 2001, Wang et al., 2002, Sandau et al., 2001). Reactive oxygen species (ROS) produced by the mitochondria is also an important oxygen sensing tool in the cell. Although the mechanism behind this process is not completely clear, studies done on the subject provide useful data. Increased levels of ROS in the cell, even under normoxic conditions, lead to stabilization of HIF-α. However, whether ROS interferes with hydroxylation of PHD enzymes to prevent HIF degradation is not very clear. Although ROS is important in the hypoxic response of the cell, it has been shown that the oxidative phosphorylation does not play a role in HIF-α stabilization. In addition, it has also been shown that mitochondria are important in the detection of hypoxia, but are not very sensitive to anoxia. Under hypoxic conditions, complex III of the electron transport chain leads to ROS production which mediates detection of hypoxia and HIF stabilization. Instead, PHD enzymes are shown to be responsible for detection of anoxia (Brunelle et al., 2005, Guzy et al., 2005, Mansfield et al., 2005).

The viability of a cell and organism depends on several factors. As discussed above, oxygen is one of them. In addition to oxygen, the cell also needs energy to be able serve its functions. The compartment of the cell responsible for generation of energy in the cell is the mitochondrion. The series of events and cycles that lead to energy production and biosynthesis is called metabolism. Examples of these pathways include Krebs cycle (also called TCA cycle), pentose phosphate pathway and glutamine pathway. Details of the TCA cycle are illustrated in **Figure 1**. Similar to other biochemical events in the cell, metabolism needs to follow a balance since a high or low metabolic rate in the cell can have pathological consequences. Oxidative phosphorylation and glycolysis constitute important parts of metabolism and are pathways which lead to adenosine 5´-triphosphate (ATP) production. Details of glycolysis are illustrated in **Figure 2**. Under normoxic conditions, glucose is converted

into pyruvate which then enters the TCA cycle in the mitochondria. This results in the production of nicotinamide adenine dinucleotide (NADH). NADH is then used in oxidative phosphorylation for ATP production. Oxidative phosphorylation yields 18 ATPs whereas glycolysis produces only two ATPs. Therefore, oxidative phosphorylation is a more efficient means of energy production. Under hypoxic conditions, the metabolic pathway gets altered and pyruvate is converted into lactate instead. Cancer cells have increased glycolysis and use the lactic acid pathway rather than oxidative phosphorylation under normoxic conditions, therefore named aerobic glycolysis. This phenomenon is also called the Warburg effect which was discovered by Otto Warburg in 1924 (Kondoh et al., 2005, Vander Heiden et al., 2009, Dang and Semenza, 1999, DeBerardinis et al., 2008, Gottlieb and Tomlinson, 2005, Warburg, 1930, Warburg, 1956).

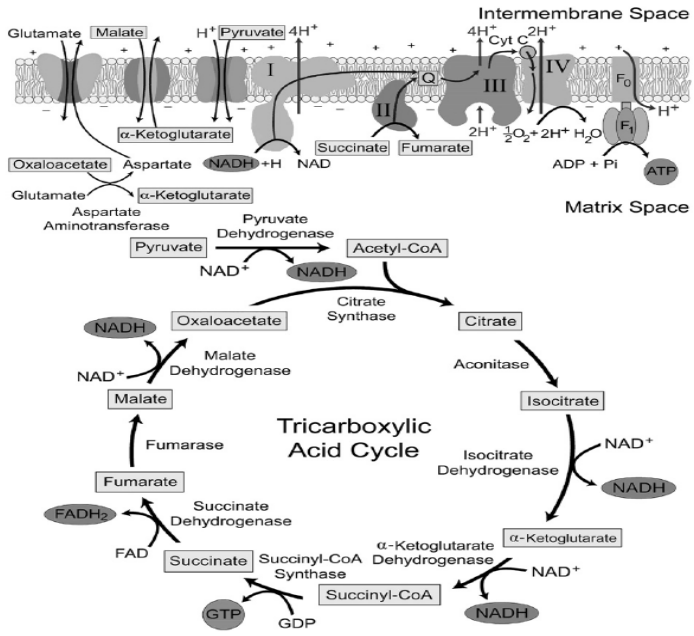


Figure 1. TCA cycle

Details of the TCA cycle is illustrated. The diagram is taken from (Gunter et al., 2010).

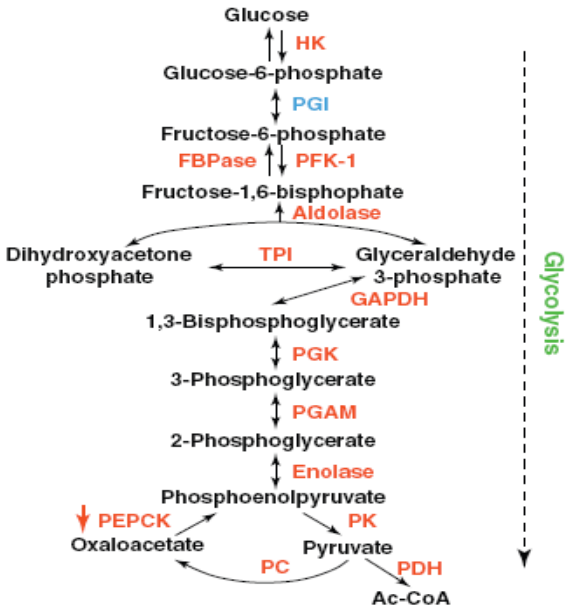


Figure 2. Glycolysis

Steps involved in glycolysis is illustrated. Figure taken from (Guan and Xiong, 2010)

(HK: Hexokinase, PGI: Phosphoglucose isomerase, PFK: Phosphofructokinase, TPI: Triose phosphate isomerase, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, PGK: Phosphoglycerate kinase, PGAM: Phosphoglycerate mutase, PK: Pyruvate kinase)

1.4. Relation between hypoxia and metabolism

The relationship between hypoxia and metabolism is a complex concept and studies show that as well as hypoxia having a regulatory effect on metabolism, metabolism can also lead to hypoxic responses (Ratcliffe, 2007). The effect of hypoxia on metabolic enzyme gene expression metabolism is mediated by HIF-α, mainly by HIF-1α. Changes in metabolism due to hypoxia are seen both as physiological responses, such as the production of lactic acid in the muscles while exercising due to switching to anaerobic metabolism in response hypoxic conditions in the muscle, and as part of a pathological mechanism. Tumours have reduced oxygen levels. As the size of a tumour increases, the cells get further away from the blood vessels which are essential oxygen supplies. Therefore, the hypoxia-metabolism relationship is important

at a pathological level in tumours. When the tumour gets hypoxic, HIF-α is stabilized as a consequence. Constitutive HIF-α expression leads to the increased expression of the glucose transporters, *GLUT1* and *GLUT2* (Denko, 2008). In addition, expression of enzymes involved in glycolysis is also upregulated by HIF. This is important in terms of maintaining the high glycolytic rate in the tumour cells and providing them with the required amount of ATP production (Brahimi-Horn et al., 2007, Denko, 2008, Kelly et al., 2008). In addition to glucose transporters, *hexokinase* (*HK*), *lactate dehydrogenase A* (*LDHA*) and *pyruvate dehydrogenase kinase* (*PDK*) which are also involved in glycolysis are also regulated by HIF (Semenza, 2010). Details of this will be discussed later.

One of the first and most important studies which shed light onto the effect of metabolism on induction of HIF and pseudohypoxia was undertaken by Gimenez-Roqueplo et al., (2001) on hereditary paragangliomas which are benign tumours of the nervous system with neuroectodermal origin and are mostly found in the carotid body. The study showed that in hereditary paraganlioma, the R22X mutation in the *SDHD* gene impaired the activity of complex II of the electron transport chain in the mitochondria. The *SDHD* gene codes for succinate dehydrogenase subunit D. SDHD is a TCA cycle enzyme converting succinate to fumarate and it forms a small subunit of cytochrome *b* in the succinate-ubiquinone oxidoreductase complex. There are four subunits of succinate dehydrogenase and all four of them form part of the complex II of the electron transport chain. SDHC and SDHD form two proteins at the inner mitochondrial membrane. SDHA which is also called flavoprotein and SDHB which forms the iron sulphur subunit both extend into the mitochondrial matrix. Mutations in the genes coding for the A, B, C or D subunits of succinate dehydrogenase were observed to cause a pseudohypoxic response by activating hypoxic pathways and lead to stabilization of HIF-1α and HIF-2α in addition to causing hereditary pheochromacytomas and paragangliomas (HPGL) (Gimenez-Roqueplo et al., 2001,

Gottlieb and Tomlinson, 2005, Eng et al., 2003, Burnichon et al., 2010). *SDH* (succinate dehyrogenase) gene also acts as a tumour suppressor. Missense and protein truncating mutations in this gene cause accumulation of succinate in the mitochondria. High levels of succinate that accumulate in the mitochondria can then enter the cytoplasm and interfere with the function of the PHD enzymes and their interaction with HIF. This leads to stabilization of HIF-α in the cells since unhydroxylated HIF is not recognized by the pVHL for degradation in this case (Selak et al., 2005). Another important mitochondrial enzyme which is involved in HIF stabilization is fumarate hydratase (FH), also called fumarase. FH is a tumour suppressor and converts fumarate to malate in the TCA cycle. Mutations in the *FH* gene prevent this conversion of fumarate to malate leading to an accumulation of fumarate in the mitochondria. Accumulated fumarate then moves into the cytoplasm and acts as a potential inhibitor of PHD enzymes. Compared to succinate, fumarate has a higher affinity for the PHD enzymes and binds to them more strongly (King et al., 2006, Koivunen et al., 2007, Hewitson et al., 2007). A recent study by O'Flaherty et al., (2010) which investigated the relationship between FH, HIF-1α and mitochondrial function reported that HIF-1α stabilization due to inhibition of HIF prolyl hydroxylation was seen in *Fh1*$^{-/-}$ MEFs. This was corrected by introducing cytoplasmic FH into the cells although the mitochondrial dysfunction in the cell was still present. This suggests accumulation of fumarate due to lack of FH in the cell inhibits HIF hydroxylases and this leads to HIF-1α accumulation. This result is important since it shows that HIF-1α accumulation is an effect of fumarate accumulation rather mitochondrial metabolic dysfunction (O'Flaherty et al., 2010).

1.5. *Fumarate hydratase* and its role in disease and cancer

Similar to *SDH*, mutations in *FH* have pathological consequences. It has been shown that fumarate hydratase deficiency, which is a recessive genetic disorder,

occurs as a result of in-frame deletions or missense mutations. On the other hand, leiomyomatosis patients usually show missense mutations which cause N-terminal changes or truncations of FH (Tomlinson et al., 2002). Further studies on *FH* have shown that germline mutations in *FH* develop hereditary leiomyomatosis and renal cell cancer (HLRCC) which is inherited in an autosomal dominant way (Pollard et al., 2005). HLRCC patients present with benign smooth muscle tumours which are found in the uterus or on the skin. Patients can also present with type II papillary cancer which is a type of malignant solid tumour of the kidney that is aggressive in character. Benign renal cysts and leiomyosarcomas are also seen in a small number of HLRCC patients. *FH* mutations are also observed in cancers such as breast cancer, lymphomas, leukaemias, prostate cancer and cancers of unknown origin (Lehtonen et al., 2006). The presence of renal cysts may be an indication of a pre-cancerous stage and this is one of the questions which will be addressed in this thesis. When leiomyomatomas and type II papillary cancers in HLRCC are analysed, it is seen that these tumours express high levels of HIF-1α and HIF-2α. However, these two proteins are not expressed at the same levels. HIF-2α expression level is slightly less compared to HIF-1α levels. *In vitro* experiments have shown that when *FH* was inhibited or knocked out in A549 (adenocarcinoma cells of the human alveolar basal epithelium) or Caki-1 (proximal tubule mimicking cells) cells, it was seen that glucose levels in the cell increased four to six fold. This result is also indicative of the higher level of glycolysis in the cells which lack *FH*. When FH was inhibited and fumarate levels were increased in UMRC2 renal cancer cells, HIF stabilization was observed in the cells. Conversely, when 2-OG levels were increased in UMRC2 cells, the level of HIF-1 and HIF-2 proteins in the cells decreased. The same study also provides evidence that would suggest that PHD activity is inhibited by fumarate. Inhibited PHD enzymes, therefore, cannot hydroxylate the prolyl residues on HIF. This prevents HIF from being recognized by pVHL for degradation (Isaacs et al., 2005). Another proposed cause of HIF stabilization is the increase in the level of ROS in response to inhibiting and/or knocking out FH.

Increased ROS levels in the cell cause a decrease in the level of Fe(II) in the cell. As described before, Fe(II) is required for prolyl hydroxylation of HIF by PHD enzymes. Therefore, when the levels of Fe(II) in the cell are not adequate, HIF stabilization occurs. In turn, increased increase HIF levels exacerbate glycolysis in the cell. This leads to a further increase in ROS due to increased glycolysis (Sudarshan et al., 2009). However, it has recently been shown that HIF-1α stabilization is a direct effect of fumarate accumulation and is independent of mitochondrial dysfunction (O'Flaherty et al., 2010).

1.6. Hypoxia inducible factors in cancer

Clinical studies show that hypoxia is an important factor in determining the prognosis of different types of cancers. Expression of hypoxia inducible factors and their target genes are associated with a shorter survival time in patients with cancers such as adenocarcinoma of the breast and cervical cancers. In addition to upregulation of HIF expression, tumour hypoxia also causes necrosis in the solid tumours which is also associated with poor prognosis in cancer (Jubb et al., 2010).

The literature shows that HIF-1α and HIF-2α are both important in cancer but that they play different roles in tumourigenesis. Studies on different colon cancer cell lines show that the expression of both HIF-1α and HIF-2α is tissue specific. Furthermore, while HIF-1α is expressed in all cell stages of colon cancer, expression of HIF-2α is downregulated as the tumour progresses. This implies that HIF-2α might have a tumour suppressor function while HIF-1α favours tumourigenesis (Imamura et al., 2009). The oncogenic role of HIF-1α has also been shown in breast cancer and prostate cancer. In both these cancers HIF-1α is up regulated. Increased HIF-1α level is shown to activate the PIP3K/Akt-1/mTor signalling pathway which regulates proliferation, cell cycle, glycolysis and leads to cancer (Kimbro and Simons, 2006, Lum

et al., 2007). PTEN mutations and activation of the MAPK signalling pathway are also suggested to be two important ways which allow HIF-1α to mediate its oncogenic function. Tumours which are positive for HIF-1α are also radio- and chemoresistant. Therefore, targeting HIF-1α in tumours can be important in improving the response to treatment and survival in cancer patients (Poon et al., 2009).

Although HIF-2α expression is not upregulated in all types of cancer, high levels of HIF-2α has been associated with resistance to treatment, metastasis and poor prognosis in neuroblastoma, ccRCC and non-small cell lung carcinoma. During development neural crest, epithelium of lung and kidneys, bone marrow and endothelium show HIF-2α expression. Cancer types which show elevated HIF-2α levels occur in these organs. This implies that HIF-2α has a cell type specific effect in cancer (Qing and Simon, 2009). Expression of HIF-2α has also been suggested to cause stem cell like characteristics in some cells of various tumours and therefore to promote metastasis, chemoresistance and an aggressive tumour phenotype. This suggests that HIF-2α can also be important in terms of the cancer stem cell theory (Heddleston et al., 2010).

1.7. Role of HIF target genes in cancer

As explained previously, HIF is a transcription factor which regulates expression of a significant number of genes. Since HIF stabilization is seen in diseases such as cancer, understanding which genes are expressed as result of HIF upregulation is important and may act as a key to understanding the mechanisms of malignancies. Genes that are expressed under the regulation of HIF are called HIF target genes. Examples of HIF target genes include *carbonic anhydrase 9* (*CA9*), *erythropoietin, vascular endothelial cell growth factor* (*VEGF*) and many others. *CA9* expression is upregulated when *HIF* is expressed in the cell. Studies show that *CA9* is especially

important in clear cell renal cell carcinoma. It is important in the regulation of pH levels in and around the tumour cells. Clinical studies show that patients with higher CA9 levels do not respond to therapy well and therefore have poor prognosis. On the other hand, benign tumours show very low levels of CA9 (McGuire and Fitzpatrick, 2009, Bratslavsky et al., 2007). The level of CA9 is also an important indicator of whether a renal cyst is benign or malignant. Studies show that renal cysts which express high levels of CA9 (more than 1000 pg/ml) are malignant. On the other hand, low expression of CA9 is seen in benign renal cysts (Li et al., 2009b).

Another gene which is induced by HIF is *Bcl-2/adenovirus E1B 19 kDa-interacting protein 3* (*BNIP3*). The protein product of *BNIP3* is found on the outer part of the mitochondrial membrane and is important for regulation of necrosis and apoptosis of the cell. Contradicting results have been published on the levels of BNIP3 in different cancers. Although some cancers such as colon cancer and pancreatic cancer have low levels of BNIP3, upregulation of *BNIP3* was seen in non-small cell lung cancer, prostate cancer, cervical cancer and breast cancer. In renal cell cancers which develop due to *VHL* mutations, BNIP3 is also upregulated in normoxia but is still due to pseudohypoxic expression of HIF. In these cancers where *BNIP3* expression is induced, the patients have poor prognosis. In cancers where the BNIP3 level is low, it has been shown that *BNIP3* is silenced epigenetically (Koop et al., 2009, Mellor and Harris, 2007, Shaida et al., 2008).

Pyruvate kinase M2 *(PKM2)* is one of the important HIF target genes involved in cancer. It is normally expressed in embryos. Pyruvate kinase, which is an enzyme involved in glycolysis, has four isoforms: M1, M2, R and L. Of these four isoforms, PKM2 is the only one which can bind to phosphotyrosines (Christofk et al., 2008). This is important in terms of the relationship between PKM2 and the Warburg effect. Studies have shown that when PKM2 binds to phosphotyrosines, the cell chooses to use (generate ATP) by glycolysis instead of oxidative phosphorylation. Although glycolysis

yields less ATP compared to oxidative phosphorylation, it provides the cell with the opportunity of biosynthesis. This is important since these cells can then proliferate. This mechanism is essential for the survival of cancer cells, especially under hypoxic conditions. Therefore, expression of PKM2 is important in cancer cell survival (Ferguson and Rathmell, 2008, Hitosugi et al., 2009). Increased levels of PKM2 are seen in various cancers such as squamous cell carcinoma of the tongue, colon cancer and dermatological malignancies. PKM2 levels also provide a useful way of differentiating malignancy from a benign tumour, especially in pancreas and kidney cancers (Wong et al., 2008, Zhang et al., 2004).

Lipocalin 2 (LCN2) is induced by hypoxia (Jiang et al., 2008). It is a glycoprotein which is 24 kDa in size. LCN2 is an important protein because of its ability to bind metalloproteinase MMP-9, Fe(II) and its role in apoptosis. Increased levels of LCN2 have also been detected in several types of cancer such as chronic myeloid leukaemia and many solid tumours. Studies on mice show that increasing *LCN2* expression in hematopoietic cells which also express BRC-ABL leads to development of leukaemia. On the other hand, inhibiting LCN2 in those cells prevents induction of leukaemia. LCN2 is also important in breast cancer development and high levels of the protein make the cancer more invasive (Leng et al., 2009, Arlinghaus and Leng, 2008, Leng et al., 2008, Berger et al., 2010).

Hypoxia also leads to upregulation of *hexokinase 2* (*HK2*). It is known that increased levels of HIF-1α under hypoxic conditions activate the HK2 promoter. HK2 is an important enzyme which is in involved in glycolysis. It converts glucose to glucose 6-phosphate. Increased HK2 levels in the cell drive the glycolytic pathway which has been shown to promote proliferation. This has pathological consequences. A study on hepatocellular carcinoma had shown that under hypoxic conditions, HK2 levels were significantly increased in the tumours. Increased HK2 levels were associated with an aggressive and progressive type of hepatocellular carcinoma (Gwak et al., 2005,

Mathupala et al., 2001). In tumours, HK2 is expressed in the outer membrane of the mitochondria where it forms a complex with voltage dependent anion channels (VDAC). This allows the glucose-6-phosphate to be transported to the cytoplasm via the VDACs very efficiently. This allows HK2 to upregulate glycolysis in the tumour cells. Due to these properties, HK2 is an important contributor to the Warburg effect. In addition, it has also been suggested that HK2 leads to immortalization of cancer cells through its interactions with the Akt protein (Mathupala et al., 2009) .

Pyruvate dehydrogenase kinase 1 (PDK1) is one of the four members of the pyruvate dehyrogenase kinase (PDK) family. PDK enzymes have regulatory role on the pyruvate dehydrogenase complex (PDC) by phosphorylating it, which converts pyruvate to acetyl Coenzyme A. Under hypoxic conditions, expression of *HIF-1α* causes upregulation of PDK1 enzymes. Increased PDK levels inhibit the function of PDC and therefore prevent the cell from entering the TCA cycle by inhibiting the production of acetyl coenzyme A by PDC. This makes the cell go into glycolysis instead of the TCA cycle. Therefore, overexpression of PDK is involved in the Warburg effect. High levels of PDK1 have been seen in many different cancer cell lines such as renal cancer, breast cancer and colon cancer cell lines. In addition, clinical studies show that high levels of PDK are associated with poor prognosis squamous carcinomas of the head and neck (Wigfield et al., 2008, McFate et al., 2008).

Myc, which is located at 8q24, is an important oncogene which codes for the Myc oncoprotein. Many cancers such as lung cancer, colon cancer, breast cancer, ovarian cancer and cervical cancer show increased Myc expression. Myc interacts with many other proteins in the cell. Therefore its expression affects different pathways. Myc mediates these effects by binding to Myc-Myc associated protein X (MAX) and regulating the transcription of genes involved in the cell cycle, proliferation, metabolism and immune response (Dang et al., 1999). Hypoxia is important in regulating Myc expression. HIF-1α and HIF-2α show different effects on Myc. HIF-1α expression

suppresses Myc expression. Conversely, HIF-2α increases Myc levels (Dang et al., 2008).

Other HIF target genes include *NF-κB* family, *p53* and *AP-1*. The *NF-κB* family is an important part of the immune system and is involved in regulation of inflammatory responses. It has also been shown to be important in cancer due to its involvement in regulation of apoptosis. p53 is an important tumour suppressor. Mutations in p53 are involved in many different types of cancer. AP-1 is involved in transcriptional regulation and proliferation of cells. Therefore, it is important in tumourigenesis (Kenneth and Rocha, 2008). HIF expression is also important in regulation of innate immunity (Nizet and Johnson, 2009).

1.8. MicroRNAs in hypoxia and cancer

Micro RNAs (miRNAs) are an important class of RNAs which have a regulatory role on the expression of a wide range of genes. miRNAs are relatively small molecules which are 20-25 base pairs in size. They undergo several modifications in the nucleus and cytoplasm before they can affect gene expression. miRNAs are transcribed in the nucleus by the RNA polymerase II enzyme. This form of RNA is called the primary microRNA (pri-miRNA). pri-miRNAs have hairpin structures, polyA tails and cap structures. They are also several kilobases in size which is much bigger as compared to the miRNAs. pri-mRNAs lose their stem loop after being processed by Drosha in the nucleus. Drosha is a type of endonuclease which forms a complex with the DGCR8 molecule. This complex recognizes the hairpin structures of the pri-RNA and forms percursor miRNA (pre-miRNA) after processing it. Pre-miRNAs are then transported from the nucleus by exportin 5 protein. Pre-miRNA is processed further in the cytoplasm by Dicer (Inui et al., 2010, Kim, 2005). The single stranded miRNA produced by Dicer then binds to the RNA-induced silencing complex (RISC). RISC contains an

Argonaute protein in it which is important in recognition of the target mRNAs. The miRNA-RISC complex binds to the 3' untranslated region (UTR) of the mRNA which leads to the degradation of that mRNA (Pratt and MacRae, 2009). Studies have shown that miRNAs are important in cancer in addition to having a physiological role in the body. Overexpression or downregulation of certain miRNAs has been associated with different types of cancer such as colon cancer, lung cancer, breast cancer, pancreatic cancer and haematological malignancies. For instance, a decreased level of let-7 miRNA is seen in lung cancer patients. On the other hand, increased levels of miR-17 are associated with B cell lymphomas (Shenouda and Alahari, 2009). miRNAs mediate their oncogenic effects by affecting different proteins and pathways which are important in proliferation, development and other vital cellular events. Myc is one of these proteins. It has been suggested that downregulation of some miRNAs activate Myc to initiate tumourigenesis (Chang et al., 2008). Myc was also shown to have regulatory effects on miRNA levels. Gao et al., (2009) demonstrated that Myc downregulates miR23a and miR23b levels which leads to the activation of glutaminase in mitochondria and therefore provides the cell with more glutamine. The glutamine metabolism derived by decreased levels of miR23a and miR23b favours proliferation of the cells and tumourigenesis (Gao et al., 2009).

The relationship between hypoxia and miRNAs has attracted much attention over the past few years. It has been suggested that HIF-1α expression is regulated by various miRNAs such as miR17-92 and miR-20b. In addition, HIFs have been shown to upregulate certain miRNAs such miR210, miR23 and miR24. Under hypoxic conditions, both HIF-1α and HIF-2α bind to the HRE to initiate transcription of miR210. Studies suggest that increased levels of miR210 in the cell lead to expression of genes which cause angiogenesis. High levels of this miRNA are also suggested to prevent differentiation of stem cells, inhibit the DNA damage repair systems in the cell, affect the cell cycle, and increase glycolysis. All of these contribute to tumourigenesis (Huang

et al., 2010). Various studies indicate that miR210 levels in the tumour can be used both as a diagnostic and prognostic marker in some cancers. For example, high levels of miR210 are associated with poor prognosis in head and neck squamous cell carcinoma (Gee et al., 2010). It has also been shown to be a biomarker in pancreatic cancer and a prognostic marker in breast cancer (Ho et al., 2010).

1.9. Mouse models

As discussed in this thesis, a significant proportion of the studies reported in the literature that analyse the role of *FH, HIF-1α, HIF-2α and VHL* in disease, are conducted using *in vitro* models. Although the studies provide valuable data about the function of these genes, *in vivo* models are required in order to have more realistic representation of their role in living organisms. In order to address this issue, several mouse models have been generated where targeted gene inactivation was performed. Haase et al., (2001) have investigated the *in vivo* role of VHL by targeted inactivation of *Vhl* (also known as *vhlh*) in mouse. They have used the Cre recombinase (a type of DNA recombinase which is obtained from bacteriophage *P1* and mediates recombination at the *loxP* sites) BLN-hyTK targeting vector to generate conditional VHL null allele and null allele mice. The conditional VHL null allele, the VHL promoter and the first exon were flanked by two *loxP* sites. The null allele had only one *loxP* site. The phenotypic analysis of the mutant mice showed that mice with *2-loxP* allele were healthy. However, when mice were heterozygous for *1-loxP* allele, which mimics autosomal dominant VHL mutations in human, they developed cavernous hemangiomas of the liver. This is suggested to be due to increased *Vegf* expression in these mutant mice. When the null allele mice were crossed with albumin-cre mice to delete *Vhl* in hepatocytes, it was seen that *Hif-1α* and *Hif-2α* stabilization look place. *Vegf, Epo* and *Glut1* which are HIF target genes were also upregulated in these mice (Haase et al., 2001, Shao et al., 2002). Rankin et al., (2006) generated a more complex mouse model to study the role of *Vhl, Hif-1α* and *Hif-2α* deficiency and the relationship

between these genes in hepatocytes and renal proximal tubules. They have used the phosphoenolpyruvate kinase carboxykinase (PEPCK) promoter to express the Cre-recombinase in the renal proximal tubules and hepatocytes. By using this mechanism they have deleted *Vhlh, Vhlh/Hif-1α* and *Vhlh/Hif-2α* in a tissue specific manner. In these mice, deletion of *Vhlh* caused cyst development in the kidneys. This phenotype was rescued in *Vhlh/Hif-2α* deletion whereas *Vhlh/Hif-1α* deleted tubules still developed cysts. This suggests that *VHL* and *HIF-2α* might both be critical in renal cyst development (Rankin et al., 2006). The consequences of deleting both *Hif-2α* and *Vhlh* in kidney and liver were analysed using the same mouse model. Results of this study indicated that conditional *Hif-2α* and *Vhl* deletion ameliorated the hemangioma development, therefore indicating that *Hif-2α,* not *Hif-1α*, is involved in regulation of *Vegf* expression (Rankin et al., 2008). Shao et al., (2002) have reported another effective mechanism which can be used target genes in a kidney tissue specific way. They have reported the use of a Cre recombinase that was expressed under the Ksp-cadherin (Ksp) transcription factor. Ksp expression was seen both in embryonic and adult mice. This group has generated Ksp1.3/Cre transgenic mice in which the first Ksp1.3/Cre expression was seen in embryonic day 15.5 (E15.5) in the metanephros and ureter. Expression becomes specific to the collecting ducts and renal tubules by E17.5. In adult mice, expression was seen in collecting ducts and thick ascending limbs (Shao et al., 2002). Therefore, this study suggests that Ksp-Cre mechanism can be used for kidney specific gene targeting. This mechanism was used to inactivate the *Fh1* gene in the mouse kidney where it was reported to cause a cystic phenotype in the kidneys (Pollard et al., 2007).

In this thesis we have utilised a variety of mouse models in which the genes *Fh1*, *Hif-1α* and *Hif-2α* have been conditionally knocked out by combination with Ksp-cre. In all cases, the functional part of the gene has been flanked by loxP sites (therefore termed ‘floxed’) and when crossed with Ksp-cre strain the Cre recombinase will act in

the kidney to excise that portion of the genes(s) within the loxP site. In this way, it is hoped that we can determine the function of the genes in the kidney, specifically in the development of renal cysts.

The main aims of this project can be summarised as following:

1. To delete the *Fh1*, *Hif-1α* and *Hif-2α* genes in the mouse kidneys using the Ksp-cre mechanism.
2. To analyse the effect of conditional inactivation of *Fh1* combined with either *Hif-1α* or *Hif-2α* on the phenotype of the kidneys.
3. To identify the roles of *Fh1*, *Hif-1α* and *Hif-2α* genes in the mechanism of the cystic phenotype observed in the knockout kidneys.
4. To identify target genes and pathways that are involved in development of the cystic phenotype observed.

2. MATERIALS AND METHODS

2.1. Origins of targeted and transgenic mice

Fh1 conditional knockout mice were intercrossed with Ksp1.3/Cre transgenic mice as described previously (Pollard et al., 2007). Progeny were then intercrossed with mice bearing conditional alleles for either *Hif-1α* (Ryan, 2000) or *Hif-2a* (Gruber et al., 2007). Mice were maintained in accordance with Home Office guidelines and licensing regulations. In this thesis comparisons of renal tissue for different genotypes have been made between littermates.

Fh1, Hif-1α and Hif-2α genes were knocked out in a kidney-specific manner in mice to investigate the role of these genes in renal pathology, specifically in renal cyst development. To knock out genes in the kidney, a Cre recombinase that was expressed under the Ksp-cadherin (Ksp) transcription factor promoter was used. The first Ksp1.3/Cre expression is seen in embryonic day 15.5 (E15.5) in the metanephros and ureter and then expression becomes localised to the collecting ducts and renal tubules by E17.5. In adult mice, expression is seen in the collecting ducts and thick ascending limbs (Shao et al., 2002).

Not all tubules in the kidneys will have these genes knocked out since the Cre-loxP mechanism is not expressed under the Ksp-cadherin in every tubule in the kidney. However, for simplicity the kidneys which were homofloxed (carrying two floxed alleles of the relevant gene) will be referred to as knockout kidneys (*$Fh1^{-/-}$, $Hif\text{-}1\alpha^{-/-}$, $Hif\text{-}2\alpha^{-/-}$, $Fh1^{-/-}/Hif\text{-}1\alpha^{-/-}$ and $Fh1^{-/-}/Hif\text{-}2\alpha^{-/-}$).*

2.2. Immunohistochemistry

Mouse kidneys were fixed in neutral-buffered 10% formalin for 24 hour, dehydrated and processed using standard methods for paraffin embedding and sectioning. Sections were cut at 5μm. Immunohistochemistry was performed on sections using

reagents from the EnVision™+ kit (DAKO) following a standard protocol. Briefly, sections were taken through two changes of xylene and a graded series of alcohols to water. Antigen retrieval was performed by pressure cooking in pre-heated 1X Antigen Retrieval solution pH 6 (DAKO) for 30 minutes. Sections were washed in PBS and then blocked with manufacturer's peroxidase block for 10 minutes. Subsequently, the slides were washed with PBS-Tween and incubated with primary antibody, diluted in the DAKO antibody diluent, for forty five minutes. After the staining, the slides were washed repeatedly with PBS-Tween and then incubated in HRP polymer for thirty minutes. Following repeated washes in PBS-Tween, DAB+ substrate-chromogen was diluted in DAB+ substrate buffer (one drop of DAB+ substrate-chromogen per 1 ml of DAB+ substrate buffer), and the tissues on the slides were exposed to DAB+ for up to five minutes until the staining was visible under the light microscope. After five minutes, the slides were washed with tap water to remove DAB+, counterstained with haematoxylin for two minutes and then washed again under a running tap.

Sections were then dehydrated through a graded series of alcohols and changes of xylene and finally mounted in DPX under glass coverslides.

Table 1: Details of the antibodies used for IHC staining

Antibody	Company	Cat. Number	Dilutions Used For Staining
FH	Autogen Bioclear	NE054	1:1000
γH2AX(Ser139)	Cell Signalling	9718	1:200
Ki67	Novus Biologicals	NB110-89719	1:1000
CCND1	Abcam	Ab16663	1:1000
Cleaved Caspase-3	Cell Signalling	9661	1:500

2.3. Haematoxylin and Eosin Staining

As for the immunohistochemistry above, the mouse kidneys were fixed in neutral-buffered 10% formalin for 24 hours, dehydrated and processed using standard methods for paraffin embedding and sectioning (5μm). Sections were stained with haematoxylin and eosin (H&E) as follows. Sections were rehydrated by taking slides through two changes of xylene and a graded series of alcohols to distilled water. The slides were then stained with Mayer's haematoxylin (Dako) for 6 minutes, rinsed in tap water for 20 minutes, stained with 1% aqueous eosin (Surgipath) for 1 minute and then washed. The sections were then dehydrated through a graded series of alcohols, changes of xylene and finally mounted by in DPX under glass coverslides.

2.4. Semi-Quantitative Scoring of Stained Tissue

The stained kidney sections were scored using a semi-quantitative scoring method. The technique was taught by Dr. Mona El-Bahrawy. The H&E stained sections were assessed for the presence or absence of cysts. Cysts with a diameter less than 0.5 mm were classified as micro cysts and cysts with diameter bigger than 0.5 mm were classified as macro cysts.

Sections stained with the antibodies explained above were quantified by counting the number of nuclei with positive staining. For each slide, 10 non-cystic, 10 micro cystic and 10 macro cystic tubules were chosen randomly. For each tubule, the total number of nuclei and number of positive nuclei were counted. After counting the number of nuclei, the mean and standard deviation of positive nuclei per tubule was calculated. The mean values were used to perform 2-tailed t-tests to compare the expression of the protein of interest between different types of cysts and normal tubules in addition to comparing the expression in tubules with different genotypes. Excell programme was used to perform the t-test analysis on the mean values

calculated. An alternative and more sensitive statistical method to analyse the data would be analysis of variance. Analysis of variance takes into account the variation between the different variables used in the experiment design. Therefore, the statistical analysis done using the analysis of variance method is better representative of the population of interest (Kirkwood, 2005).

2.5. DNA Isolation and Genomic PCR

The genotypes of mice used in this study were determined twice, first at weaning, from ear clips obtained for this purpose and for identification and second at the time of culling and harvesting of kidneys when tail tips were collected. DNA was extracted from both ear clips and tails of mice and genotypes were determined by PCR.Tissue was digested in buffer with 5% proteinase K (100mM EDTA (pH=8.0), 50 mM TRIS-HCL (pH=8), 100mM NaCl and 1% SDS) and incubated overnight at 55 °C. DNA was extracted by previously described protocols and re-suspended in DEPC treated water (Pollard et al., 2007). PCR reaction to amplify the *Fh1* gene was performed by using the following reagents per reaction supplied by Qiagen.

Table 2: Reagents for genomic PCR (Qiagen)

Reagent	**Volume in 50 µl reaction**
H_2O	31.5 µl
Buffer (x10)	5.0 µl
Q Solution	10.0 µl
Mg^{2+} (25 mM)	0.0 µl
dNTPs	1.0 µl
Primer Mix	1.0 µl
Taq polymerase	0.5 µl
DNA	1 µl
Total	**50.0 µl**

The sequences of primers used to amplify the conditionally knocked out *Fh1* are as follows:

Forward primer: ACCCTGCTAGGTGTCACCAC

Reverse Primer: CCTGGCACTGCAGACTACAA

The reaction was run using the following cycle: Step 1 at 95°C for 3 minutes, Step 2 at 95°C for 30 seconds, Step 3 at 60°C for 45 seconds, Step 4 at 72°C for 45 seconds, The Step 2 was repeated for 29 times and the reaction was held at 4°C.

PCR reaction to amplify the *Hif-1α and Hif-2α* genes was performed by using the following reagents per reaction supplied by Bioline Company.

Table 3: Reagents for genomic PCR (Bioline)

Reagent	**Volume in 50 µl reaction**
H_2O	36.5 µl
Buffer (x10)	5.0 µl
Mg^{2+} (50 mM)	2.0 µl
dNTPs	4.0 µl
Primer Mix	1.0 µl
Taq polymerase	0.5 µl
DNA	1 µl
Total	**50.0 µl**

The following primers were used to amplify the *Hif-1α* gene*:*

Forward primer: GCAGTTAAGAGCACTAGTTG

Reverse primer: GGAGCTATCTCTCTAGACC

The primers used to amplify *Hif-2α* are:

Forward primer (for *Hif-2α* 2flox): CAGGCAGTATGCCTGGCTAATTCCAGTT

Reverse primer (for *Hif-2α* 2flox): CTTCTTCCATCATCTGGGATCTGGGACT

Reverse Primer for knockout: GCTAACACTGTACTGTCTGAAAGAGTAGC

Ksp-Cre transgene primers are as following:

Forward primer: GCAGATCTGGCTCTCCAAAG

Reverse primer: AGGCAAATTTTGGTGTACGG

Details of the PCR which was run to amplify the *Hif-1α, Hif-2α and Ksp-Cre* genes are described below:

Step 1 at 95 °C for 3 minutes, Step 2 at 95 °C for 30 seconds, Step 3 ate 58 °C for 45 seconds and Step 4 at 72 °C for 45 seconds. Then step 2 was repeated 29 times and the reaction was held at 4 °C.

2.6. RNA Extraction

Mouse kidneys to be used for RNA were bisected and the tissue snap frozen when the animals were culled. The tissue was then kept at -80°C until RNA was extracted. Prior to RNA extraction kidneys were cut into quarters and the tissue pieces were then smashed using the tissue smasher. During the procedure, the tissue was kept on dry ice at all times. The smashed tissue was put into 1 ml of Trizol (Sigma) and

drawn several times through a syringe using a 21 gauge needle to homogenise the tissue. Samples were kept at room temperature for five minutes. RNA was extracted using a standard protocol and was resuspended in DEPC treated water.

2.7. cDNA Preparation

cDNA was prepared using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. In order to prepare cDNA, reaction mastermix was prepared using RT Buffer, dNTP, multiscribe reverse transcriptase, 10xRT Random Primer, RNAse inhibitior and nuclease free water in the following ratios:

Table 4: Reagents for cDNA preparation

Reagent	Amount in 10 µl Reaction
10 x RT Buffer	2.0 µl
dNTP	0.8 µl
Multiscribe Reverse Transcriptase	10 µl
10 x RT Random Primers	2.0 µl
RNase Inhibitor	1.0 µl
Nuclease Free Water	3.2 µl
Total volume	**10 µl**

2000 ng of RNA was added to each reaction and the total reaction was made up to 20µl by adding nuclease free water. The cDNA reaction was performed by PCR under following thermal cycler condition:

Step 1 at 25°C for 10 minutes, Step 2 at 37°C for 120 minutes, Step 3 at 85°C for 5 seconds and then the completed reaction was held at 4°C.

2.8. Real Time Quantitative PCR

cDNA obtained as a result of the cDNA reaction described above was used in the Real Time Quantitative PCR reaction. All the reagents used were produced by Applied Biosystems. A reaction mix was prepared using the following:

Table 5: Reagents for RT-Q-PCR reaction

Reagent	Volume in 8 µl reaction mix
Mastermix	5.0 µl
Nuclease free water	2.0 µl
Exogenous control (i.e. β-Actin)	0.5 µl
Taqman Probe	0.5 µl

2 µl of cDNA was added to the reaction mix (total reaction volume of 10 µl) and the reaction was run by using the following conditions:

Holding stage: 95°C for 20 seconds

Cycling stage: 95°C for 1 second; 60 °C for 20 seconds (40cycles)

Mouse β-actin was used as an endogenous control because it is a ubiquitously expressed gene and therefore acts as a reference point in quantifying the relative expression of the gene of interest (Bustin, 2000).

2.9. MicroRNA Reverse Transcription

In order to perform microRNA assays, TaqMan® Small RNA Assays kit was used. For the reverse transcription reaction the following master mix was prepared:

Table 6: Mastermix reagents for miRNA assay

Reagent	**Master mix volume per 15 µl reaction**
100mM dNTPs (with dTTP)	0.15 µl
RNase Inhibitor, 20 U/µL	0.19 µl
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00 µl
10 x Reverse Transcription Buffer	1.50 µl
Nuclease-free water	4.16 µl
Total volume	**7.00** µl

The reaction mix, with a total volume of 15 µl, also contained 3 µl of primer and 5 µl of RNA (with 10 ng of RNA content). Reverse transcription was under following conditions:

Table 7: PCR conditions for Reverse Transcription for miRNA

Step Type	**Time (Minutes)**	**Temperature(°C)**
Hold	30	16
Hold	30	42
Hold	5	85
Hold	∞	4

2.10. MicroRNA RT-Q-PCR

Reverse transcription product obtained from the microRNA reverse transcription reaction described above was used subsequently in the Real Time Quantitative PCR reaction. All the reagents used were produced by Applied Biosystems. A reaction mix (per reaction) was prepared as follows:

Table 8: Mastermix reagents for RT-Q-PCR for miRNA

Reagent	**Volume in 20 µl** **Single reaction**
TaqMan® Universal PCR Master Mix II (2x)	10 µl
TaqMan® Small RNA Assay (20x)	1 µl
Product from RT reaction	1.33 µl
Nuclease-free water	7.67 µl
Total reaction volume	**20** µl

The reaction was run using the following cycle:

Table 9: **PCR conditions for RT-Q-PCR for miRNA**

Step	Enzyme activation	PCR	
	Hold	Cell Cycle (40 cycles)	
		Denature	Anneal/Extend
Temp (°C)	95	95	60
Time	10 mins	15 sec.	60 sec.

DataAssist™ Software was used to analyse the results. Analysis was based on the ∆CT values. The expression levels of the microRNA of interest were calculated by comparing its ∆CT to the ∆CT value of the endogenous control. Therefore, the results obtained reflect a relative expression value.

2.11. Protein Preparation for Western Blotting

Mouse kidneys to be used for protein were bisected and the tissue snap frozen when the animals were culled. The tissue was then kept at -80°C until protein was prepared. During the procedure, the tissue was kept on dry ice at all times. Kidneys were cut into quarters and the tissue pieces were then smashed and homogenized in 7 M SDS urea by using a 21 G needle and 1 ml syringe in 1.5 ml Eppendorf tubes. The protein samples were then sonicated and rotated for 30 minutes at 4 °C. This was followed by centrifuging the samples at 14,000 rpm for 15 minutes at 4°C. The

supernatant was transferred into new Eppendorf tubes and used for Western blotting after denaturing the protein samples as explained below.

2.12. Western Blotting

Western blotting was performed according to a standard protocol. Details are outlined briefly below.

The running gel (7.5%) was prepared by using the following reagents:

Table 10a: Reagents for running gel for western blotting

Reagents	Amount used
30% Acrylamide	3 ml
Lower Buffer (pH8.8) (4xTris/HCl/SDS)	3 ml
MilliQ H_2O	6 ml
10% APS	120 µl
TEMED	12.5 µl

The stacking gel was prepared by using the following reagents:

Table 10b: Reagents for stacking gel for western blotting

Reagents	Amount used
30% Acrylamide	0.65 ml
Upper Buffer (pH6.8) (4xTris/HCl/SDS)	1.25 ml
MilliQ H_2O	3.05 ml
10% APS	31 µl
TEMED	6 µl

25 µl of 4xLysate Buffer was added to 75 µl of the protein lysate. The sample was vortexed for 20 seconds and heated at 95 °C for 10 minutes. After the denaturing the protein lysates, the samples were centrifuged for two minutes and vortexed before loading onto the gel.

10 µl of each sample was loaded per well and 5 µl of the rainbow marker (Fermentas). The protein lysates were run for sixty minutes at 20 Amp per gel in 1x Running buffer (25 mM Tris-HCl (pH8.3), 200mM glycine, 0.1% SDS).

A standard protocol was followed for the transfer of proteins onto Immobilon P membrane (pore size 0.45 µm). The transfer was run for 60 minutes at 100 Volts in 1x Transfer buffer (10 mM Tris-HCl, 100 mM glycine, 10% methanol, 0.005% SDS).

After transferring the gel to the membrane, the membrane was cut based on the molecular weight of the protein being investigated and cut membranes were then blocked with 5% Marvel Milk in PBS-Tween) for thirty minutes to one hour.

The primary antibody dilution was prepared in 5% Marvel Milk and the membrane was incubated with the primary antibody overnight. Alternatively, if the

signal of the antibody was strong, the primary antibody incubation was done for ninety minutes. The membranes were rinsed and washed repeatedly with PBS-Tween with rocking at room temperature.

The secondary antibody dilutions were prepared at appropriate concentrations in 5% Marvel Milk. The membranes were incubated with the secondary antibody for forty five minutes to an hour. After the incubation, the membranes were rinsed and washed repeatedly with PBS-Tween with rocking at room temperature.

Antibody incubations were followed by exposing the membranes to substrate for 5 minutes prior to exposure to developing film to obtain the results. Luminol/Enhancer solution was mixed with equal volume of Stable Peroxide Buffer. Three different types of Stable Peroxide Buffer were used: PICO, DURA or FEMTO. The choice of the Stable Peroxidase Buffer depending on the strength of the signal expected from the antibody used and the intensity of exposure required. For mild exposure, PICO is used. DURA is preferred for mild exposure and FEMTO is used when a strong signal is expected.

2.13. Microarray Analysis

Whole-genome gene expression microarray was performed by Dr Dilair Baban at WTCHG. Whole-genome gene expression analysis were performed using Illumina Single Colour MouseWG-6_V2_0_R0_11278593_A BeadChip with direct hybridization assay (Illumina, Essex, UK) according to the manufacturer's directions starting with 300 ng total RNA from each sample. For each genotype (*Fh1*$^{-/-}$, *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ and wild type), kidneys from 4 mice aged between 15-17 weeks were used. Biotinylated cRNA was prepared using the Illumina TotalPrep-96 RNA Amplification Kit (# 4393543 Ambion, Inc., Austin, TX). This briefly includes a first- and second-strand reverse transcription step, followed by a single *in vitro* transcription (IVT) amplification that

incorporates biotin-labelled nucleotides. Subsequent steps include array hybridization, washing, blocking, and streptavadin-Cy3 staining. Fluorescence emissions by Cy3 were quantitatively detected by BeadArray Scanner for downstream analysis. GenomeStudio version 1.0.6 was used for data extraction from images generated. This software provides results in standard file formats that can be readily processed with most commercial expression-analysis software programs, for more secondary or tertiary analysis through the use of plug-ins integrated within the GenomeStudio module architecture. Data imported to GeneSpring GX 11.0.2 (Agilent Technologies, Inc, Santa Clara CA) normalized with Shift to 75 percentile and baseline transformed to median of all samples for detailed analysis to identify significantly differentially expressed genes.

Genome wide microarray analysis was employed in order to quantify the relative abundance of mRNA transcripts between the various genotypes of the mice. As the greatest phenotypic differences were observed between the *Fh1*$^{-/-}$, *Fh1*$^{-/-}$/*Hif-1a*$^{-/-}$ and wild type mice we decided to compare gene expression profiles of these genotypes. Gene expression array experiments generate complex 'gene lists' i.e. genes that are differentially expressed between each group. Therefore we utilised 2 independent software packages (GSEA and KEGG) that assign genes within each gene list with respect to biological function and pathways. This process is termed 'Gene Ontology' and we were able to utilise these Gene Ontology programs to identify significant differences in biological pathways and processes between the genotypes.

3. RESULTS

3.1. Effects of *Fh1*, *Hif-1α* and *Hif-2α* knockout on kidney phenotype in mice

3.1.1. General phenotypic analysis of the kidneys

To investigate the effect of deleting different combinations of *Fh1*, *Hif-1α* and *Hif-2α* on kidney phenotype in mice, these genes were knocked out in a kidney tubule specific manner as explained in the Material and Methods section. UKCCCR guidelines for the welfare for animals were followed at all times. The terms 'knockout kidney' and '*Fh1*$^{-/-}$*, Hif-1α*$^{-/-}$*, Hif-2α*$^{-/-}$*, Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ *and Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys' refer to kidneys where in some of the tubules, alleles of these genes (that are homofloxed), have been excised using the Ksp-Cre-*loxP* mechanism.

The wild type kidneys show normal morphology without any cysts, hydronephrosis, tumour formation or any other form of pathology. The size of the wild type kidneys are within normal size limits. These normal characteristics are observed at different developmental stages of mice varying from 9 weeks to 33 weeks old mice.

The phenotype of *Fh1*$^{-/-}$, *Hif-1α*$^{-/-}$, *Hif-2α*$^{-/-}$*, Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys have been analysed. The genotypes studied generated no phenotype outside the kidney. Confirmation of the genotypes of these kidney samples by immunohistochemistry, and genomic PCR is shown in **Figure 3**. In order to have better interpretation of the immunoohistochemistry analysis, identifying where in the kidney the Ksp-cre is expressed will be useful. This can be achieved by staining the proximal and distal tubules in addition to collecting ducts with tubule specific markers such as aquaporin 2 and Tamm-Horsfall protein (Pollard et al., 2007). It was also confirmed by

Western blotting as shown in **Figure 4**. Haematoxylin and eosin (H&E) stained kidney sections from each genotype is shown in **Figure 5**.

3.1.2. Phenotypic analysis of *Fh1*$^{-/-}$ kidneys

The phenotype of the kidneys changes when the *Fh1* gene is knocked out. Although the size of the kidneys in which *Fh1* has been knocked out are normal in size until up to 20 weeks compared to wild type kidneys, the gross morphology of the kidneys is different to the wild type kidneys. The most obvious and important characteristic of the *Fh1* knocked out kidneys is that they show a mild to moderate degree of cyst formation and dilation in both the proximal and distal convoluted tubules. The majority of the cysts are observed in the cortex of the kidneys. In addition to the cysts, *Fh1* deficient mouse kidneys have inflammatory areas. The effect of genotype on the phenotype of the kidneys varies depending on the age of mice. The pathological changes in kidneys are observed at older ages (older than 13 weeks). Kidneys have normal morphology without any cysts or tubular dilations at nine weeks of age. However, at thirteen weeks of age, *Fh1* knockout kidneys start showing mild tubular dilation. At this stage, approximately 10% of the surface area of the kidney sections analysed consists of cysts. These cysts were classified into two main groups based on their size. Cysts with a diameter of less than 0.5 mm were called micro cysts and cysts with a diameter bigger than 0.5 mm were classified as macro cysts. The majority of cysts in the *Fh1* knockout kidneys are micro cysts. This cystic phenotype is also observed in eighteen week-old mice. By the time mice reach twenty two weeks of age, the cystic appearance and other pathological changes become apparent in the kidneys. 10-25 % of the surface area of the kidney sections consists of cysts which are both micro and macro in character. The latest stage at which the kidneys of *Fh1* knocked out mice were analysed was at thirty three weeks of age. At this time point, the kidneys show the most significant cyst formation and tubular dilation. Micro and macro cysts

make up 25-50% the kidney. The main trend seen while analysing the correlation between *Fh1*$^{-/-}$ genotype and the phenotype of the mouse kidneys is that the pathological changes (mainly referring to cyst formation) in the mouse kidneys occur in a progressive manner. Despite having the *Fh1* knocked out, the cystic phenotype is not apparent in mice less than 12.5 weeks of age old mice. As the mice develop, cysts and tubular dilations become more pronounced. Although it is possible to identify this trend from the kidneys analysed, the ages at which the phenotypic changes were observed might not be exact ages of mice when the cyst formation or progression starts. This is due to the fact that the morphological changes in the kidneys were not analysed at each week during the development of mice, but at the defined time points, mainly because of ethical and also practical reasons. It is also costly to keep mice for a long period of time. Previous studies show and justify that analysing kidneys at 33 weeks of age is late enough to see the pathological phenotype (Pollard et al., 2007). However, this does not affect the conclusion of the analysis since the main aim was to see difference in morphology of the kidneys rather than identifying definitive time points in development associated with cyst formation in kidneys.

3.1.3. Phenotypic analysis of *Hif-1α*$^{-/-}$ kidneys

Hif-1α, which mediates the response to hypoxia, was also knocked out in order to see its effect on the phenotype of the mouse kidney. In the first instance, we investigated if kidneys deficient for *Hif-1α* developed abnormal pathology. For this purpose we examined kidneys from *Hif-1α* fl/fl Ksp-Cre$^{+/-}$ mice at 12 weeks, 24 weeks and 33 weeks. As *Fh1* fl/fl Ksp-cre$^{+/-}$ mice present with moderate cystic disease by 24 weeks, the hypothesis before the analysis of the kidney was to see a cystic phenotypic effect due to *Hif-1α*$^{-/-}$ genotype. However, the morphological analysis of the kidneys with *Hif-1α* gene knocked out revealed that these kidneys are free of pathology. They are normal in size compared to wild type kidneys and do not show any cystic formation,

hydronephrosis, proximal or distal convoluted tubule cyst formation. In addition, this normal phenotype is seen at all developmental stages of mice up to thirty three weeks which is the oldest age of mice used in this study. Although this study does not completely rule out any potential phenotype which might occur after the age of thirty three weeks, our results are highly suggestive, if not conclusive, that knocking out *Hif-1α* gene in mouse kidneys does not result in a pathological phenotype in the kidney whereas knocking out of *Fh1* does.

3.1.4. Phenotypic analysis of *Hif-2α*$^{-/-}$ kidneys

Hif-2α was also knocked out in kidneys of experimental mice in this study to investigate any correlation between the *Hif-2α*$^{-/-}$ genotype and any pathological changes in the kidney. As in the case of kidneys deficient for *Hif-1α*, *Hif-2α* deficient kidneys were mice were healthy and did not display any abnormal pathology. Kidneys with *Hif-2α*$^{-/-}$ genotype showed a similar phenotype to kidneys with wild type genotype. No obvious changes in the size and gross anatomy of the kidneys were observed. They were also completely free of cysts and tubular dilatation.

3.1.5. Phenotypic analysis of *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys

The question which is addressed in this study is the effects of *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ genotypes on the phenotype of the kidneys. The most striking phenotypic change was seen in kidneys of *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ mice. These kidneys have abnormal gross anatomy due to cysts and dilatations in the proximal and distal convoluted tubules. The cysts are mostly located in the cortical region of the kidney. The characteristics of the cysts are different when compared to those observed in *Fh1*$^{-/-}$ kidneys. The number of cysts is significantly higher in the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys. Approximately 60-75 % of the surface of the kidney sections consists of cysts which

are a mixture of both micro cysts and macro cysts at an age of older than 14 weeks. Progression of the cystic phenotype in the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys is directly proportional to the age of the mice. At younger ages such as thirteen weeks, the cysts are smaller in number and are mostly micro in character. As the mice get older, such as twenty four weeks of age, the cysts are spread over a larger area of the kidney and both macro cysts and micro cysts are seen. An example of an *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$kidney from a twenty four week old mouse is illustrated in **Figure 5**. Therefore, the main conclusion from this part of the study is that knocking out *Hif-1α* as well as *Fh1* exacerbates the cystic phenotype seen in the kidneys and makes this phenotype more aggressive. The number, size and distribution of the cysts in the kidney increase as the mice get older.

The pathology seen in *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys is similar to that of *Fh1*$^{-/-}$ and different from the characteristics seen in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys. Although both micro and macro cysts are present in *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys, most of the cysts are micro in character. The overall percentage of cysts is also less when compared to the percentage seen in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys. The highest percentage of the surface area of the kidney section occupied by cysts due to this genotype is 25-50% in mice older than 14 weeks. However, this percentage varies according to age of mice. For instance, approximately 10% of a kidney from 13 weeks old mice consists of cysts. On the other hand, the distribution of the cysts in the kidney gets higher as the mice get older. In contrast to the 13 weeks old mice, 25-50% of the kidney is covered with cysts in 28 weeks old mice.

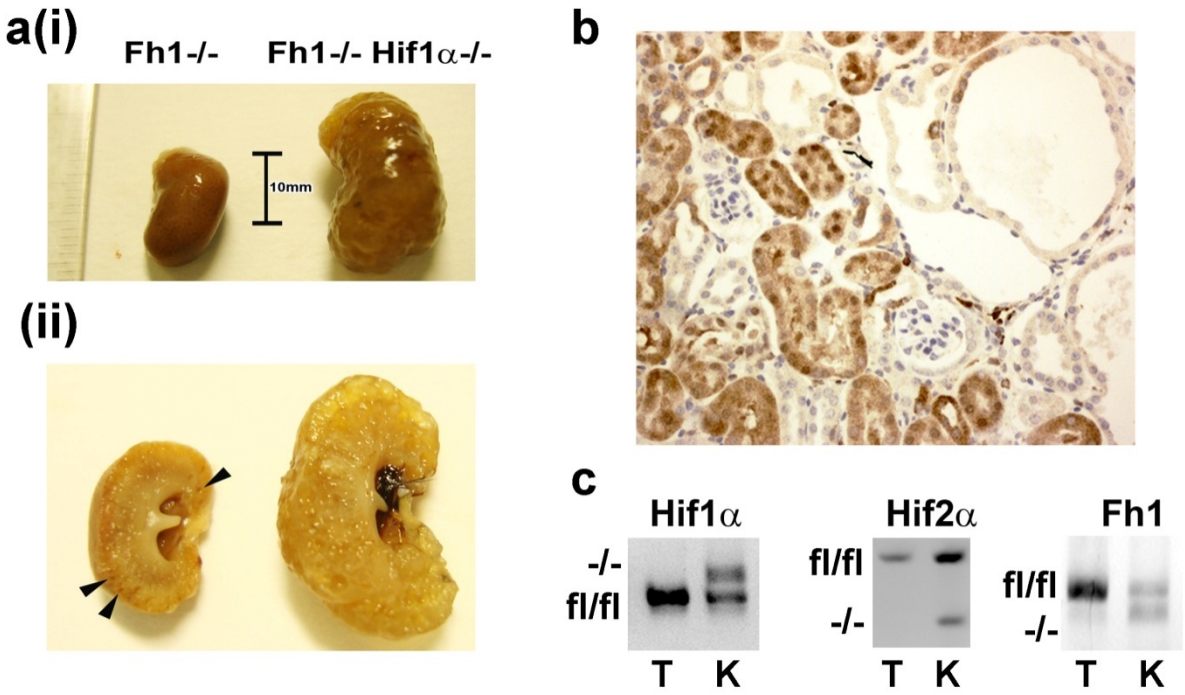


Figure 3: Validation of mouse models for deletion of *Fh1*, *Hif-1α* and *Hif-2α* in the kidney

The gross phenotype of the *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/ *Hif-1α*$^{-/-}$ kidneys at 30 weeks of age is shown in **(ai)** and **(aii)**. The dissected kidneys show the gross cysts which are mostly located in the cortex **a(ii),** some of which are indicated by arrows. ***(*b)** IHC shows FH staining on *Fh1*$^{-/-}$ kidney. It is seen that the tubules in the kidneys which are *Fh1*$^{-/-}$ are negative for FH staining. However, since *Fh1* is not knocked out in all the tubules in the kidney, some tubules are positive for FH. **(c)** The genomic PCR, which was performed by extracting DNA from the tails (T) and kidneys (K), confirm the genotypes of the mice used and the presence of the floxed alleles that are deleted in the kidney by the Ksp-cre, but not the tails.

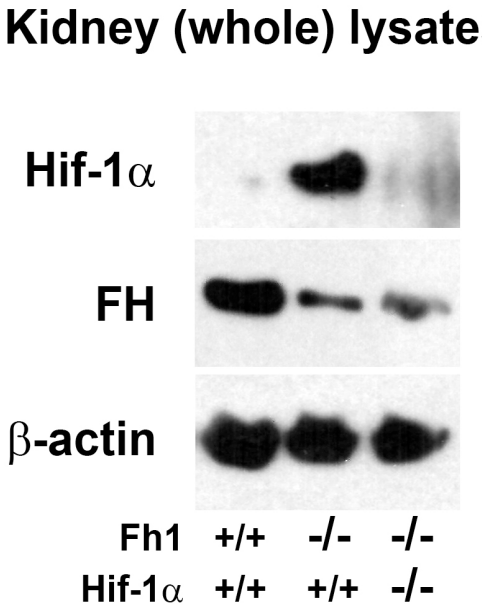


Figure 4: Western blot analysis using whole kidney lysates

Kidney lysates were prepared from wild type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys. Western blot analysis shows that the wild type kidney expresses FH protein but not Hif-1α. In the *Fh1*$^{-/-}$ kidney, FH level is significantly downregulated and HIF-1α expression is induced. In the *Fh1*$^{-/-}$*/Hif -1α*$^{-/-}$ kidney, both FH protein and Hif-1α expression are down regulated. β-actin, which is the endogenous control, is expressed in all three samples. Protein loading in the western blot is also reflected by β-actin expression level.

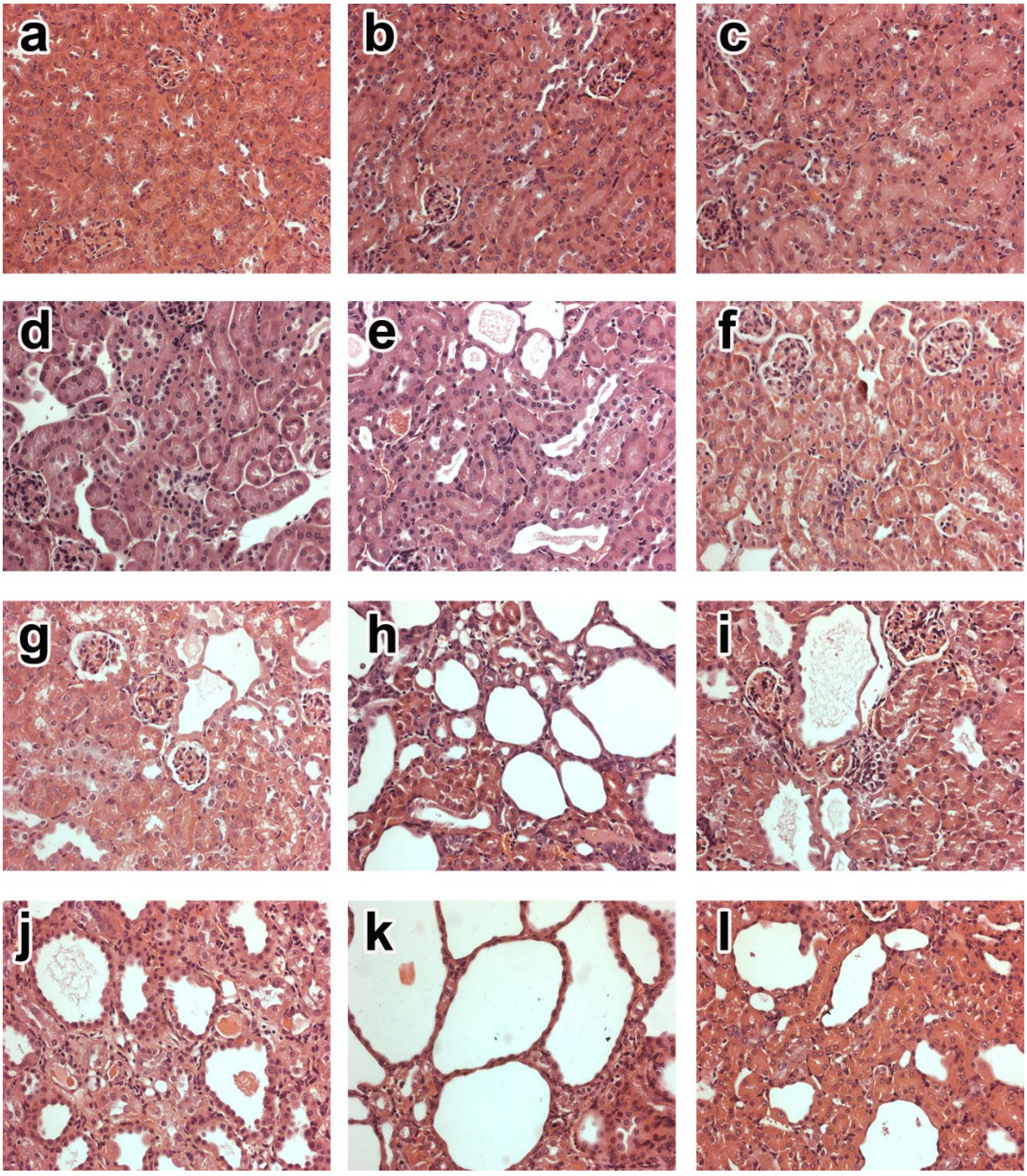


Figure 5: H&E stained kidney sections

(a-c) show wild type, *Hif-1α*$^{-/-}$ and *Hif-2α*$^{-/-}$ kidneys respectively, at 30 weeks of age. These kidneys are free of cysts and do not show any tubular dilatation. **(d-f)** *Fh1*$^{-/-}$, *Fh1*$^{-/-}$/ *Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$/ *Hif-2α*$^{-/-}$ kidneys respectively at 13 weeks of age. *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/ *Hif-1α*$^{-/-}$ show tubular dilatations and microcysts. However, *Fh1*$^{-/-}$/ *Hif-2α*$^{-/-}$ kidney does not have any cysts or tubular dilatations. **(g-i)** show *Fh1*$^{-/-}$, *Fh1*$^{-/-}$/ *Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$/ *Hif-2α*$^{-/-}$ kidneys respectively at 17 weeks of age. At this stage, the cystic phenotype of the kidneys becomes more obvious. Micro cysts are seen in all three kidneys.

However, macro cysts are only present in the *Fh1*$^{-/-}$*/ Hif-1α*$^{-/-}$ kidney. **(j-l)** show *Fh1*$^{-/-}$*, Fh1*$^{-/-}$*/ Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$*/ Hif-2α*$^{-/-}$ kidneys respectively at 24 weeks of age. At this later stage, the cysts are bigger in all three kidneys. However, the greatest exacerbation of the cystic phenotype is seen in the *Fh1*$^{-/-}$*/ Hif-1α*$^{-/-}$ kidney.

3.2. Effects of *Fh1*, *Hif-1α* and *Hif-2α* expression on proliferation in the kidneys

As illustrated in the previous section, transformation of a normal proximal convoluted tubule or a distal convoluted tubule into a cystic tubule requires dilation of the tubule and an increase in diameter. One of the possible explanations for a mechanism of cyst formation in the kidneys analysed in this study can be hypothesized to be a proliferation effect. Previously loss of *Fh1* has been shown to increase proliferation (Pollard et al., 2007). Therefore, sections of kidneys for all the different genotypes (genotypes stated previously) were stained for Ki67 using immunohistochemistry. Ki67 protein is a proliferation marker which is expressed in the nucleus of a proliferating cell. It can be detected in the cell at all phases of the cell cycles except G_0 which is a phase when the cell is not dividing and therefore not proliferating (Scholzen and Gerdes, 2000).

Proliferation in *Fh1*$^{-/-}$, *Hif-1α*$^{-/-}$, *Hif-2α*$^{-/-}$, *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys are analysed and examples of Ki67 stained sections of kidneys from each genotype are shown in **Figure 6.** For each slide, 10 non cystic, 10 micro cystic and 10 macro cystic tubules were chosen randomly. For each tubule, the total number of nuclei and the number of positive nuclei were counted. The mean and standard deviation of positive nuclei per tubule was calculated and was used to perform 2-tailed t-tests to make comparisons in the expression of Ki67 between different types of cysts (micro and macro) and normal tubules within a genotype, and between different genotypes.

3.2.1. Ki67 expression in wild type and Fh1$^{-/-}$ kidneys

Ki67 expression in *Fh1*$^{-/-}$ kidneys was analysed by immunohistochemistry (**Table 11a**). It was hypothesized that the *Fh1*$^{-/-}$ kidneys will have higher proliferation rate compared to wild type kidneys since knocking out the *Fh1* gene results in

proliferative cyst formation in the kidneys (as previously shown) (Pollard et al., 2007). Ki67 expression is seen in one or two nuclei per tubule wild type kidneys and the positive staining is not present in all of the tubules. Examples of Ki67 expression in wild type mouse kidney at twenty four weeks of age is shown in **Figure 6**. In the *Fh1*$^{-/-}$ kidneys, Ki67 expression is observed as two or more positive nuclei per tubule. In addition, most of the positive staining was seen in dilated tubules.

Positive and total nuclei per tubule were counted in both wild type and *Fh1*$^{-/-}$ kidneys and mean values were calculated as described above. In order to test whether there was a significant difference in proliferation between kidneys with different genotypes analysed in this study, two tailed t-test analysis was performed on these values. Ki67 expression was also compared between tubules with different phenotypes. In *Fh1*$^{-/-}$ kidneys with ages varying from nine weeks to thirty three weeks, Ki67 expression was significantly higher in micro cystic tubules (p-value= 9.9×10^{-5}) and macro cystic tubules (p-value=0.027) compare to non-cystic tubules. Therefore, this result is highly suggestive of a difference in proliferation between cystic and non-cystic epithelium in tubules when *Fh1* is knocked out in the kidneys. This corroborates the previous description of *Fh1* knockout kidneys and therefore we utilised these parameters and methods for all subsequent analyses of the *Fh1/Hif* crosses (Pollard et al., 2007). Comparison of proliferation between micro cysts and macro cysts, which was done by pooling data from all the cysts across all the ages, showed that there was not any statistically significant difference between the two different types of cysts (p-value=0.11).

3.2.2. Ki67 expression in *Hif-1α*$^{-/-}$ and in *Hif-2α*$^{-/-}$ kidneys

As described previously a t-test analysis was performed to identify any significant difference in proliferation due to different parameters in kidneys such as

genotype, presence or absence of cysts and age. When kidneys with *Hif-1α*$^{-/-}$ and in *Hif-2α*$^{-/-}$ genotypes were analysed, it was seen that the one or two nuclei per tubule were positive for Ki67. This positivity does not correlate with tubular dilatation or cystic formation since *Hif-1α*$^{-/-}$ and in *Hif-2α*$^{-/-}$ genotypes did not lead any cystic phenotype in the kidneys. Due to the absence of micro and macro cysts in these two different groups of kidneys, t-test analysis could not be performed. The pattern of Ki67 positivity observed in kidneys with *Hif-1α*$^{-/-}$ and *Hif-2α*$^{-/-}$ genotypes is identical to the Ki67 positivity pattern seen in wild type kidneys. Therefore, it can be suggested that knocking out *Hif-1α* and *Hif-2α* genes does not cause a direct proliferation effect in proximal or distal convoluted tubules of the kidneys. Other evidence supporting this conclusion is the absence of cysts in these kidneys.

3.2.3. Ki67 expression in *Fh1*$^{-/-}$ / *Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$ / *Hif-2α*$^{-/-}$ kidneys

The overall Ki67 positivity in *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidneys was higher when compared to wild type, *Fh1*$^{-/-}$, *Hif-1α*$^{-/-}$ and *Hif-2α*$^{-/-}$ kidneys. The semi-quantitative scoring of the kidneys showed that there are more than three Ki67 positive nuclei per tubule and that this number went up to thirteen per nuclei, although there was variation between tubules. Although there is Ki67 positivity in non-cystic tubules too, dilated tubules had higher number of Ki67 positive nuclei. In addition, staining is more intense in some of the cystic tubules, although this is not observed in all of the cystic tubules. An example of Ki67 stained *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidney from a twenty two weeks old mouse with the characteristics as explained above is shown in **Figure 6.**

When the Ki67 expression is compared between different types of cysts and between cystic and non-cystic tubules, it is seen that the statistical analysis of the data is suggestive of significant differences between certain variables. The mean number of positive nuclei was significantly higher in macro cysts (p value= 0.032) and micro cysts

(p value= 0.035) compared to the non-cystic tubules in *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidney. This is indicative of higher proliferation in both macro and micro cystic tubules compared to the non-cystic tubules despite having the same genotype. When the mean positive nuclei in macro cystic tubules is compared to the micro cystic tubules, a statistical difference was observed between the two groups (p value= 0.025) as well.

Tubules of *Fh1*$^{-/-}$/*Hif-2α*$^{-/-}$ kidneys are also positive for Ki67. Approximately four nuclei per cystic tubule expressed Ki67 although this number can vary between cystic and non-cystic tubules. Ki67 positivity is mostly concentrated in the cystic area of the kidney and non-cystic tubules have less Ki67 positive nuclei with an average of one or two positive nuclei per tubule. The level of Ki67 expression per cystic tubule is not as high as the expression level in *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidneys.

3.2.4. Comparison of Ki67 expression based on different parameters

The kidneys analysed were divided into two groups according to the age of the mice they were taken from: one of the groups consisting of mice younger than 14 weeks and the second group of mice older than fourteen weeks. Both of the groups contained mice with the following genotypes: wild type, *Fh1*$^{-/-}$, *Hif-1α*$^{-/-}$, *Hif-2α*$^{-/-}$, *Fh1*$^{-/-}$ / *Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$ / *Hif-2α*$^{-/-}$. Kidneys which came from mice younger than fourteen weeks show both cystic and non cystic tubules. However, the cysts are only micro in character and no macro cysts are present in these kidneys. When the Ki67 expression is compared between non-cystic tubules and micro cystic tubules in mice less than fourteen weeks of age, it was seen that the proliferation was significantly higher in the micro cysts as compared to the non-cystic tubules (p value= 0.024). When kidneys were analysed from mice older than fourteen weeks, macro cysts in addition to the micro cysts and non-cystic tubules were observed. The difference in Ki67 expression was higher in macro cysts compared to the expression levels in non-cystic tubules (p

value= 0.00047). The same pattern is also observed for the micro cysts (p-value= $1.85x10^{8}$). In addition, when the expression levels in macro cysts were compared to the levels in micro cysts, Ki67 expression was higher in macro cysts than in micro cysts (p value= 0.013). These results suggest that in addition to cystic tubules having higher proliferation compared to non-cystic tubules, macro cysts also have higher proliferation compared to micro cysts.

When Ki67 expression levels were compared between kidneys with different genotypes, none of the p values suggested a statistically significant difference in Ki67 expression between the different genotypes stated above.

3.3. Effects of *Fh1*, *Hif-1α* and *Hif-2α* expression on DNA damage (γH2AX expression levels)

H2AX is a histone protein which has an important role in DNA damage response. When a DNA double strand break takes place, serine 139 at the carboxyl terminal of H2AX gets phosphorylated in response to DNA damage. The phosphorylated form of the protein is called γH2AX. Therefore, γH2AX also acts as a damage marker for double stranded DNA (Fernandez-Capetillo et al., 2004). DNA damage is suggested to have role in tumour formation and increasing proliferation. H2AX has also been suggested to be involved in hypoxia response and to cause endothelial cell proliferation as a consequence (Celeste et al., 2003, Economopoulou et al., 2009). After observing the increased proliferation, specifically in the cystic tubules as compared to non-cystic tubules, and in the light of the studies mentioned above, it was hypothesized that DNA damage could be important in explaining why certain genotypes lead to cyst formation in kidneys. Furthermore, a recent study by Yogev et al., identified a direct role of FH in the DNA damage pathway (Yogev et al., 2010). DNA damage was analysed by immunohistochemistry (IHC) in kidneys from mice of different ages and different

genotypes such as wild type, *Fh1*$^{-/-}$, *Hif-1α*$^{-/-}$, *Hif-2α*$^{-/-}$, *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-2α*$^{-/-}$ using γH2AX as a marker for double stranded DNA damage (**Table 11b**). Sections of kidneys from each genotype stained with γH2AX are shown in **Figure 7.** Again, for each slide, 10 non cystic, 10 micro cystic and 10 macro cystic tubules were chosen randomly. For each tubule, the total number of nuclei and the number of positive nuclei were counted. The mean and standard deviation of positive nuclei per tubule was calculated and was used to perform 2-tailed t-tests to make comparisons in the expression of γH2AX between different types of cysts (micro and macro) and normal tubules within a genotype, and between different genotypes.

3.3.1. Expression of γH2AX in wild type and *Fh1*$^{-/-}$ kidneys

IHC was used to detect DNA damage in wild type and *Fh1*$^{-/-}$ kidneys using γH2AX as a marker (nuclear staining). Since it is known that *Fh1*$^{-/-}$ kidneys show a cystic phenotype compared to the wild type kidneys, it was hypothesized that γH2AX expression would be higher in *Fh1*$^{-/-}$ kidneys. Wild type kidneys which were free of any cysts and dilated ducts were negative for γH2AX. An example of a wild type kidney from a twenty four week old mouse stained for γH2AX is shown in **Figure 7.**

Occasional γH2AX positivity is seen in *Fh1*$^{-/-}$ kidneys. Although some of the dilated tubules and cysts are positive for γH2AX, not all cysts have γH2AX expression. When distal convoluted tubules are compared with the proximal convoluted tubules, distal convoluted tubules have a higher number of positive nuclei than the proximal convoluted tubules. The intensity of the γH2AX staining is higher around the cystic tubules. γH2AX staining in a *Fh1*$^{-/-}$ kidney from a 22 week-old mouse is shown in **Figure 7**.

In order to analyze the γH2AX expression patterns more accurately and in more detail, the method used for semi-quantitative analysis of Ki67 expression was also

used for γH2AX. Numbers of nuclei stained positively for γH2AX per cystic and non-cystic tubule were counted and the mean value for each kidney was calculated. Two tailed t-test analysis was performed to identify any correlation between different genotypes, the consequent phenotype and γH2AX expression. In *Fh1*$^{-/-}$ kidneys, the number of nuclei expressing γH2AX is significantly higher in micro cystic kidneys compared to the non-cystic kidneys (p-value = 0.0024). When macro cysts are compared to non-cystic tubules, t-test analysis shows no significant difference (p value=0.079) in DNA damage inferred by the number of nuclei that stained with γH2AX. Also, no significant difference is seen when γH2AX expression is compared between macro cysts and micro cysts (p value= 0.38).

3.3.2. Expression of γH2AX in *Hif-1α*$^{-/-}$ and in *Hif-2α*$^{-/-}$ kidneys

When *Hif-1α*$^{-/-}$ and in *Hif-2α*$^{-/-}$ kidneys were analysed, the main feature they had in common was the absence of cysts. A similar pattern of γH2AX staining (mostly negative) was observed for both of the genotypes. Staining was seen mostly in distal convoluted tubules with only occasional positivity present in proximal convoluted tubules. It was also seen that these kidneys had the same staining characteristics regardless of the age of the mice. Although some of the tubules had positive staining, the intensity of the staining was weak. t-test analysis was not performed for the kidneys with *Hif-1α*$^{-/-}$ and in *Hif-2α*$^{-/-}$ genotypes since they did not have any cysts. When absence of cysts is evaluated together with the non specific and low intensity staining in those samples, it can be suggested that *Hif-1α*$^{-/-}$ or *Hif-2α*$^{-/-}$ does not cause any DNA damage which can be detected with γH2AX. However, it not possible to completely rule out a possible DNA damage effect since markers other than γH2AX can also be used to detect DNA damage.

3.3.3. Expression of γH2AX in *Fh1*$^{-/-}$ / *Hif-1α*$^{-/-}$ and Fh1$^{-/-}$ / Hif-2α$^{-/-}$ kidneys

As explained previously, the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ genotype results in an exacerbation of cyst formation in the kidneys. The cystic phenotype is more aggressive when compared to the number and character of the cysts in *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys. The γH2AX staining follows a similar pattern. Staining is mostly concentrated in the cystic epithelium and the intensity of the staining is also greater around the cysts compared to the non-cystic tubules. γH2AX staining in a *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidney from a seventeen weeks old mouse is shown in **Figure 7.** The staining pattern is the same at different ages in kidneys with this genotype. Nuclei positive for γH2AX are also mostly seen in cysts in *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys and the intensity of the staining is increased around the cysts.

T-test analysis for the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys show that micro cysts (p value= 0.0068) have significantly higher γH2AX positivity than the non-cystic tubules. There is also a significant difference in γH2AX expression between macro cystic and non-cystic tubules (p-value = 0.015). When expression in micro cysts was compared with the expression in macro cysts, the mean for γH2AX expression was higher in macro cysts than in micro cysts (p value= 0.015). However, when the same analysis was done for the *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys, it was seen that there was not any significant difference in γH2AX between micro cysts and non-cystic tubules (p-value= 0.078).

3.3.4. Comparison of γH2AX expression based on different parameters

As for the analysis performed for Ki67, the kidneys analysed were divided into two groups according to the age of the mice from which they were taken: one group consisting of mice younger than 14 weeks and the second group of mice older than fourteen weeks. Both of the groups contained mice with the following genotypes: wild type, *Fh1*$^{-/-}$*, Hif-1α*$^{-/-}$ **,** *Hif-2α*$^{-/-}$**,** *Fh1*$^{-/-}$ */ Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$ */ HIF-2α*$^{-/-}$. Kidneys which come from mice younger than fourteen weeks have both cystic and non cystic tubules. However, no macro cysts were present in these kidneys. In 'younger than fourteen

weeks old' group, DNA damage marker expression is higher in micro cysts compared to the non-cystic tubules (p-value = 0.01). Kidneys from mice older than fourteen weeks old have higher γH2AX expression both in macro cysts (p value=0.0022) and micro cysts (p value=0.0007) compared to non-cystic tubules. There was also a significant increase in expression level in macro cysts compared to micro cysts (p-value= 0.041). When γH2AX positivity was compared between different genotypes, it was seen that there was no significant difference in γH2AX expression between *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ genotypes (p value= 0.1 for macro cysts and p value= 0.7 for micro cysts). The same result was also true for the comparison between the *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/ Hif-2α*$^{-/-}$ genotypes (p value=0.42 for micro cysts). Comparing *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ genotype with *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ showed no significant difference either (p-value=0.55). This is similar to what was observed in Ki67 expression comparisons. In that any differences seem to be solely due to loss of *Fh1*.

Finally we analysed HLRCC renal papillary tumours (n=3) for γH2AX and saw a large increase in γH2AX in tumour tissue compared to normal tissue **Figure 8.**

In the light of the results explained above, we can conclude that *in vivo* loss of *Fh1* (in renal tubules) increases H2AX and therefore suggestive of a role in DNA damage pathway.

After analysing proliferation and DNA damage in the kidneys, apoptosis was also investigated using Caspase-3. There were one or two positive nuclei per kidney section regardless of the genotype of the kidneys and the age of the mice the kidneys were taken from. This shows that although the IHC has worked successfully, there is no significant apoptosis in the kidneys analysed in this study. This result is similar to the previously reported studies on role of apoptosis in cystic kidneys (Pollard et al., 2007). Therefore, the data on apoptosis is not reported in further detail in the thesis.

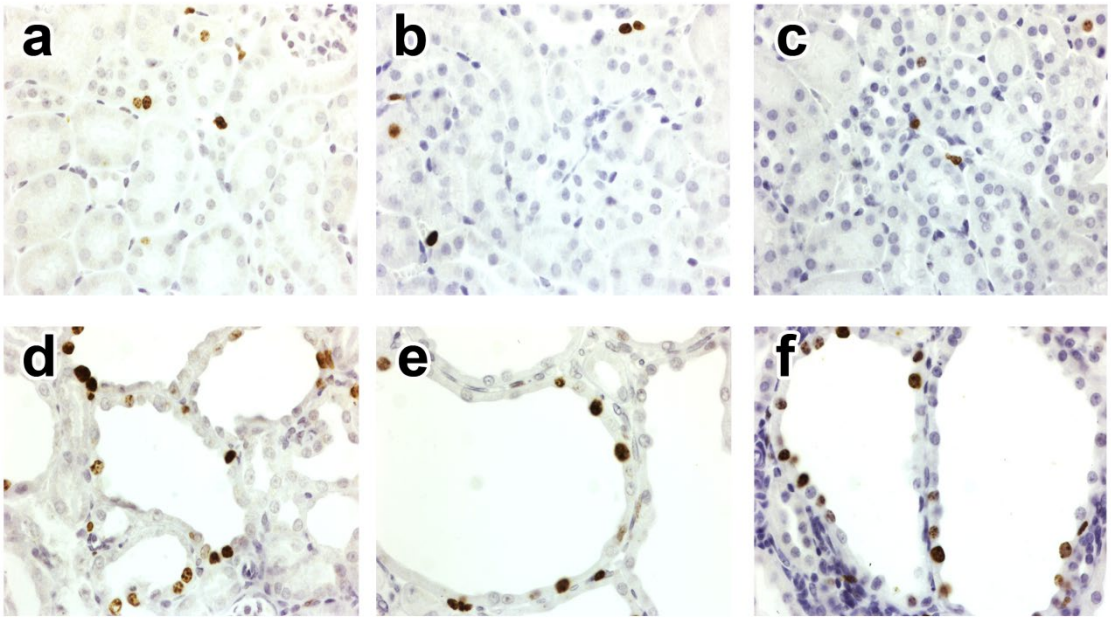


Figure 6: Ki67 stained sections of kidneys

(a-c) show wild type, *Hif-1α*$^{-/-}$ and *Hif-2α*$^{-/-}$ kidneys respectively, at 30 weeks of age. These kidneys display occasional positivity with strong intensity. **(d-f)** show *Fh1*$^{-/-}$, *Fh1*$^{-/-}$ / *Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$/ *Hif-2α*$^{-/-}$ kidneys respectively, aged between 20-22 weeks. These cystic kidneys are positive for Ki67. Expression is mostly located in the epithelial cells lining the cysts.

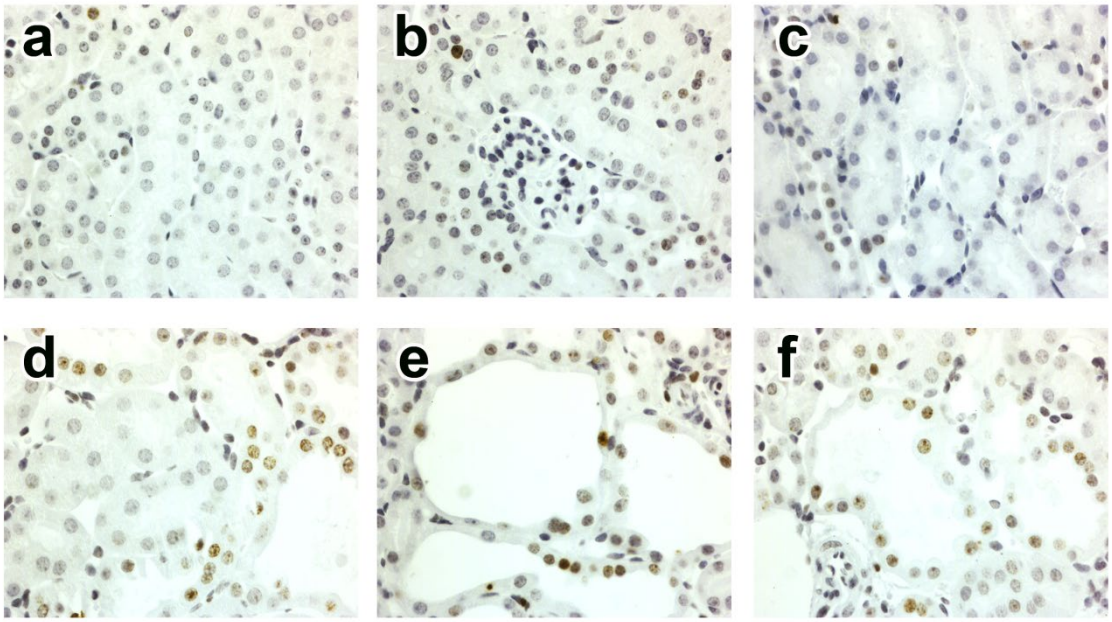


Figure 7: Kidney sections showing γH2AX staining

(a-c) show wild type, *Hif-1α*$^{-/-}$ and *Hif-2α*$^{-/-}$ kidneys respectively, at 30 weeks of age. There is no γH2AX in these kidneys which are free of cysts. **(d-f)** show *Fh1*$^{-/-}$, *Fh1*$^{-/-}$/ *Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$/ *Hif-2α*$^{-/-}$ kidneys respectively at aged between 16-18 weeks. The γH2AX expression is present in those kidneys which are cystic and is mostly located in the epithelial cells lining the cysts. The intensity of the staining also increases in these epithelial cells.

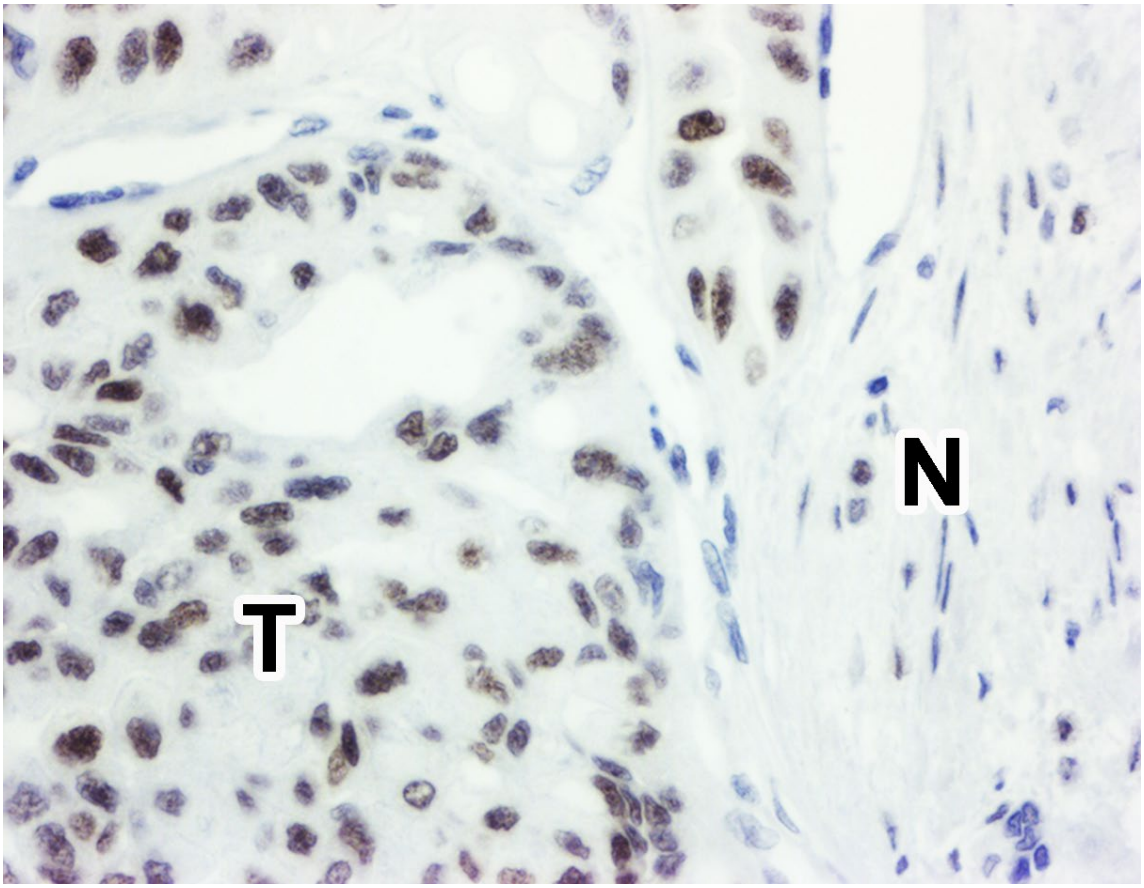


Figure 8: Human Type II papillary cancer stained with γH2AX

(Magnification x400) The tumour is marked as 'T' and the normal tissue is marked as 'N'. γH2AX is expressed widely in the human Type II papillary cancer tissue which has FH mutations. However, it is not expressed in the normal tissue.

						MACRO CYSTIC			MICRO CYSTIC			NON CYSTIC		
GENOTYPE						POS NUCLEI			POS NUCLEI			POS NUCLEI		
FH	HIF1	HIF2	KSP	SEX	AGE	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev
fl/+	fl/fl	+/+	-	F	12.5							125	0.5	0.71
fl/+	fl/+	+/+	+	M	24							131	0.80	13.10
fl/fl	fl/+	+/+	+	F	9							173	1.3	1.25
fl/fl	+/+	fl/+	+	F	13				356	2.9	3.03	105	0.4	0.7
fl/fl	+/+	fl/+	+	F	13							114	1.1	1.45
fl/fl	+/+	fl/+	+	F	13				356	2.9	3.03	105	0.4	0.7
fl/fl	fl/+	+/+	+	M	17				255	2.3	3.06	101	0.5	0.85
fl/fl	fl/+	+/+	+	F	22				356	3.44	2.24	100	0.3	0.67
fl/fl	flf/+	+/+	+	M	24	546	17	10.03	356	3.9	3.14	146	0.1	0.32
fl/fl	fl/+	+/+	+	F	32-33	627	3.2	3.96	375	2.3	2.06			
fl/+	fl/fl	+/+	+	F	13							120	0.6	0.97
fl/+	fl/fl	+/+	+	F	18							122	0.8	1.03

GENOTYPE						MACRO CYSTIC POS NUCLEI			MICRO CYSTIC POS NUCLEI			NON CYSTIC POS NUCLEI		
FH	HIF1	HIF2	KSP	SEX	AGE	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev
fl/+	fl/fl	+/+	+	M	22							113	0.3	0.67
fl/+	+/+	fl/fl	+	F	12.5							143	0.5	0.71
+/+	+/+	fl/fl	+	F	27-28							197	0.3	0.48
fl/fl	fl/fl	+/+	+	F	9							131	0.7	0.95
fl/fl	fl/fl	+/+	+	F	13				178	1.4	1.43	101	0.5	0.71
fl/fl	fl/fl	+/+	+	F	17	929	8.63	3.07	329	2.5	1.72			
fl/fl	fl/fl	+/+	+	F	22				348	4.6	4.53	119	0.3	0.95
fl/fl	fl/fl	+/+	+	M	24	1849	15.50	8.51	410	4.8	4.34			
fl/fl	fl/fl	+/+	+	M	32-33	1887	5.6	3.69	397	1.9	2.13			
fl/fl	+/+	fl/fl	+	F	12.5							158	0.4	0.7
fl/fl	+/+	fl/fl	+	M	13				230	1.2	1.4	96	0.3	0.67
fl/fl	+/+	fl/fl	+	M	18				513	4.6	2.91	132	0.3	0.67

GENOTYPE						MACRO CYSTIC			MICRO CYSTIC			NON CYSTIC		
						POS NUCLEI			POS NUCLEI			POS NUCLEI		
FH	HIF1	HIF2	KSP	SEX	AGE	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev
fl/fl	+/+	fl/fl	+	M	18				348	4	2.05	87	0.4	1.2
fl/fl	+/+	fl/fl	+	M	18				519	5.4	8.54	116	0.3	0.48
fl/fl	+/+	fl/fl	+	F	27-28	981	2	3.39	534	2.8	2.53			

Table 11a: IHC scoring details for Ki67 staining.

The age in weeks and sex of the mice (F= female, M=male) and genotypes of the kidneys used in IHC are shown in the table above. As described in the Methods, for each kidney section, 10 tubules of each phenotype (normal, micro cystic and macro cystic) were identified randomly. Within each of these tubules (normal, micro cystic and macro cystic) the total number of nuclei was counted and is included. Also, shown in the table is mean number of nuclei positive for Ki67, together with the standard deviation, per tubule. This allows for a comparison of different cystic phenotypes within one genotype at different ages and also between genotypes.

GENOTYPE						MACRO CYSTIC			MICRO CYSTIC			NON CYSTIC		
						POS NUCLEI			POS NUCLEI			POS NUCLEI		
FH	HIF1	HIF2	KSP	SEX	AGE	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev
fl/+	fl/+	+/+	+	M	24							90	0.00	9.00
fl/+	fl/fl	+/+	-	F	12.5							134	1.8	3.16
fl/fl	fl/+	+/+	+	F	9							120	0.36	0.5
fl/fl	+/+	fl/+	+	F	13							183	0.9	1.29
fl/fl	+/+	fl/+	+	F	13				275	4	2.49	115	0.3	0.48
fl/fl	fl/+	+/+	+	M	17				197	2	1.7	119	0.5	0.85
fl/fl	fl/+	+/+	+	F	22				149	2.9	1.45	111	0.3	0.67
fl/fl	flf/+	+/+	+	M	24	542	0.5	1	188	2.2	3.26	117	0.1	0.32
fl/fl	fl/+	+/+	+	F	32-33	625	2.4	0.89	269	0.9	0.88			
fl/+	fl/fl	+/+	+	F	18							124	0.5	0.85
fl/+	fl/fl	+/+	+	F	13							106	2.5	3.24
fl/+	fl/fl	+/+	+	M	22							102	2.4	4.1

						MACRO CYSTIC			MICRO CYSTIC			NON CYSTIC		
GENOTYPE						POS NUCLEI			POS NUCLEI			POS NUCLEI		
FH	HIF1	HIF2	KSP	SEX	AGE	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev
fl/+	+/+	fl/fl	+	F	12.5							112	1.5	2.22
+/+	+/+	fl/fl	+	F	27-28							168	1.2	1.87
fl/fl	fl/fl	+/+	+	F	9							110	0.6	0.7
fl/fl	fl/fl	+/+	+	F	13				118	1.8	1.87	101	0.2	0.42
fl/fl	fl/fl	+/+	+	F	17	1003	8.13	4.49	310	3	2.94			
fl/fl	fl/fl	+/+	+	F	22				151	2.9	1.85	112	0.3	0.95
fl/fl	fl/fl	+/+	+	M	24	972	3.70	2.67	143	1.5	1.27			
fl/fl	fl/fl	+/+	+	M	32-33	1517	5.2	4.42	279	1.6	1.58			
fl/fl	+/+	fl/fl	+	F	12.5							121	1.3	1.89
fl/fl	+/+	fl/fl	+	M	13				182	3.9	2.73	115	0.3	0.48
fl/fl	+/+	fl/fl	+	M	18				295	1.3	1.63	110	0.3	0.48
fl/fl	+/+	fl/fl	+	M	18				360	0.6	0.69	128	0.3	0.48

GENOTYPE						MACRO CYSTIC POS NUCLEI			MICRO CYSTIC POS NUCLEI			NON CYSTIC POS NUCLEI		
FH	HIF1	HIF2	KSP	SEX	AGE	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev
fl/fl	+/+	fl/fl	+	F	18				262	0.9	1.19	147	0.2	0.42
fl/fl	+/+	fl/fl	+	F	27-28	1341	2	1.26	319	2	1.49			

Table 11b: IHC scoring details for γH2AX staining

The age in weeks and sex of the mice (F= female, M=male) and genotypes of the kidneys used in IHC are shown in the table above. As described in the Methods, for each kidney section, 10 tubules of each phenotype (normal, micro cystic and macro cystic) were identified randomly. Within each of these tubules (normal, micro cystic and macro cystic) the total number of nuclei was counted and is included. Also, shown in the table is mean number of nuclei positive for γH2AX, together with the standard deviation, per tubule. This allows for a comparison of different cystic phenotypes within one genotype at different ages and also between genotypes.

3.4. Microarray Analysis

The striking cystic phenotype observed due to the combined renal-specific deletion of *Fh1* and *Hif-1α* led to further in-depth investigations. Genome wide microarray analysis was employed in order to quantify the relative abundance of mRNA transcripts between the various genotypes of the mice. As the greatest phenotypic differences were observed between the $Fh1^{-/-}$, $Fh^{1-/-}$/$Hif\text{-}1a^{-/-}$ and wild type mice we decided to compare gene expression profiles of these genotypes.

Raw data obtained from the microarray experiment was initially analysed by using the LIMMA bioinformatics programme to determine the differential expression between different genotypes (Smyth, 2005). For each genotypic comparison, upregulated and downregulated genes were identified. The genes in both groups which were statistically differentially expressed (p-value less than 0.05) were identified both ranked based on fold changes in their expression.

After analysing the differential gene expression between different genotypes as described above, Gene Set Enrichment Analysis (GSEA) programme was used to generate biological processor gene groups by using the raw data from the microarray experiment (Subramanian et al., 2005). Two different GSEA analyses were performed by sorting the raw data in different ways. By default, the GSEA programme assigns genes together in groups on the basis of whether they are upregulated or downregulated. Therefore, the output files generated show genes associated with each other in the upregulated or in the downregulated gene list. However, this may not be a completely true reflection of biological events since in any biological pathway some genes can be upregulated and some will be downregulated. In order to be able to see all the differentially expressed genes which are associated with each other regardless of being upregulated or downregulated, the gene lists in raw data were prepared again

by ranking the genes based on absolute t-statics which only takes into account the differential expression of the gene rather than the negative or positive expression levels of the genes.

Gene expression array experiments generate complex 'gene lists' i.e. genes that are differentially expressed between each group. The gene lists described here were used to create biological processor and Kegg (Kyoto Encylopedia of Genes and Genomes) pathway lists. The biological process list is created by the GSEA programme by grouping the genes according to biological pathways they are involved in and then ranking them based on statistical significance of their expression within that subset (Joslyn et al., 2004). The Kegg pathway analysis shows the role of the differentially expressed genes in higher order function and groups the genes together based this function and the cellular pathway they are involved in (Kanehisa and Goto, 2000).

In summary, the microarray analysis showed that in a comparison between *Fh1*$^{-/-}$ kidneys and wild type kidneys, genes involved in glycolysis and the immune response are upregulated, whereas genes involved in oxidative phosphorylation are downregulated. In the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys, cell cycle genes (especially *Ccnd1*) and immune response genes were upregulated compared to wild type kidneys. Comparison of *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys to *Fh1*$^{-/-}$ kidneys showed that cell cycle control genes, genes involved in glutathione metabolism and cell organelle organization and filament structure are significantly upregulated.

Tables of the significantly differentially expressed genes and pathways are attached in the Appendix.

3.4.1. *Fh1*$^{-/-}$ versus wild type comparison

The LIMMA gene lists show that there are 1194 genes which were significantly upregulated in the *Fh1* knocked out kidneys compared to the wild type kidneys. When these genes are ranked on the basis of average expression levels, it is seen that the highest upregulated gene was 15.4 times higher in the knock-out kidneys than in the wild type kidneys. The lowest average expression level in this list of significantly differentially expressed gene list is 7.4. Since over a thousand genes are significantly up regulated, only those genes ranked highest in the list will be discussed in more detail. A summary of these results are provided in **Table 12**.

The highest ranked upregulated genes include *S100 calcium binding protein A11* (*calgizzarin* or also called *S100a11*), *lactate dehydrogenase A* (*Ldha*), *enolase 1* (*Eno1*), *phosphoglycerate kinase* (*Pgk1*), *glutathione S-transferase pi-1* (*Gstp1*) and *glutathione S-transferase mu 2* (*Gstm2*), *phosphofrutokinase* (*Pfk*), *solute carrier family 2* (*facilitated glucose transporter*) *membrane 1* (*Slc2a1*) and *pyruvate kinase M2* (*Pkm2*). An important aspect of these highest upregulated genes is that most of them are involved in metabolic pathways. Indeed many of the upregulated genes in the remainder of the list are involved in metabolic pathways. Examples of these genes include *aldehyde dehydrogenase 1 family L2* (*Ald1l12*), *hexokinase 2* (*Hk2*), carbonic *anhydrase 15* (*Ca15*) and *aldolase 3 C* isoform (*Aldoc*).

Many of the upregulated genes are also involved in the immune system. Some examples of these genes include *lipocalin 2* (*Lcn2*), various interferon induced transmembrane protein coding genes, *interferon regulatory factor 1* (*Irf1*) and *CD47*.

In addition to the metabolism and immune response, related genes, genes which are involved in oncogenesis are also significantly up regulated. Examples are *mitogen activated protein kinase kinase kinase 1* (*MAPKKK*1), *mitogen activated protein kinase*

kinase kinase 3 (*MAPKKK3*), *Rab8b* which is a member of the RAS oncogene family, other members of the RAS family of oncogenes and *GATA binding protein 3* (*Gata3*).

There are 527 genes which are significantly downregulated in the kidneys in which *Fh1* is deleted compared to the wild type kidneys. The most significantly downregulated gene on the list is expressed 16.1 times less than in the knocked out kidneys compared to the wild type kidneys. The most significantly downregulated genes in the list include *serine peptidase inhibitor*, *Kazal type 3* (*Spink3*), *kidney expressed gene 1*(*Keg1*), *ferritin heavy chain 1* (*Fth1*), *insulin-like growth factor binding protein 7* (*Igfbp7*), *ubiquitin B* (*Ubb*), *glutathione peroxidise 3* (*Gpx3*), *NADH dehydrogenase* (ubiquinone) *1 alpha subcomplex 4* (*Ndufa4*), *isocitrate dehydrogenase 3* (*NAD+*) *gamma* (*Idh3g*), *ornithine decarboxylase*, *structural 1* (*Odc1*), *fructose bisphosphatase 1* (*Fbp1*) and *NADH dehydrogenase* (*ubiquinone*) *1 subcomplex unknown, 2* (*Ndufc2*). Similar to the results seen in the upregulated gene list, most of the downregulated genes are involved in metabolic pathways. This suggests that knocking out the *Fh1* gene up regulates some metabolic genes while down regulating other metabolic genes.

GSEA biological processor analysis, which was done taking into account both up and downregulated genes, shows that there are statistically significant biological processor gene groups. In other words, although there is a high number of genes which are statistically significantly differentially expressed between *Fh1* knock-out kidneys and wild type kidneys, these genes cannot be grouped into statistically significant groups with related biological functions when up or down regulation in their expression level is not taken into account. However, when the statistical significance boundary is released to a p-value of less than 0.1 rather than rather than 0.05, it is seen that 21 gene groups are identified. The most significant of the groups is formed by genes involved in epidermis development. The other examples from those 21 gene groups include response to nutrient levels, carbohydrate catabolic process, glucose metabolic response which are related to mitochondrial function and metabolism. In

addition, a few of those 21 gene groups were immune related groups such as defence response and inflammatory response. When the most significantly differentially expressed genes are analysed without being grouped based on biological processor function, the highest ranked ones include *aldo-keto reductase family 1 member B8* (*Akr1b8*), *aldehyde dehydrogenase 1 family member L2* (*Aldh1l2*), *aldehyde dehydrogenase family 1 subfamily A1* (*Aldh1a1*), *tribbles homolog 3* (*Trib3*), *lipocalin 2* (*Lcn2) , glucose phosphate* isomerase 1 (*Gpi1*), *cyclin-dependent kinase inhibitor 1A* (*Cdkn1a*), *FOS-like antigen 2* (*Fosl2*), *Eno2, Pdk1, Egln3* and *Pkm2*.

GSEA biological processor analysis was also done by using upregulated and downregulated genes lists separately. Analysis of upregulated genes show that they form 123 gene groups where each group consists of genes with similar biological processor function. These 123 gene groups contain groups with different functions. However majority of the most significantly upregulated groups are immune system related. For instance 5 out of the 10 highest ranked biological processor groups are involved in immune response. These groups are inflammatory response, defence response, immune system process, leukocyte activation and immune response gene groups. There are many other significantly upregulated immune system related gene groups in the rest of the list as well. Examples of some of these include regulation of lymphocyte activation, humoral immune response, T cell activation and immune system development gene groups. Genes which are commonly and most significantly upregulated in these groups are l*ysozyme* (*Lyz*), *chemokine (C-X-C motif) receptor 4* (*Cxcr4*), *transforming growth factor beta 1* (*Tgfβ1*), *chemokine (C-X3-C motif) ligand 1* (*Cx3cl1)*, *thymus cell antigen 1 theta* (*Thy1*), *inhibin beta-A (Inhiba*) and *interleukin 7* (*IL7*). Two other very important gene groups which are very significantly upregulated and ranked in the top 10 of the significantly upregulated biological processor gene groups include regulation of cell proliferation and positive regulation of cell proliferation groups. The most significantly upregulated genes which are involved in proliferation are

cyclin-dependent kinase inhibitor 1A (*P21*) (*Cdkn1a*) and *T-cell, immune regulator 1* (*Tcirg1*). Analysis also suggest that various cell signalling pathways such as small GTPase mediated signal transduction pathway, Ras protein signal transduction pathway, Rho protein signal transduction pathway and positive regulation signal transduction biological processor gene groups are upregulated in the kidneys when *Fh1* is knocked out. Harvey rat sarcoma oncogene subgroup R (*Rras*), *ras homolog gene family member B* (*Rhob*), *Rho GDP dissociation inhibitor* (*Gdi) gamma* (*Arhgdig*) and *RAS guanyl releasing protein 1* (*Rasgrp1*) are significantly and commonly upregulated in these pathways.

Although many gene groups are significantly upregulated in the *Fh1* deficient kidneys compared to wild types, only 3 biological processor gene groups are downregulated in the *Fh1* knock out kidneys. These are energy derivation by oxidation of organic compounds, cellular respiration and anion transport groups. Specific genes from these groups are *peroxisome proliferative activated receptor gamma coactivator 1* α (*Ppargc1α*), *solute carrier family 25* (*mitochondrial carrier, brain*), *member 14* (*Slc25a4*), *chloride intracellular channel 1* (*Clic1*) and *surfeit gene 1* (*Surf1*).

GSEA Kegg pathway analysis was performed by using the two differently ranked gene lists as described above. When the significantly differentially expressed gene list, without taking up or downregulation of the genes in to account, was used 3 significant pathways are analysed: glycolysis and gluconeogenesis, carbon fixation and complement and coagulation cascade pathways. *Pkm2, Aldoa, Pgk1*, *transketolase* (*Tkt*) and *Aldoc* are significant and common in glycolysis and gluconeogenesis and carbon fixation pathways. Identification of Kegg pathways showed that 50 are significantly upregulated. T cell receptor signalling pathway, renal cell carcinoma and MAPK signalling pathways are the top 3 significantly upregulated pathways. Some of the upregulated genes in these pathways are (*Slc2a1*), *HIF-1α*, *Egln3*, *phosphoinositide-3-kinase*, *regulatory subunit 3 γ* (*Pik3r3*), *DNA-damage-inducible*

transcript 3 (*Ddit3*), *Pdk1* and *Rras*. Glycolysis and gluconeogenesis, VEGF signalling, Notch signalling, p53 signalling, actin cytoskeleton, cell cycle and several immune related pathways such as Toll like receptor signalling, B cell receptor signalling and natural killer cell cytotoxicity pathways are significantly up regulated. On the other hand, 7 pathways are significantly downregulated. The two most significantly downregulated pathways are oxidative phosphorylation and valine, leucine and isoleucine degradation pathways.

3.4.2. *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ versus wild type comparison

A comparison of gene expression between *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and wild type kidneys (**summarised in Table 13**) shows that 2816 genes were significantly upregulated in double knockout kidneys. The most significantly and highest upregulated gene is *ribosomal protein S8* (*Rps8*). The majority of genes in the most highly expressed 60 genes code for a type of ribosomal protein. Examples of some of these genes are *ribosomal protein S4 X-linked* (*Rps4x*), *ribosomal protein large p2* (*Rplp2*), *ribosomal protein S16* (*Rps16*), *ribosomal protein S6* (*Rps6*) and *ribosomal protein S9* (*Rbs9*). There are a few other ribosomal protein genes which are significantly up regulated. The full list can be seen in the appendix. Different types of *glutathione transferase* genes are also another group of genes make most of the genes that are ranked highest in gene list along with the ribosomal protein genes. *Glutathione S-transferase mu 2* (*Gstm2*), *glutathione S-transferase pi-1* (*Gstp1*), *glutathione S-transferase omega 1* (*Gsto1*) and *glutathione S-transferase theta 3* (*Gstt3*) genes are expressed up to 14 times higher in the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ cells compared to the wild type cells. In addition, *eukaryotic translation initiation factor 5* (*Eif5*), *catenin (cadherin associated protein) α1* (*Ctnna1*), *tubulin α1B* (*Tuba1b*) and *cyclin D1* (*Ccnd1*) are among the highest upregulated genes in the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ cells. Many genes which are involved in

transcriptional regulation and the immunes system are also highly up regulated. These will be discussed in more detail while explaining the GSEA analysis.

When genes that are downregulated in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ compared to wild type cells are analysed, it is seen that 1989 genes are significantly downregulated. It is seen that the most of the significantly downregulated genes are expressed in the mitochondria and are involved metabolism. *Mitochondrially encoded ATP synthase 6* (mt-ATP-6) is the downregulated 16 fold in the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ cells and is the most downregulated gene in the list. Other significantly downregulated genes which encode for *mitochondrial proteins are mitochondrial ATP synthase protein 8* (*mtDNA_ATP8*), *ATP synthase H+ transporting mitochondrial F1 complex, beta subunit* (*Atp5b*), *ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F* (*Atp5j*), *cytchrome c oxidase unit 8A* (*Cox8a*) and *translocase of inner mitochondrial membrane 8 homolog b* (*Timm8b*) are only a few examples of the significantly downregulated mitochondrial genes. Kidney specific genes such as *myo-inositol oxygenase* (*Miox*) and *kidney expressed gene 1*(Keg1) are also very highly downregulated. *Glutathione reductase 1*(*Gpx1*), *serine peptidase inhibitor Kazal type3* (*Spink3*), *ferritin heavy chain 1* (*Fth1*) and *insulin like growth factor binding protein 7* (*Igfbp7*) are also seen ranked in the top 10 of the most downregulated genes. Many genes which are involved in different metabolic pathways are also significantly downregulated. Due to larger number of these genes, names of only a few will be mentioned here but the pathways they are involved in will be discussed later on in the thesis. Examples of these genes include *isocitrate dehydrogenase 3 γ* (*Idh3g*), *superoxide dismutase 2* (*Sod2*), *ornithine decarboxylase 1* (*Odc1*) and *fructose bisphosphatase 1* (*Fbp1*) genes.

GSEA biological processor analysis, which was done taking into account both up and downregulated genes in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and wild type cells, shows there are 4 statistically significantly expressed biological processor gene groups. The most significant of these is the aromatic compound metabolic process group. The

differentially expressed genes in this group include *methylenetetrahydrofolate dehydrogenase* (*Mthfd2*), *transforming growth factor β 2* (*Tgfβ2*), *fatty acid hydroxylase* (*Fah*) and *aldehyde dehydrognase family5 member A1* (*Aldh5a1*). The other significantly differentially expressed biological processor groups are rhythmic process, humoral immune response and excretion.

GSEA analysis which was done to sort the up and downregulated biological processor gene groups show that 136 gene groups were significantly upregulated in the double knock out cells compared to the wild type cells. Positive regulation of cell proliferation, defence response and cell proliferation groups were the most significantly upregulated biological processor groups. *Cdkn1a* is the most significantly upregulated gene which is involved in positive regulation of cell proliferation. *Cyclin dependent kinase inhibitor 2B* (*Cdkn2b*), *cyclin-dependent kinase inhibitor 3* (*Cdkn3*), *Myc* and *surfactant associated protein D* (*Sftpd*) are also highly upregulated and are involved in regulation of proliferation. A considerable number of upregulated biological processor groups were involved in cycle. A few important examples include cell cycle process, cell cycle phase, mitosis, M-Phase, cell cycle check point, interphase and cell division biological processor groups. *Cdkn1a*, *Cdkn2b*, *annilin actin binding protein* (*Anln*), *non-SMC condensin I complex subunit H* (*Ncaph*), *Tgfb1* and *microtubule associated protein homolog Tpx2* are among the most significantly upregulated genes which are involved in the cell cycle. Immune system related biological processor groups are also significantly up regulated. These groups have genes such as *Sftpd*, *B cell linker* (*Blnk*), *triggering receptor expressed on myeloid cells 2* (*Trem2*) and *Gata3* which are involved in immune system process, leukocyte activation, lymphocyte activation, inflammatory response and regulation of cytokine biosysnthesis. Groups of genes which are important for cell structure such as actin filament based process, microtubule based process and anatomical structure morphogenesis are also significantly up regulated. In addition, many signalling pathway groups including small GTPase mediated signal

transduction, Ras protein signal transduction and cell surface receptor linked signalling transduction are significantly upregulated in the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ cells as well.

There are 16 biological processor groups which are significantly downregulated in the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ cells compared to wild type cells. All of these groups are metabolism related except one which is the cellular respiration group. This group has genes such as *pyruvate dehydrogenase* (*lipoamide*) *beta* (*Pdhb*), *Surf1* and *peroxisome proliferative activated receptor, gamma, coactivator 1 alpha* (*Ppargc1a*). Biological processor groups which are involved in metabolism include nitrogen compound catabolic process, amino acid and derivative metabolic process and carboxylic acid metabolic process.

Kegg pathway analysis, which was done without taking up or down regulation of the genes into account, showed 7 Kegg pathways were differentially expressed between *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ wild type cells. These pathways include metabolic pathways such as pentose phosphate pathway and glutathione metabolic pathway. Metabolism of xenobiotics by cytochrome P450, complement and coagulation pathways, cell communication and ECM receptor interaction pathways are other significantly differentially expressed Kegg pathways.

Kegg pathway analysis which was done to identify upregulated and downregulated pathways show that 48 Kegg pathways are significantly upregulated in the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ cells. The most significantly upregulated Kegg pathways are cell cycle and metabolism of xenobiotics by cytochrome P450 pathways. *Cdkn1a* shows the highest expression level in the cell cycle. The other highly expressed cell cycle genes are *Cdkn2b*, *growth arrest and DNA-damage-inducible 45 alpha* (*Gadd45a*) and *cyclin B1* (*Ccnb1*). *Alcohol dehydrogenase 7* (*Adh7*) is the significantly expressed gene in metabolism of xenobiotics by cytochrome P450 pathway. Other genes which are highly expresses in this pathway include *microsomal glutathione S-transferase* 1(*Mgst1*) and

several other *glutathione S-transferase* genes. Several signalling pathways which are involved in different cancers also up regulated. These pathways are renal cell carcinoma, small cell lung cancer, colorectal cancer, chronic myeloid leukaemia, pancreatic cancer and basal cell carcinoma. *Cdkn1a*, *Cdkn2b*, *Myc* and several immune system and cell cycle related genes are commonly upregulated in these cancer pathways. In addition, signalling pathways such as the WNT signalling pathway, ERB signalling pathway, MAPK pathway, p53 signalling pathway and immune related pathways such as the Toll like receptor signalling pathway are also significantly upregulated.

On the other hand, 9 Kegg pathways are downregulated in the double knockout cells. The most significantly downregulated pathways are oxidative phosphorylation, valine, leucine and isoleucine degradation pathway and citrate cycle. Other significantly downregulated Kegg pathways are metabolic pathways like propanoate, arginine and proline metabolism pathways. In addition, PPAR pathway is also significantly downregulated.

3.4.3. *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ versus *Fh1*$^{-/-}$ comparison

A comparison of gene expression between *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$ was performed and is summarised in **Table 14**. LIMMA analysis of the differentially expressed genes between *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$ identified 61 genes which are upregulated in the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ cells but not in the *Fh1*$^{-/-}$ cells. *Ccnd1* shows the highest expression level in the double knockout cells. It is followed by the *phosphogluconate dehydrogenase* (*Pgd*) expression. Other significantly upregulated genes belong to various different biological groups and a few examples *Include Mgst1, Rab15* from the RAS oncogene family, *Gata3*, *keratin 7* (*Krt7*), *keratin 18* (*Krt1-18*), *keratin 23* (*Krt1-23*) and *Adh7*.

There are 21 genes which are downregulated in *Fh1*$^{-/-}$/*HIF-1α*$^{-/-}$ compared to the *Fh1*$^{-/-}$ cells. *Ldha* has the lowest expression level. It is followed by *Pgk1* in the list. *Fibroblast growth factor* 1 (*Fgf1*), *endothelin receptor type B* (*Ednrb*), *Pdk1*, *Gpi1*, *chemokine (C-X-C motif) ligand 9* (*Cxcl9*), *pyridoxal (pyridoxine, vitamin B6) phosphatise* (*Pdxp*) and *Hif-1α* are the other significantly down regulate genes in the double knockout kidneys.

GSEA biological processor analysis, which was done taking into account both up and downregulated genes in *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$ kidneys, show that 5 biological processor groups were differentially expressed. Humoral immune response is the most significantly differentially expressed group. *Blnk*, *beta galactoside alpha 2,6 sialyltransferase 1* (*St6gal1*), *bone marrow stromal cell antigen* 1(*Bst1*) and *B-cell CLL/lymphoma 2* (*Bcl2*) are the a few of the most important and differentially expressed genes in this biological processor group. Response to hypoxia is another biological processor group which shows significant expression levels in the *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$. *Hif-1α*, *nicotinic cholinergic receptor alpha polypeptide 4* (*Chrna4*), *BCL2/adenovirus E1B interacting protein 3* (*Bnip3*), *vascular endothelial cell growth factor A* (*Vegfa*), *procollagen lysine (2-oxoglutarate 5-dioxygenase 2)* (*Plod2*), *Egln1* and *Epas1* constitute the most important hypoxic response genes identified in the group. Receptor mediated endocytosis, ectoderm development and epidermis development are the other group of genes that show significant differential expression.

The second GSEA analysis, performed to identify the upregulated and downregulated biological processor groups, shows that 27 biological processor groups are significantly upregulated in the double knockout kidneys. These groups of genes can be classified into two main classes depending on their function. The first class consists of cell cycle related biological processor groups which make up most of the 27 significantly upregulated gene groups. Cell cycle phase, mitotic cell cycle, M-phase of cell cycle, interphase and cell cycle check point biological processor groups are examples of the upregulated cell cycle groups. *Cdkn1a*, *Cdkn2b*, *cyclin-dependent*

kinase inhibitor 3 (*Cdkn3*), *baculoviral IAP repeat-containing 5* (*Birc5*), *cell division cycle 23, yeast, homolog* (CDC23), *Rad52*, *DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (CHL1-like helicase homolog, S. cerevisiae)* (*Ddx11*) and *polymerase (DNA directed), delta 1, catalytic subunit* (*Pold1*) are examples of the most significantly upregulated genes in the cell cycle relate gene groups stated above. Another important class of biological processor groups which showed significant upregulation are important for construction of cell structure and cell development. These groups are involved in epidermis development, ectoderm development, negative regulation of cellular component organization and biogenesis, establishment of organelle localization, actin cytoskeleton organization and biogenesis, organelle localization and microtubule cytoskeleton organization and biogenesis. *Caspase 14* is the most significantly upregulated gene in epidermis development and ectoderm development groups. Other most significantly upregulated genes in these two groups are *laminin gamma 2* (*Lamc2*), *laminin beta 3* (*Lamb3*), *flotillin 2* (*Flot2*), *repair cross-complementing rodent repair deficiency, complementation group 2* (*Ercc2*) and *fibroblast growth factor 7* (*Fgd7*). Genes that are commonly significantly upregulated in the biological processor groups involved in cell structure include *Birc5*, *Cdc23, shroom family member 2* (*Shroom2*), *engulfment and cell motility 1* (*Elmo1*) and *pleckstrin 2* (*Plek2*). Positive regulation of cell proliferation and humoral immunity groups are also significantly upregulated.

In contrast to the various significantly upregulated biological processor groups, only one biological processor group is significantly downregulated in the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys compared to the *Fh1*$^{-/-}$ kidneys. The most significantly downregulated genes in this group are *Hif-1α*, *Chrna4, Bnip3, Vegfa, Plod2, Cxcr4, Egln1, Epas1* and CREB binding protein (*Crebbp*).

Kegg pathway analysis which was done without taking up or downregulation of the genes in to account showed that 10 Kegg pathways were differentially expressed between *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and the *Fh1*$^{-/-}$ kidneys. Most of these pathways are metabolic

pathways and examples include metabolism of xenobiotics by cytochrome P450, pentose phosphate pathway, glycolysis and gluconeogenesis pathways.

Another Kegg pathway analysis which was done by using up and downregulated genes separately showed that 10 Kegg pathways are significantly upregulated in the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys. The most significantly upregulated pathway is the metabolism of xenobiotics by cytochrome P450. The other significantly upregulated pathways are aminoacyl tRNA synthesis, cell cycle and tight junction pathways in addition to some some cancer pathways such as small cell lung cancer, thyroid cancer and basal carcinoma pathways. The most significantly upregulated genes in these pathways are *Ccnd1*, *Myc*, integrin alpha 3 (*Itga3*), *Cdkn2b*, *wingless-type MMTV integration site 9B* (*Wnt9b*), *glycogen synthase kinase 3 beta* (*Gsk3b*), *peroxisome proliferator activated receptor gamma* (*Pparg*) and *paired box gene 8* (*Pax8*).

Seven Kegg pathways are significantly downregulated in the double knock out kidneys when compared to *Fh1*$^{-/-}$. Most of these pathways are metabolic pathways and the most significantly downregulated pathway is the oxidative phosphorylation pathway. Citrate cycle, glycolysis and gluconeogenesis pathway, propanoate metabolism and carbon fixation pathways are also downregulated. In addition, the renal cell cancer pathway is also significantly downregulated.

Comparison	Significant Genes		GO Biological Processors		Kegg Pathway	
	Upregulated	**Downregulated**	**Upregulated**	**Downregulated**	**Upregulated**	**Downregulated**
***Fh1*$^{-/-}$ vs Wt**	Ldha	Spink3	Inflammatory response	Energy derivation by oxidation of organic compounds	T cell Receptor Signalling	Oxidative phosphorylation
	Eno1	Keg1	Defence response	Cellular respiration	Renal cell carcinoma	Valine, leucine and isoleucine degradation
	Pgk1	Fth1	Leukocyte activation	Anion transport	Mapk signalling	
	Gstp1	Igfbp7	Humoral immune response		Glycolysis and gluconeogenesis	
	Gstm2	Ubb	T cell Activation		VEGF signalling	
	Pfk	Gpx3	Positive regulation of cell proliferation		Notch Signalling	
	Slc2a1	Ndufa4			p53 Signalling	
	Pkm2	Idh3g			Cell Cycle	
	Calgizarrin	Odc1				
	Hk2	Fbp1				
	Ca15					
	Aldo C					
	Ald1l12					
	Lcn2					
	CD47					
	Irf1					
	MAPKKK1					
	MAPKKK3					
	Rab8					
	Gata3					

Table 12: Summary of genes identified as significant in microarray analysis comparing *Fh1*$^{-/-}$ and wild type kidneys

The table summarizes the most important significantly upregulated and downregulated genes, gene ontology biological processors and Kegg pathways identified in the microarray analysis performed to compare expression between *Fh1*$^{-/-}$ and wild type kidneys.

Comparison	Significant Genes		GO Biological Processors		Kegg Pathway	
	Upregulated	**Downregulated**	**Upregulated**	**Downregulated**	**Upregulated**	**Downregulated**
***Fh1*$^{-/-}$ */Hif-1α*$^{-/-}$ **vs Wt**	Ccnd1	Spink3	Positive regulation of Cell proliferation	Cellular respiration	Cell cycle	Oxidative phosphorylation
	Cdkn1a	Keg1	Cell cycle check point	Nitrogen compound catabolic process	Metabolism of xenobiotics by cytchrome p450	Valine, leucine and isoleucine degradation
	Cdkn3a	Cox8	Mitosis	Amino acid and derivative process	Renal cell carcinoma	Propanoate, arginine and proline metabolism
	Cdkn2b	Miox	Interphase	Carboxylic acid metabolic process	Small cell lung cancer	PPAR pathway
	Myc	Gpx1	Cell division		Colorectal cancer	
	Tgfb1	Fth1	Actin filament based process		Chronic myeloid leukaemia	
	Gata3	Igfbp7	Microtubule based process		Pancreatic cancer	
	Anln	Sod2	Anatomical structure morphogeneis		WNT signalling pathway	
	Rps8	Fbp1	Immune system process		ERB signalling pathway	
	Rps4x	Idh3g	Leukocyte activation		p53 signalling pathway	
	Rplp2	Odc1	Lymphocyte activation		Toll-like receptor signalling pathway	
	Rp9	mt-ATP-6	Cytokine biosynthesis			
	Gsmt2	mtDNA-ATP8				
	Gstp1	Atp5b				
	Gsto1	Atp5j				
	Gstt3					
	Tuba1b					

Table 13: Summary of genes identified as significant in microarray analysis comparing *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and wild type kidneys

The table summarizes the most important significantly upregulated and downregulated genes, gene ontology biological processors and Kegg pathways identified in the microarray analysis performed to compare expression between *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and wild type kidneys.

Comparison	Significant Genes		GO Biological Processors		Kegg Pathway	
	Upregulated	**Downregulated**	**Upregulated**	**Downregulated**	**Upregulated**	**Downregulated**
***Fh1*$^{-/-}$ */Hif-1α*$^{-/-}$ **Vs** ***Fh1*$^{-/-}$**	Ccnd1	Ldha	Cell cycle phase	Response to hypoxia	Metabolism of xenobiotics by cytochrome p450	Citrate cycle
	Cdkn1a	Pgk1	M-phase of cell cycle		Cell cycle and tight junction pathway	Glycolysis and gluconeogenesis
	Cdkn2a	Fgf1	Cell cycle check point		Aminoacyl tRNA synthesis	Propanoate metabolism
	Cdkn3	Ednrb	Epidermis development		Small cell lung cancer	Carbon fixation
	Myc	Pdk1	Ectoderm development		Basal cell carcinoma	Renal cell carcinoma
	Krt7	Gpi1	Negative regulation of cellular component organization and biogenesis			
	Krt1-18	Cxcl9	Establishment of organelle organization			
	Adh7	Pdxp	Actin cytoskeleton organization and biogenesis			
	Gata3		Microtubule cytoskeleton organization and biogenesis			
	Lamc2		Positive regulation of cell proliferation			
	Lamb3		Humoral immunity			
	Flat2					
	Fgd7					

Table 14: Summary of genes identified as significant in microarray analysis comparing *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$ kidneys

The table summarizes the most important significantly upregulated and downregulated genes, gene ontology biological processors and Kegg pathways identified in the microarray analysis performed to compare expression between *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$ kidneys.

3.5. Validation of gene expression levels of RT-Q-PCR

Knocking out *Fh1* and *Hif-1α* genes separately and together resulted in the development of a cystic phenotype in mouse kidneys. This is described in detail in the previous chapters. In order to understand the mechanism leading to the observed cystic phenotype a microarray analysis was performed. The microarray results suggest that several genes are significantly upregulated or downregulated in *Fh1*$^{-/-}$and *Fh1*$^{-/-}$ */Hif-1α*$^{-/-}$ kidneys. RT-Q-PCR was performed to validate the results obtained from the microarray experiment. In addition, as discussed in the introduction, there are some genes which are suggested to be regulated by FH or hypoxia. Although some of these genes are not found to be differentially expressed in *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys according to the microarray results, expression levels of these genes were still investigated by RT-Q-PCR in order to validate the information available in literature.

A total of 15 samples were used for RT-Q-PCR analysis of the microarray samples. The genotypes of the kidneys from which the cDNAs were prepared can be seen **Table 15**. Gene expression levels obtained by RT-Q-PCR were pooled for some of the genes for samples with the same genotypes. t-test analysis was conducted for the pooled expression levels to identify whether there is a statistically significant difference in the expression level of a gene between *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples. In the pooled result graphs, sample 1 refers to *Fh1*$^{-/-}$ samples, sample 2 refers to *Fh1*$^{-/-}$ */Hif-1α*$^{-/-}$ samples and sample 3 refers to wild type samples. A summary of these analyses is provided in **Table 16**.

Table 15: Subset of kidney samples used for RT-Q-PCR analysis of the microarray samples

Genotype Group	Age	*Fh*	*Hif-1α*	*Hif-2α*	Ksp-Cre	Sex
1	13 wks	flox/flox	Wt	flox/+	+	F
1	17.5 wks	flox/flox	Wt	flox/+	+	M
1	16 wks	flox/flox	Wt	flox/+	+	F
1	17 wks	flox/flox	flox/+	Wt	+	M
1	33 wks	flox/flox	Wt	Wt	+	M
2	18 wks	flox/flox	flox/flox	Wt	+	M
2	19 wks	flox/flox	flox/flox	Wt	+	M
2	17 wks	flox/flox	flox/flox	Wt	+	F
2	18 wks	flox/flox	flox/flox	Wt	+	F
2	24 wks	flox/flox	flox/flox	Wt	+	F
2	18 wks	flox/flox	flox/flox	Wt	+	F
3	17.5 wks	flox/flox	Wt	flox/+	-	M
3	17.5 wks	flox/+	Wt	flox/flox	-	M
3	13.5 wks	Wt	flox/flox	Wt	-	M
3	12.5 wks	flox/+	Wt	flox/flox	-	F

Before quantifying the expression level of any other gene, *Fh1* and *Hif-1α* levels in the samples were quantified. The results confirm that $Fh1^{-/-}$ samples express lower levels of *Fh1*. This expression level is approximately equal to approximately half the level of *Fh1* expression seen in wild type samples. $Fh1^{-/-}/Hif\text{-}1\alpha^{-/-}$ samples show even lower levels of *Fh1* expression compared to $Fh1^{-/-}$ samples which is statistically significant (p-value = 0.049). A comparison of the expression levels in $Fh1^{-/-}$ samples and $Fh1^{-/-}/Hif\text{-}1\alpha^{-/-}$ samples to wild type samples shows that both $Fh1^{-/-}$ samples (p-

value = 0.0001) and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ samples (p-value = 1.24 x 10^{-6}) exhibit significant downregulation in *Fh1* levels compared to wild type samples.

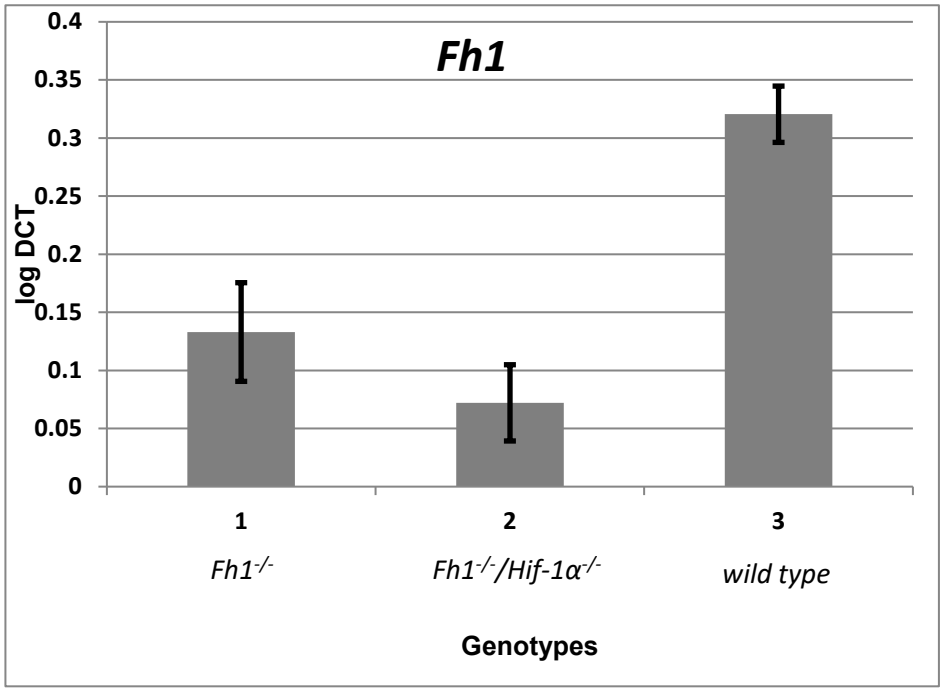


Figure 9: Comparison of *Fh1* expression in wild-type, *Fh1*$^{-/-}$ *and Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidneys

Hif-1α expression is confirmed to be downregulated in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$. On the other hand, *Fh1*$^{-/-}$ samples also show a slight downregulation of *Hif-1α* compared to the wild type samples but this is not a significant difference (p-value = 0.061). They express higher levels of *Hif-1α compared to the Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ as expected. Wild type samples express the highest level of *Hif-1α.* When *Hif-1α* expression in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples is compared to the expression levels in wild type samples, a significant downregulation in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples is seen (p-value = 1.77 x 10^{-5}). The level of *Hif-1α* expression is also significantly lower in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples when compared to *Fh1*$^{-/-}$ samples (p-value = 0.0002).

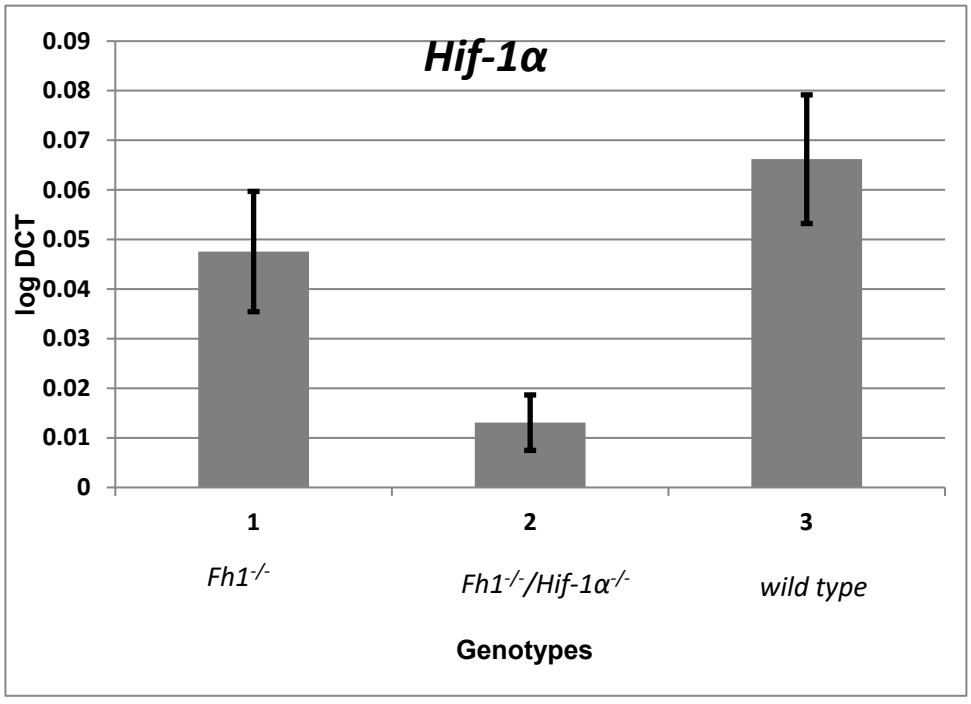


Figure 10: Comparison of *Hif-1α* expression in wild-type, *Fh1*$^{-/-}$ *and Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys

Microarray results show that *Eno2* is one of the most significantly upregulated genes in the *Fh1*$^{-/-}$ samples. RT-Q-PCR results also show that *Eno2* expression is 3 fold higher in *Fh1*$^{-/-}$ samples compared to wild type samples which is a significant difference (p-value = 0.021). *Eno2* is also upregulated gene in *Fh* $^{-/-}$*/Hif-1α*$^{-/-}$ samples but the expression level in the double knockout samples is slightly lower compared to the expression levels in *Fh1*$^{-/-}$ samples. t-test analysis show that this difference in the expression level is not significant (p-value=0.56). However, *Eno2* expression is significantly upregulated in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples compared to wild type samples (p-value= 0.00018).

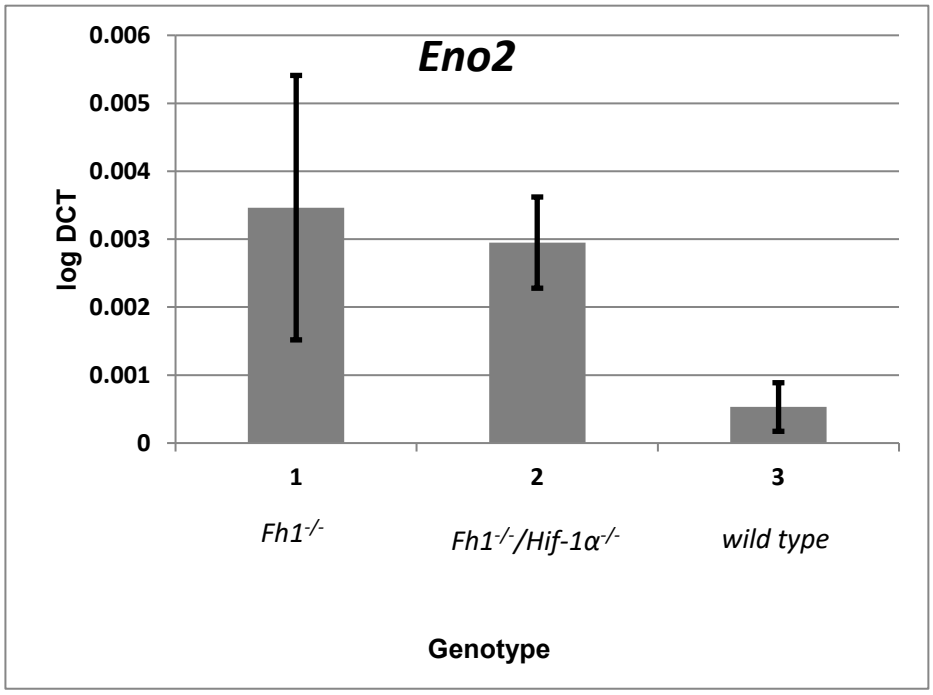


Figure 11: Comparison of *Eno2* expression in wild-type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys

Microarray results suggest that *Hk2* is another important gene which is significantly upregulated when *Fh1* is knocked out. When expression level in samples with different genotypes is compared, it is seen that *Hk2* expression is lowest in wild type samples. On the other hand, highest *Hk2* expression is seen in $Fh1^{-/-}$ samples. Expression level in double knockout samples is approximately 6 folds higher than the level in wild type samples which is a significant difference (p-value = 0.0038). In addition, expression of *Hk2* is significantly higher in $Fh1^{-/-}$ samples compared to double knockout kidneys (p-value=0.016). The most significant difference is seen between $Fh1^{-/-}$ and wild type samples where *Hk2* is significantly upregulated in $Fh1^{-/-}$ samples compared to wild type samples (p-value = 1.73×10^{-6})

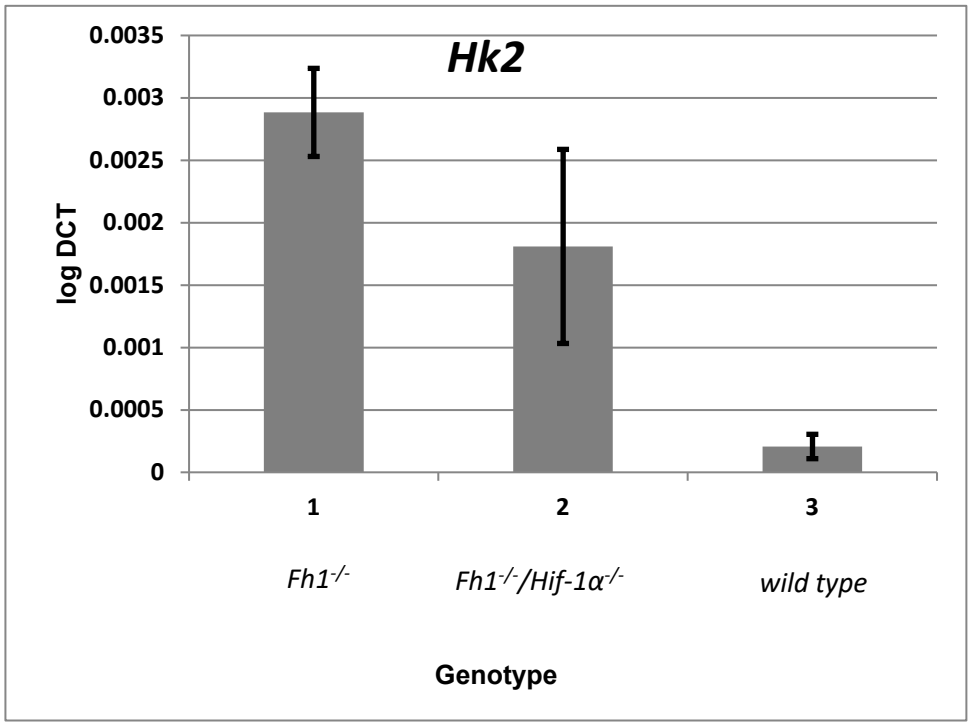


Figure 12: Comparison of *Hk2* expression in wild-type, $Fh1^{-/-}$ and $Fh1^{-/-}/Hif\text{-}1\alpha^{-/-}$ kidneys

Lcn2 levels are undetectable in wild type samples. However, *Lcn2* is upregulated in *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ samples. This result is compatible with the microarray results as well. The highest *Lcn2* expression level is seen in the double knockout kidneys. It is approximately 4 folds higher compared to the expression level in *Fh1*$^{-/-}$ kidneys which is a significant up regulation (p-value = 0.0026). Knocking out *Fh1* also causes a significant increase in *Lcn2* expression compared to the level in wild type samples (p-value = 0.0059). The most significant increase in expression level is seen *Fh1* $^{-/-}$/*Hif-1α*$^{-/-}$ samples compared to wild type samples (p-value = 0.0011).

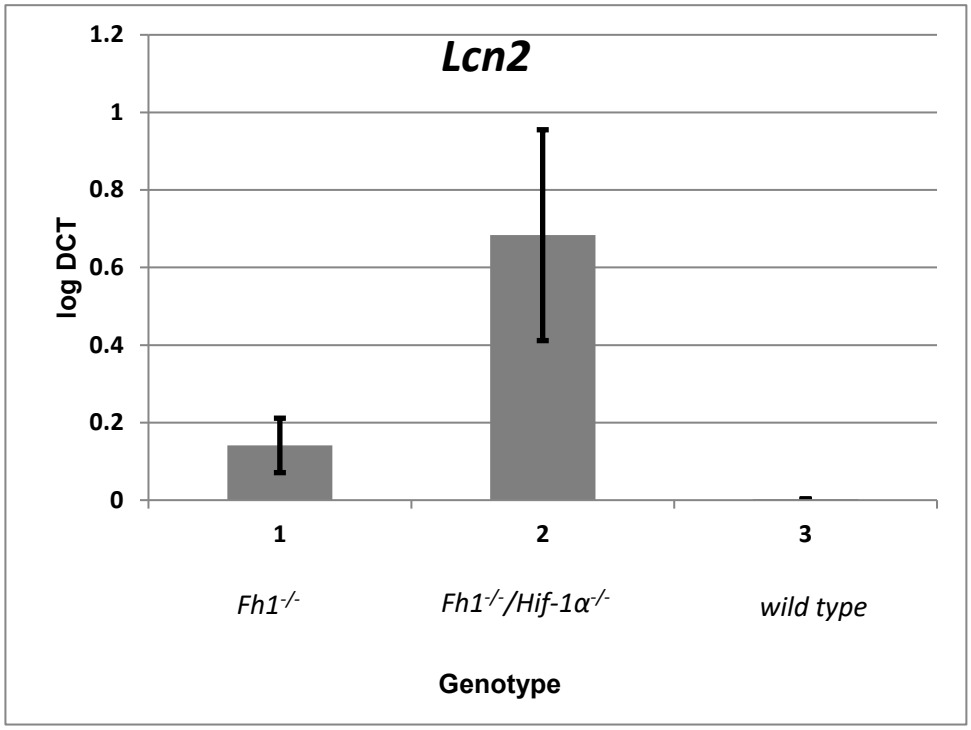


Figure 13: Comparison of *Lcn2* expression in wild-type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidneys

A pattern which is similar to *Lcn2* is also seen for *Trib3*, *Tgfa* and *Myc* expression. *Trib3* level is almost undetectable in wild type kidneys. Knocking out *Fh1* causes an increase in the *Trib3* levels which is significant compared to *Trib3* levels in wild type samples (p-value = 0.00057) . Knocking out both *Fh1* and *Hif-1α* also causes an up regulation in *Trib3* expression compared to wild type samples but this difference is at the borderline to be considered to be statistically significant (p-value = 0.051). Comparison of expression levels between in *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples show that up *Trib3* is upregulated in both samples at similar levels and there is no significant difference between them (p-value= 0.11).

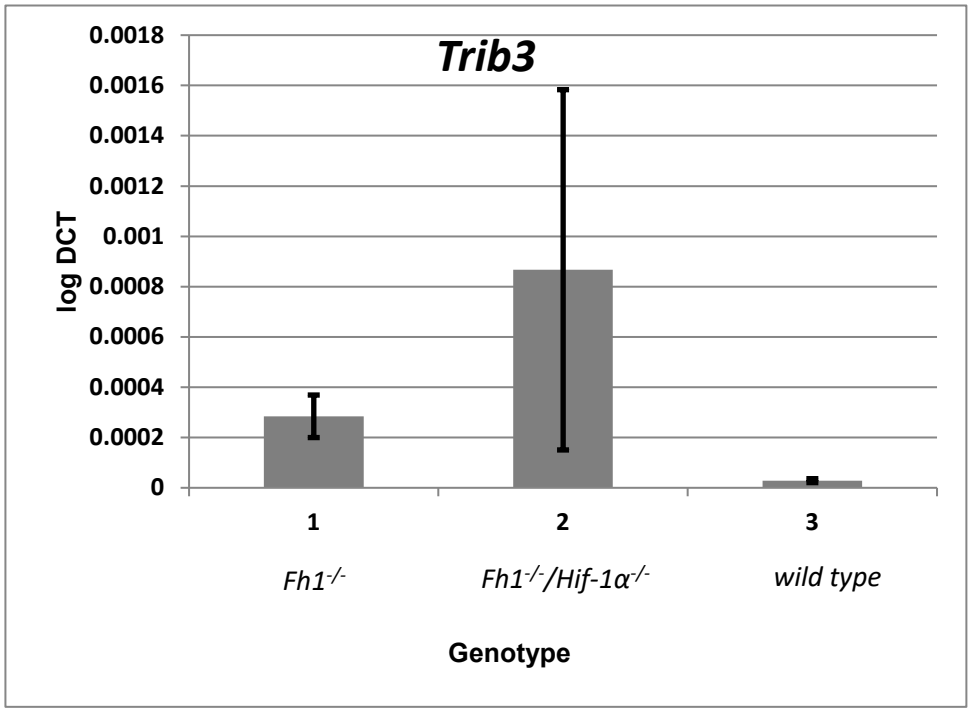


Figure 14: Comparison of *Trib3* expression in wild-type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys

As suggested by the microarray analysis, knocking out both and *Fh1*$^{-/-}$ and *HIF-1α*$^{-/-}$ leads to *Myc* up regulation. The highest Myc expression is seen in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples and the lowest expression in seen in wild type samples. Although *Fh1* $^{-/-}$ samples also express higher levels of *Myc*, expression level in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples is significantly higher compared to the expression level in *Fh1*$^{-/-}$ (p-value= 0.007). In *Fh1* $^{-/-}$ samples, *Myc* expression is significantly upregulated compared to the wild type samples (p-value = 0.019). A significant up regulation is also seen in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples compared to wild type samples (p-value = 0.0019).

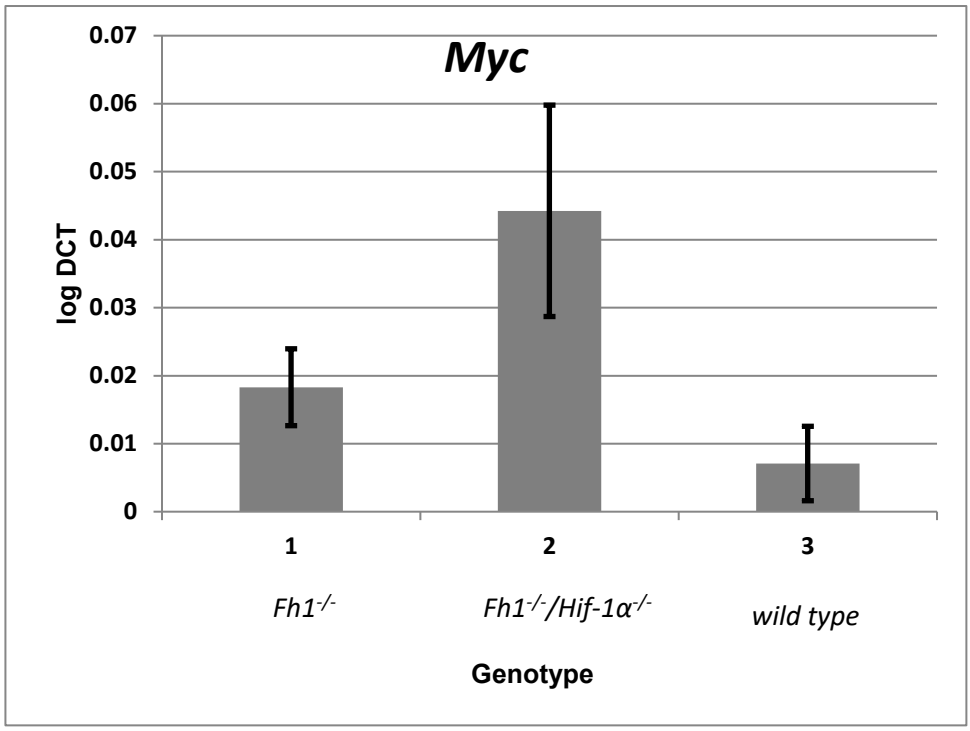


Figure 15: Comparison of *Myc* expression in wild-type, *Fh1*$^{-/-}$ *and Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys

Tgfα expression is significantly upregulated in the double knockout samples. Wild type and *Fh1* $^{-/-}$ samples show similar levels of *Tgfα* expression with no significant difference (p-value = 0.909). t-test analysis show that *Tgfα* expression is significantly higher in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples compared to *Fh1*$^{-/-}$ samples (p-value=0.00013) and wild type samples (p-value = 0.0014).

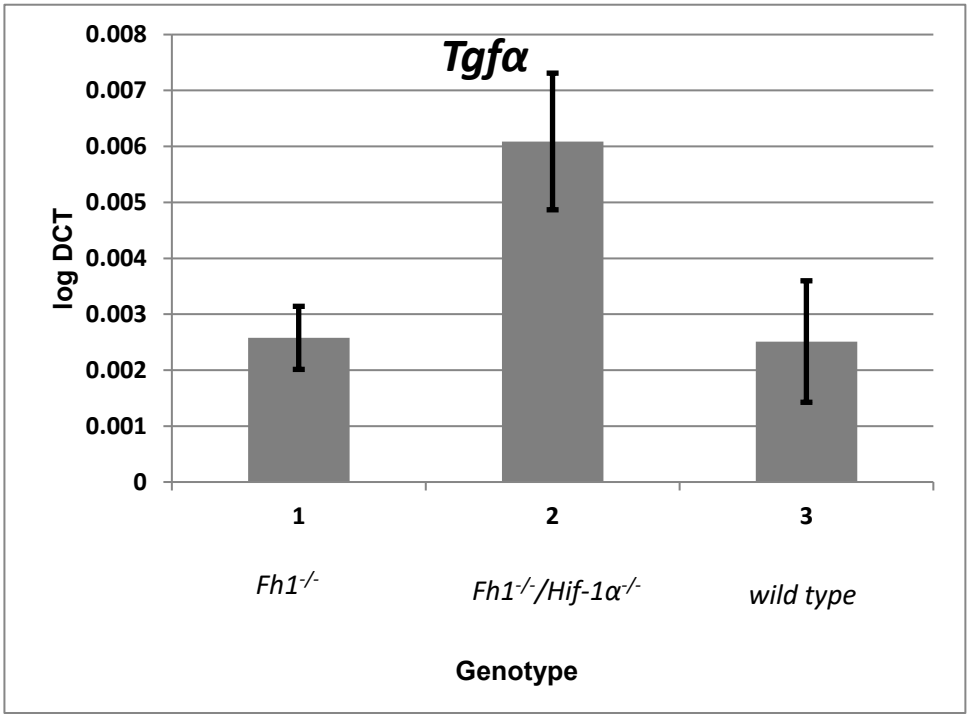


Figure 16: Comparison of *Tgfα* expression in wild-type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys

Microarray analysis show that *Ccnd1* in an important gene which is significantly upregulated in many pathways and biological processer groups in the double knockout samples. RT-Q-PCR analysis also confirm that the *Ccnd1* is significantly upregulated in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$. However, *Ccnd1* expression is 2 folds less in *Fh1*$^{-/-}$ compared to the expression level in *Fh1* $^{-/-}$*/Hif-1α*$^{-/-}$ samples. t-test analysis show that the difference in *Ccdn1* expression between in *Fh1*$^{-/-}$ samples and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples is statistically significant (p-value= 0.0026). This indicates that although knocking out *Fh1* suppresses expression of *Ccdn1,* knocking out *Hif-1α* has a rescue effect. *Ccnd1* is also significantly higher in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples compared to wild type samples (p-value = 0.0069). However, comparison between *Fh1*$^{-/-}$ and wild type samples show no significant difference (p-value = 0.22).

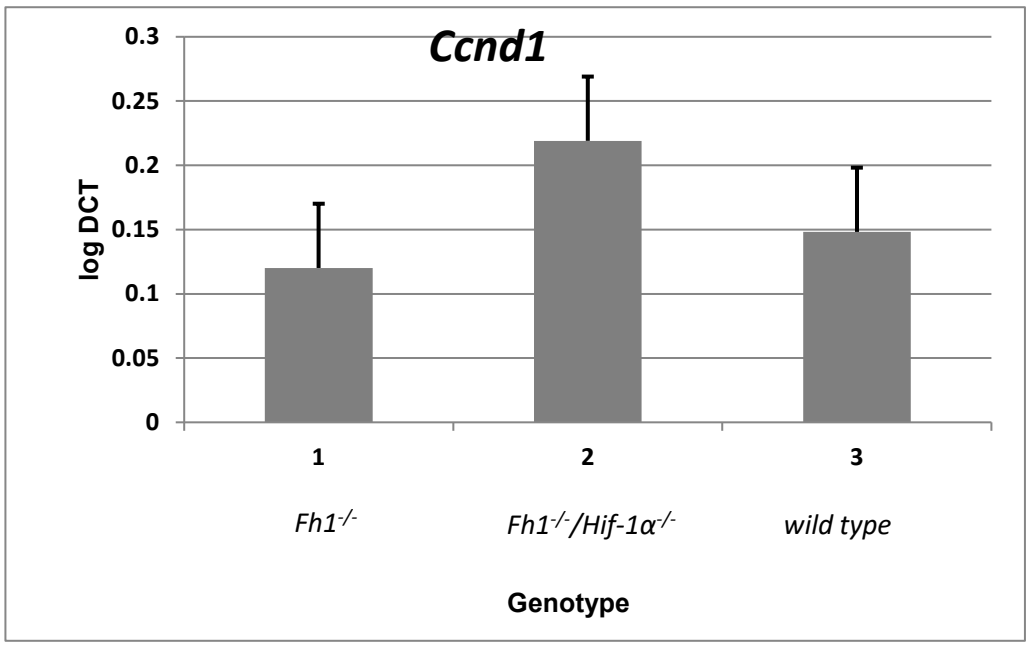


Figure 17: Comparison of *Ccnd1* expression in wild-type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys

A similar expression pattern is also seen for *Cdkn2a*. Wild type samples have undetectable expression of *Cdkn2a*. *Fh1*$^{-/-}$ samples have slightly increased expression levels compared to the wild type samples but it is not a significant difference (p-value = 0.091). On the other hand, knocking out *Hif-1α* in addition to *Fh1* increases *Cdkn2a* expression level over 6 folds compared to wild type and *Fh1*$^{-/-}$ samples. This result shows that *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ genotype causes a significant up regulation in *Cdkn2a* expression (p- value = 0.0083). This significant up regulation is also seen when *Cdkn2a* expression in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples is compared to expression level in wild type samples (p-value = 0.0052).

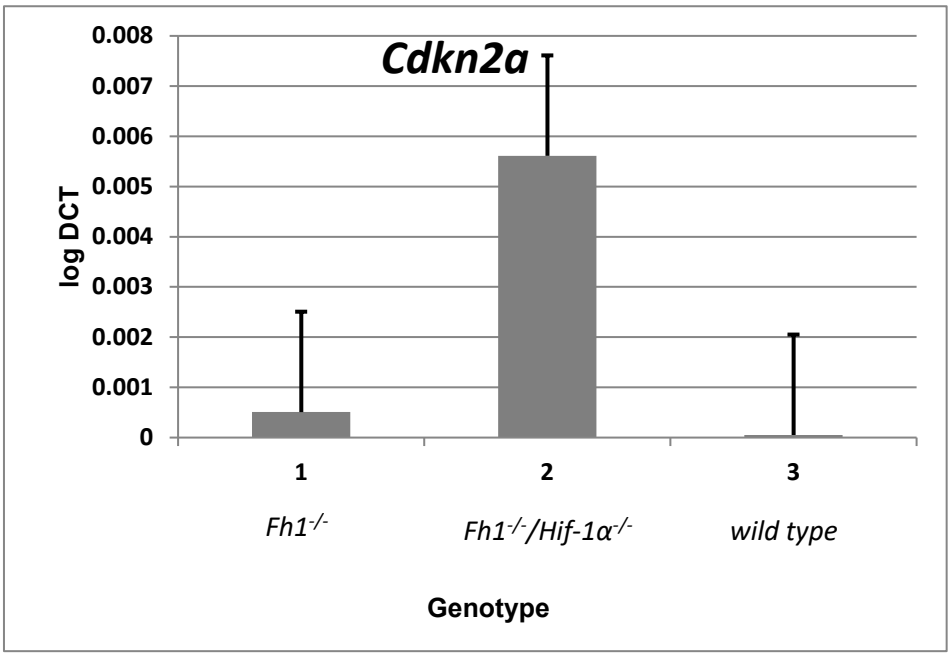


Figure 18: Comparison of *Cdkn2a* expression in wild-type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys

AldoC is another gene which shows differential expression between Fh1$^{-/-}$ and *Fh1$^{-/-}$/Hif-1α$^{-/-}$* kidneys. It is significantly downregulated in *Fh1$^{-/-}$/Hif-1α$^{-/-}$* compared to *Fh1$^{-/-}$* samples (p-value = 0.0034). However, there is no significant difference between expression levels in *Fh1$^{-/-}$* and wild type samples (p-value = 0.284). The same result is also true for the comparison between *Fh1$^{-/-}$/Hif-1α$^{-/-}$* and wild type samples (p-value = 0.163).

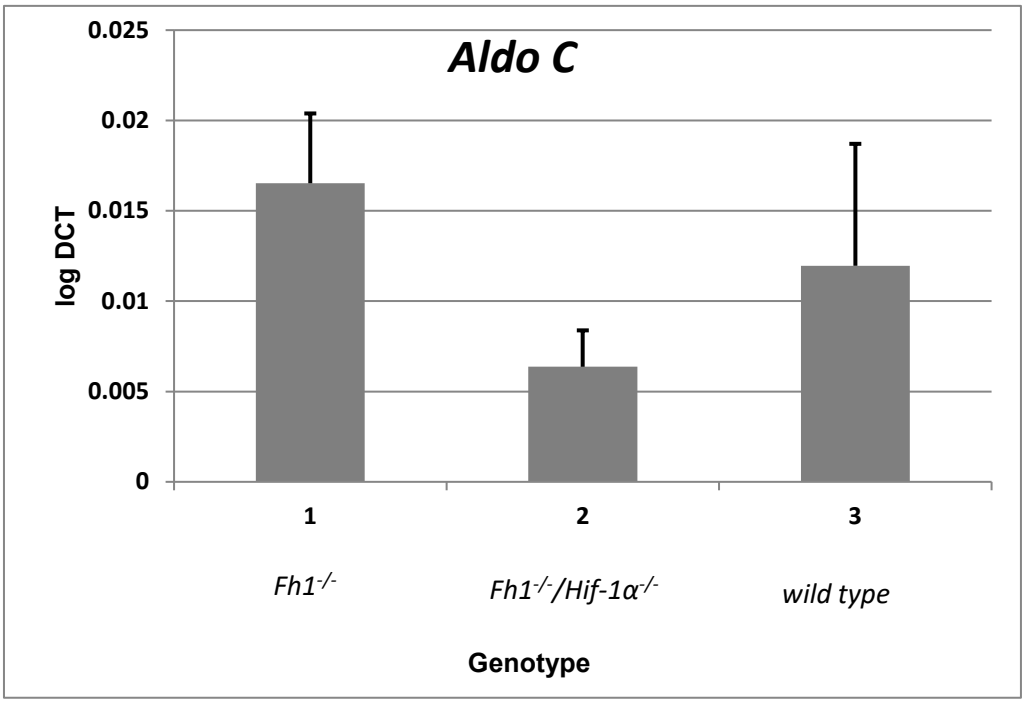


Figure 19: Comparison of *AldoC* expression in wild-type, *Fh1$^{-/-}$* and *Fh1$^{-/-}$/Hif-1α$^{-/-}$* kidneys

Pdk1 is another gene which is differentially expressed in *Fh1* $^{-/-}$ samples based on the microarray analysis. RT-Q-PCR results show that *Pdk1* expression is significantly upregulated in *Fh1*$^{-/-}$ samples compared to the wild type samples (p-value= 0.026). On the other hand, *Pdk1* expression is significantly downregulated compared to both wild type samples (p-value = 0.038) and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples (p-value = 0.003).

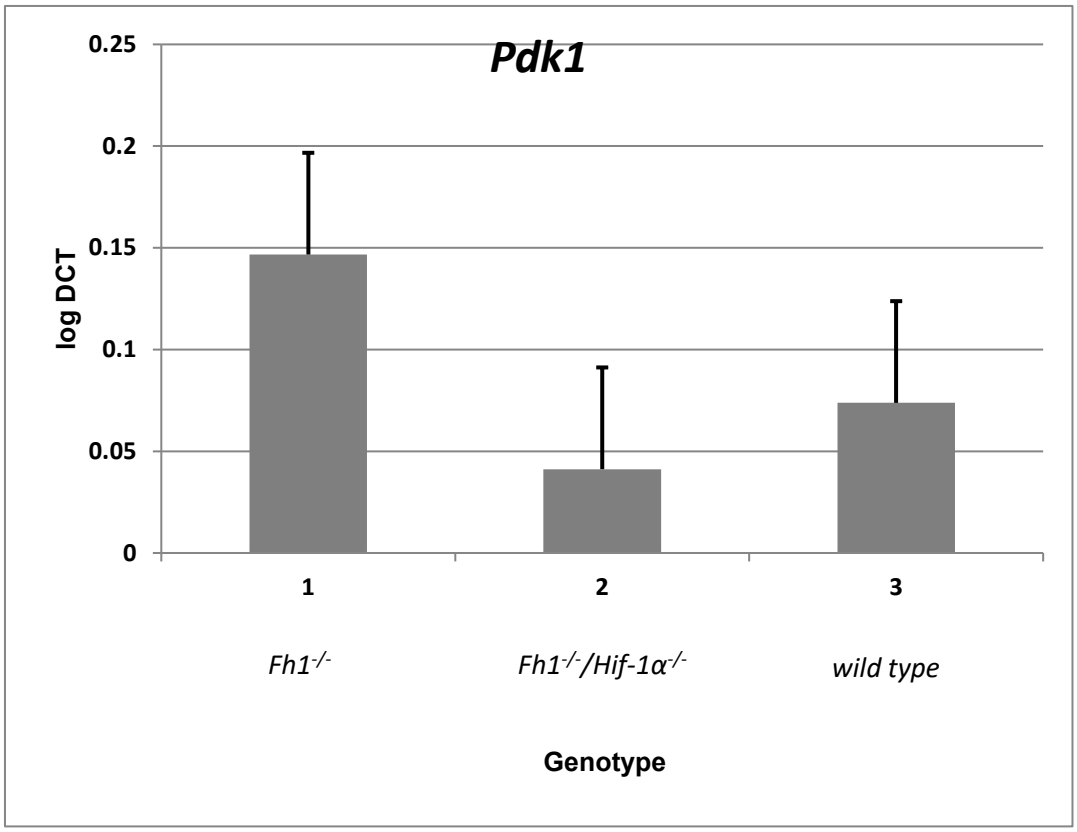


Figure 20: Comparison of *Pdk1* expression in wild-type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys

Slc2a1 is identified as one of the significantly upregulated genes in the microarray analysis. Quantitative PCR analysis also suggests that *Slc2a1* is significantly upregulated in *Fh1*$^{-/-}$ (p-value = 0.0054) and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples (p-value = 0.0032) compared to wild type samples. However, there is no significant difference in *Slc2a1* expression between *Fh1* $^{-/-}$ and *Fh1* $^{-/-}$*/Hif-1α*$^{-/-}$ samples.

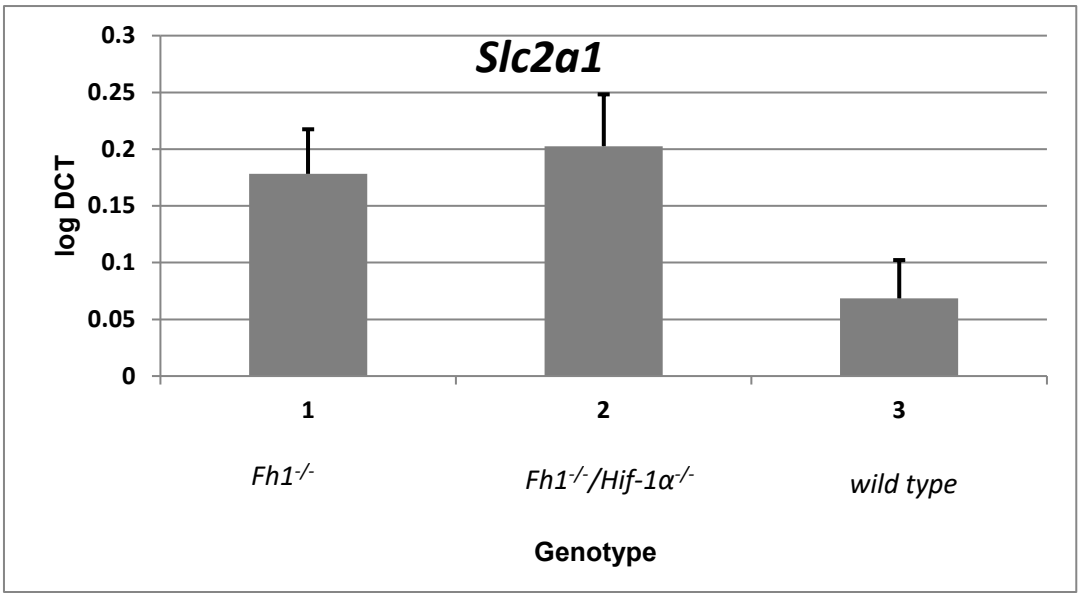


Figure 21: Comparison of *Slc2a1* expression in wild-type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys

Microarray analysis also suggests that pentose phosphate pathway is one of the differentially expressed pathways in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples. Therefore, expression of *glucose-6-phosphate dehydrogenase (G6pdx), transaldolase (Taldo1) and transketolase (Tkt),* which are three important genes involved in pentose phosphate

pathway, are investigated to see if the double knockout samples show any change in expression levels if these three genes. *G6pdx* expression is significantly upregulated in *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ samples compared to wild type samples (p-value= 0.0053). On the other hand, knocking out just *Fh1* does not cause a significant change in *G6pdx* expression level compared to expression level in wild type samples. A comparison between *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ samples shows that double knockout samples have a significantly higher *G6pdx* expression level. *Taldo1* expression is slightly downregulated in *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ samples compared to wild type samples. However, t-test analysis shows that this downregulation is not statistically significant. In addition, comparison of *Taldo1* expression between *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ does not show any significant difference either. A similar pattern is also true for *Tkt*. *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ samples express slightly higher levels of compared to wild type samples. However, this up regulation is not significant. There is no significant difference in expression of *Tkt* between *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ samples either. These results show that only *G6pdx* is the only gene out of the three pentose phosphate pathway related genes tested here which is significantly up regulated.

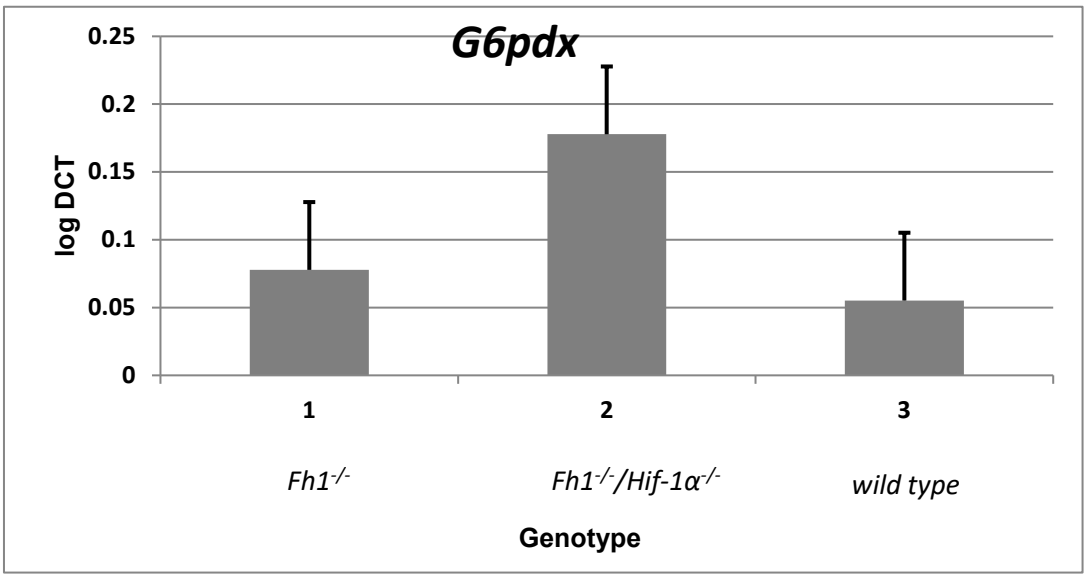


Figure 22: Comparison of G6pdx expression in wild-type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidneys

In addition to validating expression levels of genes identified in microarray analysis, expression of genes which are suggested to be hypoxia regulated are also investigated in *Fh1* $^{-/-}$, *Fh1* $^{-/-}$*/Hif-1α*$^{-/-}$ and wild type samples. One of these important hypoxia regulated genes *serpine*. *Serpine* is expression is very low in wild type samples. *Fh1*$^{-/-}$ samples have slightly higher in *Serpine* expression compared to wild type sample but it is not a significant difference. On the other hand, expression of *Serpine* is significantly upregulated in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples compared to both *Fh1*$^{-/-}$ samples (p-value= 0.0013) and wild type samples (p-value =5.8x10^{-5}).

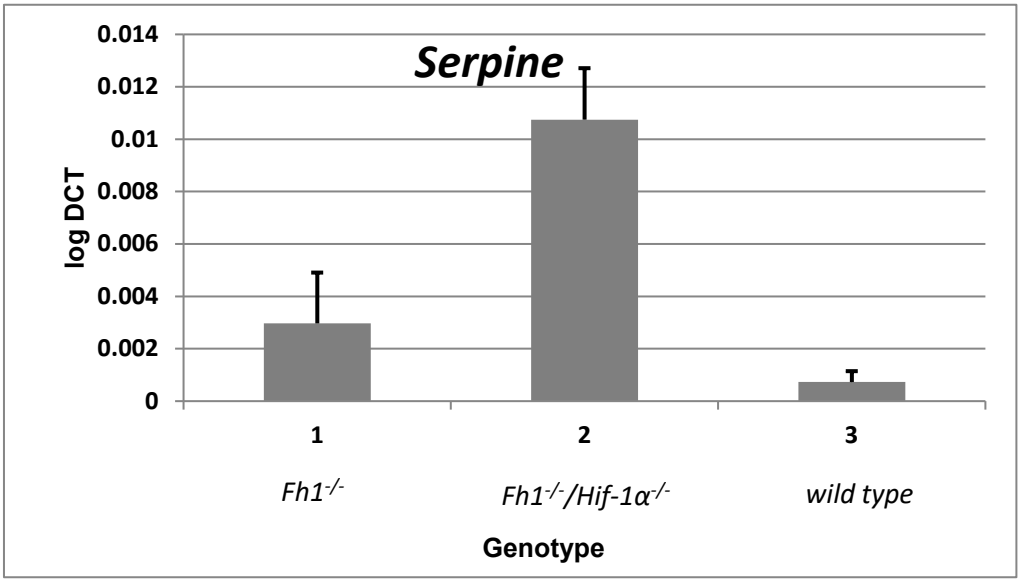


Figure 23: Comparison of *Serpine* expression in wild-type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys

Flavin containing monooxygenase 1 (*Fmo1*) is downregulated in *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ samples compared to the wild type samples. Knocking out *Fh1* significantly down regulates *Fmo1* expression (p-value = 0.0033). The same effect is also seen when *Fh1* and *Hif-1α* are knocked out together in the kidney. Although down regulation of *Fmo1* is significant in *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ samples, there is no significant difference in the level of down regulation between *Fh1* $^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ samples.

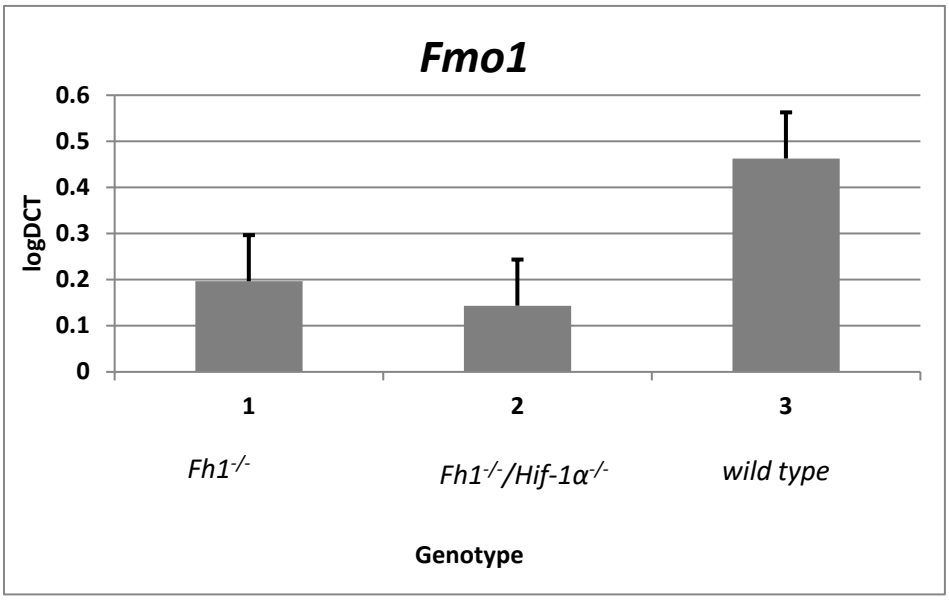


Figure 24: Comparison of *Fmo1* expression in wild-type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidneys

Carbonyl reductase 3 (Crb3) in another important gene which suggested to be important in oxidative stress in the cell. *Crb3* expression is very low in wild type samples. On the other hand, Crb3 expression is significantly upregulated in the *Fh1*$^{-/-}$ samples. However, t-test analysis show that there is no significant difference in *Crb3 expression between Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples (p-value = 0.083). On the other hand, compared to wild type samples, both *Fh1*$^{-/-}$ (p-value = 0.024) and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ (p-value = 0.026) samples show significant up regulation in *Crb3 levels.* Knocking out *Hif-1α* with *Fh1* down regulates the expression level of the gene to a level which is close to the expression level seen in wild type samples. This suggests that *Fh1* and *Hif-1α* are important in regulation *Crb3* expression, but they have different effects on the expression level.

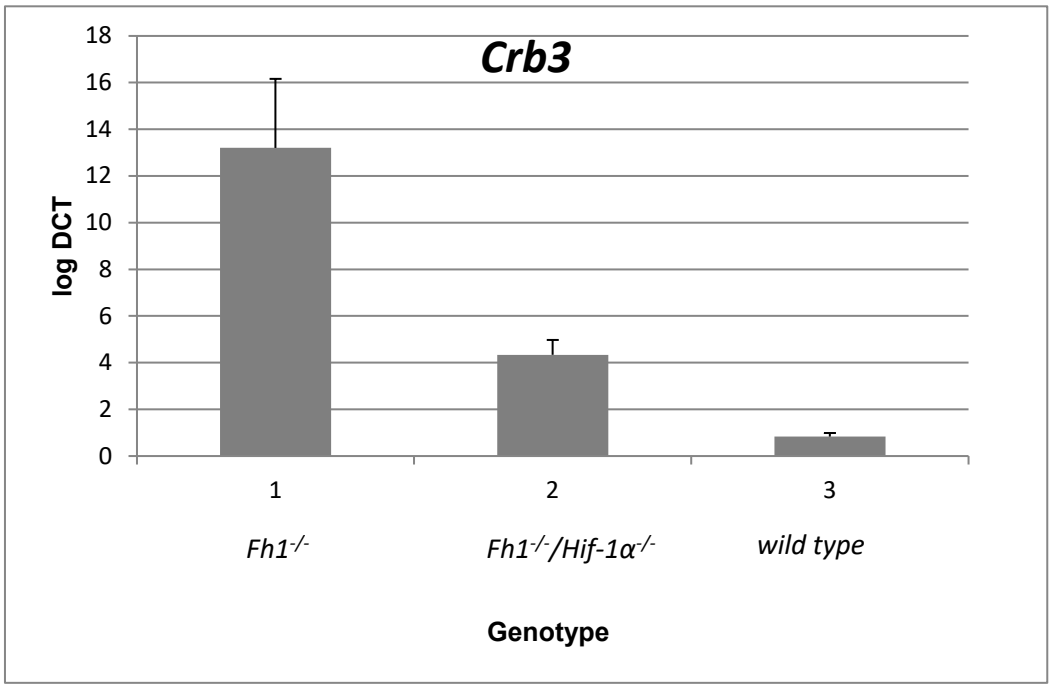


Figure 25: Comparison of Crb3 expression in wild-type, *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys

In addition to quantifying the expression levels of genes identified by the microarray analysis and some of the hypoxia regulated genes that are identified after literature review, expression microRNAs associated with hypoxia and metabolism in the samples used for microarray analysis are also quantified by using a microRNA assay. As explained in the introduction, microRNA 210 (miR210) is one of the most important hypoxia regulated microRNAs. The results are shown in the table below. The results of the assay show that *Fh1*$^{-/-}$ samples express the highest level of miR210. When the expression level in *Fh1*$^{-/-}$/*Hif1α*$^{-/-}$ samples is compared to the expression level in *Fh1*$^{-/-}$ samples, it is seen that miR210 expression is approximately 10 folds higher in the *Fh1*$^{-/-}$ samples compared to the double knockout samples and wild type samples. This indicates a significant upregulation of miR210 in *Fh1*$^{-/-}$ samples compared to *Fh1*$^{-/-}$ */Hif1α*$^{-/-}$ (p-value = 0.005) and wild type samples (p- value= 0.006). There is no difference in miR210 expression levels between the double knockout samples and wild type samples as well (p-value = 0.63).

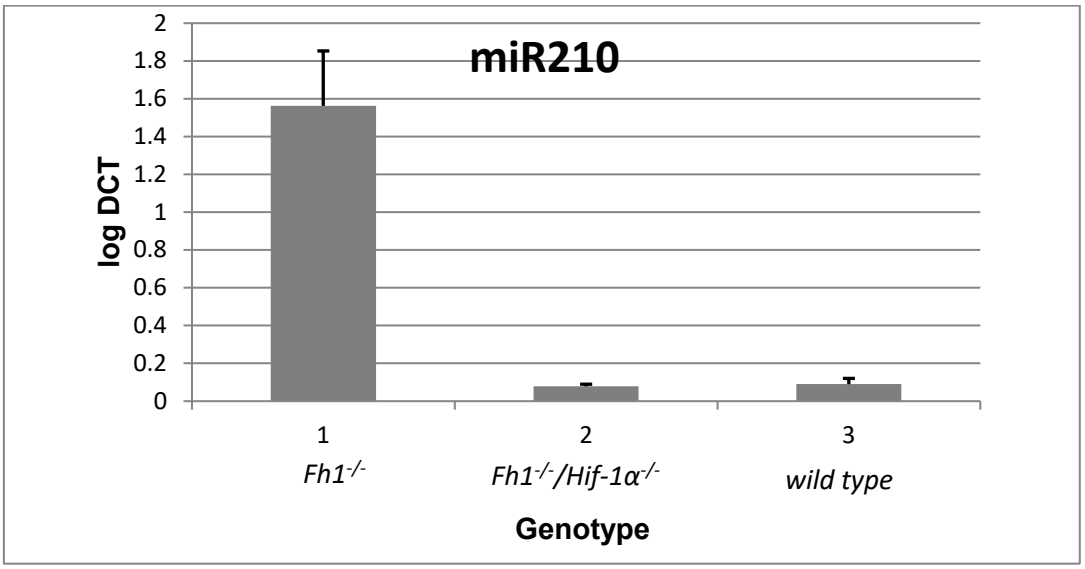


Figure 26: Comparison of *miR210* expression in wild-type, *Fh1*$^{-/-}$ *and Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys

Microarray results also show that Myc is differentially expressed between different genotypes analysed. MicroRNA 23a (miR23a) and microRNA 23b (miR23b) are regulated by Myc are important in glutamine metabolism. Therefore, the expression of these two important microRNAs was also investigated. The microRNA assay results show that miR23a is expressed in wild type, *Fh1*$^{-/-}$ samples and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ samples. The expression level is similar in all three genotypes without any significant difference. A similar result is also true for miR23b expression. Although all samples show a degree of miR23b expression, there is no significant difference in the expression level in samples *with* different genotypes.

Table 16: Ratio of expression of significant genes identified by microarray analysis comparing *Fh1*$^{-/-}$ with wild type, *Fh1*$^{-/-}$ with *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ with wild type samples

The ratios between average expression levels of significant genes identified in the microarray analysis comparing *Fh1*$^{-/-}$ with wild type, *Fh1*$^{-/-}$ with *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ with wild type samples. The statistical significance of the comparison is also indicated.

Gene	Comparison	Ratio of average expression	p-value
Fh1	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$	1.845	0.049
	Fh1$^{-/-}$ vs wt	0.415	0.0001
	Fh1$^{-/-}$/*Hif-1α*$^{-/-}$ vs wt	0.224	$1.24x^{-6}$
Hif-1α	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$	3.641	0.0001
	Fh1$^{-/-}$ vs wt	0.718	0.061
	Fh1-/-/Hif-1α$^{-/-}$ vs wt	0.197	$1.772x10^{-5}$
Eno2	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$	1.174	0.559
	Fh1$^{-/-}$ vs wt	6.530	0.022
	Fh1$^{-/-}$/*Hif-1α*$^{-/-}$ vs wt	5.560	0.0001
Hk2	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$	1.593	0.016
	Fh1$^{-/-}$ vs wt	13.940	$1.73x10^{-6}$
	Fh1$^{-/-}$/*Hif-1α*$^{-/-}$ vs wt	8.74	0.003
Lcn2	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$	0.206	0.002
	Fh1$^{-/-}$ vs wt	58.405	0.005
	Fh1$^{-/-}$/*Hif-1α*$^{-/-}$ vs wt	282.734	0.001
Trib3	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$	0.328	0.106
	Fh1$^{-/-}$ vs wt	9.971	0.0005
	Fh1$^{-/-}$/*Hif-1α*$^{-/-}$ vs wt	30.400	0.051

Gene	Comparison	Ratio of average expression	p-value
Myc	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$	0.413	0.007
	Fh1$^{-/-}$ vs wt	2.584	0.0199
	Fh1$^{-/-}$*/Hif-1α*$^{-/-}$ vs wt	6.247	0.0019
Tgfα	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$	0.423	0.0001
	Fh1$^{-/-}$ vs wt	1.026	0.909
	Fh1$^{-/-}$*/Hif-1α*$^{-/-}$ vs wt	2.424	0.001
Ccnd1	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$	0.548	0.002
	Fh1$^{-/-}$ vs wt	0.810	0.220
	Fh1$^{-/-}$*/Hif-1α*$^{-/-}$ vs wt	1.478	0.006
Cdkn2a	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$	0.090	0.008
	Fh1$^{-/-}$ vs wt	9.853	0.0914
	Fh1$^{-/-}$*/Hif-1α*$^{-/-}$ vs wt	109.036	0.005
Aldo C	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$	2.595	0.003
	Fh1$^{-/-}$ vs wt	1.381	0.284
	Fh1$^{-/-}$*/Hif-1α*$^{-/-}$ vs wt	0.532	0.163
Pdk1	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$	3.560	0.003
	Fh1$^{-/-}$ vs wt	1.988	0.026
	Fh1$^{-/-}$*/Hif-1α*$^{-/-}$ vs wt	0.558	0.038
Slc2a1	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$	0.879	0.449
	Fh1$^{-/-}$ vs wt	2.604	0.005
	Fh1$^{-/-}$*/Hif-1α*$^{-/-}$ vs wt	2.960	0.003
G6pdx	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$	0.437	0.014
	Fh1$^{-/-}$ vs wt	1.409	0.073
	Fh1$^{-/-}$*/Hif-1α*$^{-/-}$ vs wt	3.222	0.005

Gene	Comparison	Ratio of average expression	p-value
Serpine	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$	0.276	0.001
	Fh1$^{-/-}$ vs wt	4.072	0.064
	Fh1$^{-/-}$/*Hif-1α*$^{-/-}$ vs wt	14.744	5.86×10^{-5}
Crb3	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$	3.043	0.083
	Fh1$^{-/-}$ vs wt	15.794	0.024
	Fh1$^{-/-}$/*Hif-1α*$^{-/-}$ vs wt	5.189	0.026
miR210	*Fh1*$^{-/-}$ vs *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$	19.901	0.005
	Fh1$^{-/-}$ vs wt	17.375	0.006
	Fh1$^{-/-}$/*Hif-1α*$^{-/-}$ vs wt	0.873	0.637

3.6. Validation of *Ccnd1* expression by immunohistochemistry

As explained above *Ccnd1* expression is significantly upregulated in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys. In order to investigate this further, semi-quantitative *Ccnd1* expression analysis was performed in the same was as it was done for γH2AX and Ki67 expression (**Table 17**). Based on the microarray and RT-Q-PCR results, it was hypothesized that the expression level will be highest in *Ccnd1* expression level would be was seen in the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys. It was seen that there was degree of positivity in wild type kidneys as well. However, the highest expression level was seen in cystic tubules and the intensity of the staining was very strong **(Figure 27)**.

t-test analysis show that, without taking age and genotype into consideration, both macro cysts (p- value = 1.52 x10^{-23}) and micro cysts (p-value = 4.38 x 10^{-16}) express *Ccnd1* higher compared to wild type kidneys. Comparison between macro cysts and micro cysts show that macro cystic tubules have significantly higher expression of *Ccnd1* compared to micro cystic tubules (p-value = 2. 88 x10 $^{-12}$). Analysis of kidneys from mice younger than 14 weeks show that *Ccnd1* is expressed significantly higher in micro cysts compared to non-cystic tubules (p-value = 0.001). Comparison between macro cysts and non-cystic tubules was not done due to the fact that kidneys from mice aged younger than 14 weeks did not develop macro cysts in our study. Analysis of kidneys from mice older than 14 weeks of age also showed similar results. Ccnd1 expression is significantly higher in macro cysts compared to non-cystic tubules (p-value = 3.70 x 10^{-16}). An increased expression level is also seen in micro cystic tubules compared to the non-cystic tubules (p-value= 1.35x 10^{-13}). Macro cyst to micro cyst comparison also confirm that macro cysts express Ccnd1 significantly higher compared to micro cystic tubules (p-value = 1.77 x 10 $^{-10}$).

Ccnd1 expression in the kidneys was also analysed based on the genotype of the kidneys without taking age of the mice into consideration. In *Fh1*$^{-/-}$ kidneys, micro

cystic tubules express a higher level of Ccnd1 compared to non-cystic tubules (p-value = 3.54 x 10^{-6}). No comparison was done for the *Hif-1α*$^{-/-}$ and *Hif-2α*$^{-/-}$ kidneys since they did not show a cystic phenotype. Analysis of *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys also show similar results. Both macro cysts (p-value= 7.47 x 10^{-7}) and micro cysts (p-value = 7.58 x 10^{-6}) show significant up regulation in *Ccnd1* expression compared to non-cystic tubules. Macro cysts also show a higher expression level compared to micro cysts (p- value = 1.96 x 10^{-5}). In *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys, micro cyst to non-cystic tubule comparison also showed that micro cysts have significantly higher *Ccnd1* expression level (p- value = 1.86 x 10^{-5}).

Comparing expression levels between different genotypes show that *Fh1*$^{-/-}$ kidneys have significantly higher levels of *Ccnd1* in micro cysts compared to micro cysts in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys (p-value = 0.019). This result is in conflict with the microarray and RT-Q-PCR data which show that expression level is higher in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys compared to *Fh1*$^{-/-}$ kidneys. However, it should be remembered that the result of the immunohistochemistry quantification reflects the expression in micro cysts and is not conclusive about the expression level in whole kidney. Comparing the expression level in *Fh1*$^{-/-}$ micro cysts to micro cysts in *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys also show an significant up regulation in *Fh1*$^{-/-}$ micro cysts (p-value = 0.003). There is no significant difference between in the expression level in micro cysts in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys (p-value = 0.34).

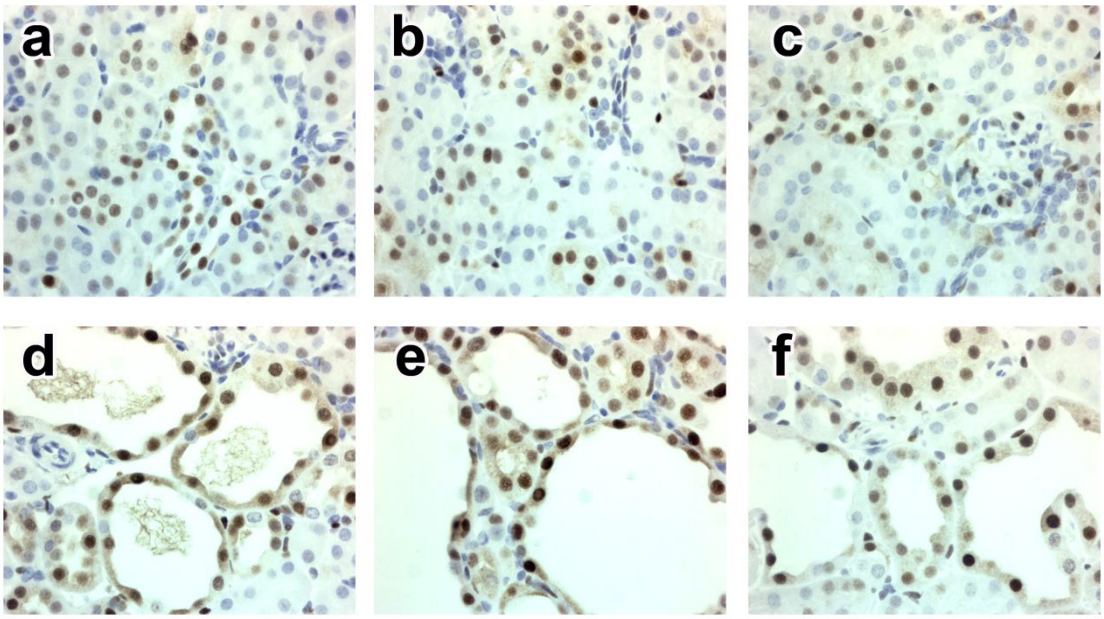


Figure 27: Ccnd1 stained kidney sections

(a-c) show wild type, *Hif-1α*$^{-/-}$ and *Hif-2α*$^{-/-}$ kidneys respectively, at 30 weeks of age. These kidneys are free of cysts. There is occasional Ccnd1 positivity in these kidneys, but it is not present widely in the tissues **(d-f)** show *Fh1*$^{-/-}$, *Fh1*$^{-/-}$/ *Hif-1α*$^{-/-}$ and *Fh1*$^{-/-}$/ *Hif-2α*$^{-/-}$ kidneys respectively at aged between 16-18 weeks. These kidneys are severely cystic and the Ccnd1 expression and intensity are higher compared to the wild type, *Hif-1α*$^{-/-}$ and *Hif-2α*$^{-/-}$ kidneys. Ccnd1 expression is mostly located in the epithelium surrounding the cysts and the intensity of the staining is higher around the cysts as well.

GENOTYPE						MACRO CYSTIC POS NUCLEI			MICRO CYSTIC POS NUCLEI			NON CYSTIC POS NUCLEI		
FH	HIF1	HIF2	KSP	SEX	AGE	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev
fl/+	fl/+	+/+	+	M	24							105	2	1.25
fl/fl	fl/+	+/+	+	F	9							95	4.2	4.24
fl/fl	+/+	fl/+	+	F	13				220	17.45	6.72	82	1.6	2.17
fl/fl	fl/+	+/+	+	M	17				176	13.75	4.84	65	1.3	1.64
fl/fl	fl/+	+/+	+	F	22				233	14.4	8.88	84	0.9	1.73
fl/fl	fl/+	+/+	+	F	24	668	70.71	42.46	401	17	7.06	85	1.4	1.71
fl/+	fl/fl	+/+	+	M	13							114	3.1	2.47
+/+	fl/fl	+/+	+	F	13							82	3	3.13
+/+	fl/fl	+/+	+	F	17							79	2.6	2.67
fl/+	fl/fl	+/+	+	M	22							75	3.5	3.34
fl/+	+/+	fl/fl	+	F	12.5							77	2.9	3.75
fl/+	+/+	fl/fl	+	F	18							72	2.5	3.20

						MACRO CYSTIC			MICRO CYSTIC			NON CYSTIC		
GENOTYPE						POS NUCLEI			POS NUCLEI			POS NUCLEI		
FH	HIF1	HIF2	KSP	SEX	AGE	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev
+/+	+/+	fl/fl	+	F	27-28							86	3.1	3.51
fl/fl	fl/fl	+/+	+	F	9							78	2.3	2.54
fl/fl	fl/fl	+/+	+	M	13				112	7.7	2.50	65	1.5	1.78
fl/fl	fl/fl	+/+	+	F	17	768	57.36	20.35	132	9.3	3.68	92	1.1	1.37
fl/fl	fl/fl	+/+	+	F	17	823	69	22.49	152	12.7	3.09	77	0.8	0.92
fl/fl	fl/fl	+/+	+	F	22	565	76	25.43	166	14.1	3.93	93	1	1.25
fl/fl	fl/fl	+/+	+	M	24	643	50.7	19.78	176	11.2	5.37	67	0.9	0.74
fl/fl	+/+	fl/fl	+	F	12.5							80	2	2.57
fl/fl	+/+	fl/fl	+	M	13				107	6.8	3.91	99	1.4	1.43
fl/fl	+/+	fl/fl	+	M	17.5				156	11.9	3.07	95	2	2.45
fl/fl	+/+	fl/fl	+	M	17.5				165	10.3	5.17	95	1.6	1.96
fl/fl	+/+	fl/fl	+	F	18				130	7.1	3.70	66	0.9	1.10

GENOTYPE						MACRO CYSTIC POS NUCLEI			MICRO CYSTIC POS NUCLEI			NON CYSTIC POS NUCLEI		
FH	HIF1	HIF2	KSP	SEX	AGE	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev	Total No. Nuclei	Mean	StDev
fl/fl	+/+	fl/fl	+	F	27-28				133	11.1	4.31	72	1.4	1.35

Table 17: IHC scoring details for Ccnd1 staining

The age in weeks and sex of the mice (F= female, M=male) and genotypes of the kidneys used in IHC are shown in the table above. As described in the Methods, for each kidney section, 10 tubules of each phenotype (normal, micro cystic and macro cystic) were identified randomly. Within each of these tubules (normal, micro cystic and macro cystic) the total number of nuclei was counted and is included. Also, shown in the table is mean number of nuclei positive for Ccnd1, together with the standard deviation, per tubule. This allows for a comparison of different cystic phenotypes within one genotype at different ages and also between genotypes

4. DISCUSSION AND CONCLUSION

As explained in the introduction of this thesis, it has been reported that *HIF* expression and mutations in *FH* are very important in the aetiology of kidney cysts and tumourigenesis (Pantuck et al., 2003, Pollard et al., 2005). *Fh1*$^{-/-}$ kidneys, which are cystic, show *Hif-1α* and *Hif-2α* stabilization. However, Hif-1α protein levels are expressed at higher levels in the kidney cysts compared to Hif-2α expression (Pollard et al., 2007). Studies on *Vhl* knockout mice show that renal cyst formation is more *Hif-2α* dependent than *Hif-1α* dependent (Rankin et al., 2006). There is strong evidence that *Hif-2α* is more important in tumourigenesis and gives a more aggressive character to the tumour compared to *Hif-1α.* For instance, in VHL mutatant tumours, HIF-2α upregulation favours tumour growth whereas HIF-1α overexpression has the opposite effect (Raval et al., 2005, Zimmer et al., 2004). In order to investigate the role of *Fh1*, *Hif-1α* and *Hif-2α* in cyst development in the kidneys, a breeding strategy was set up to generate mouse kidneys with the following genotypes: *Fh1*$^{-/-}$, *Hif-1α*$^{-/-}$, *Hif-2α*$^{-/-}$, *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$, *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ and wild type as described in the materials and methods section. The mouse model system was validated by confirming the kidney specific deletion of these genes as described in the results section.

4.1. Correlation between *Fh1*, *Hif1α* and *Hif2α* and cystic phenotype

Phenotypic analysis of the *Fh1*$^{-/-}$, *Hif-1α*$^{-/-}$, *Hif-2α*$^{-/-}$, *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$, *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ and wild type kidneys is described in detail in the results section. Briefly, *Fh1*$^{-/-}$ kidneys develop micro and macro cysts. On the other hand, no cystic phenotype is observed in *Hif-1α*$^{-/-}$ and *Hif-2α*$^{-/-}$kidneys. The combined knockout of *Hif-1α* with *Fh1* exacerbates the cystic phenotype resulting in an increase in both the frequency and size of cysts. Cysts develop progressively as the age of the mice increases. *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ kidneys

also develop cysts. However, this cystic phenotype is not as severe as that seen in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys. These results suggest that knocking out *Fh1* leads to cyst formation in the kidneys analysed, confirming the results from previous results Pollard et al., (2007) (Pollard et al., 2007). Identifying the role of *Fh1* in the cystic pathology observed in the kidneys is important since knocking out this gene causes a quite severe pathological phenotype. Knockout of *Hif-1α* or *Hif-2α* does not cause any pathological phenotype in the kidneys by 33 weeks of age. Although knocking out these genes alone does not cause cyst formation, it is seen that knocking out both *Hif-1α* with *Fh1* exacerbates the cystic phenotype. Two possible explanations for this might be: 1) Loss of *Hif-1α* expression can trigger mechanisms and pathways which are associated with HIF and changes in the HIF levels can have consequences on the expression of other genes and pathways such as HIF target genes. This would suggest a more cell autonomous effect. 2) Another explanation could be that secretions and responses from cells neighbouring the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ cells are triggered in response to the biochemical changes that take place in the double knockout cells. Since a more severe cystic phenotype is observed when *Hif-1α* is knocked out together with *Fh1* rather than knocking out *Hif-1α* alone, the main explanation for the cyst formation could be the interaction between *Fh1* and *Hif-1α.* It can be speculated that knocking out *Hif-1α* inhibits mechanisms which protect the cell from the pathological consequences of knocking out *Fh1.* If this is the case, overexpressing *Hif-1α* when *Fh1* is knocked out could potentially have a rescue effect on the cystic phenotype observed in the kidneys. On the other hand, knocking out *Hif-2α* together with *Fh1* does not cause a very severe cystic phenotype. The phenotype is similar to the phenotype seen in *Fh1*$^{-/-}$ kidneys. This suggests that knocking out *Hif-2α* is not associated with the pathological changes in the kidney. When compared to the consequences of the *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ genotype, it is seen that lack of *Hif-2α* expression in combination with lack of *Fh1* expression in the kidneys does not cause such severe pathological changes. This might suggest that the interaction between the mechanism involving *Fh1* and *Hif-2α* are not as strong as the

ones present between *Fh1* and *Hif-1α.* It could also be argued that the effect caused by knocking out *Fh1* and *Hif-1α* is sequential. Knocking out *Hif-1α* exacerbates the cystic phenotype after a pathological response is initiated by knocking out *Fh1.* This also explains why knocking out *Hif-1α* alone does not cause cysts formation.

4.2. DNA damage and Cyst formation

As discussed in detail in the results section, the DNA damage marker γH2AX is expressed in cysts with different genotypes. In *Fh1*$^{-/-}$ kidneys, DNA damage is significantly higher in micro cysts. Knocking out *Hif-1α* and *Hif-2α* genes does not cause any DNA damage in the kidneys analysed. However, a different result is seen in the *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidneys. Both micro and macro cysts in these kidneys have significant DNA damage compared to non cystic tubules. DNA damage is more significant in macro cysts compared to the micro cysts. DNA damage is also present in *Fh1*$^{-/-}$/*Hif-2α*$^{-/-}$ kidneys. Increased DNA damage is observed in cystic tubules both at earlier (younger than 14 weeks) and older ages (older than 14 weeks). This shows that DNA damage occurs early in cyst formation rather than the late stages. No significant difference was found when DNA damage is measured by γH2AX in the epithelial cells of the cysts in comparisons between *Fh1*$^{-/-}$ kidneys versus *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidneys, or *Fh1*$^{-/-}$ kidneys versus *Fh1*$^{-/-}$/*Hif-2α*$^{-/-}$ kidneys. These results indicate that loss of *Fh1, and not Hif-1α or Hif-2α,* is responsible for the increase in DNA damage we have observed here and that expression of *Fh1* in the cystic tubules might protect the cells from DNA damage. This is also supported by studies by Yogev et al., (2010) on yeast and human cell lines in which they have shown that the FH enzyme is found both in the mitochondrion and the cytoplasm and that the cytoplasmic FH is important in yeast in the response to DNA damage. These authors have observed that cytoplasmic FH moves into the nucleus following a DNA damage insult as a part of the DNA damage response mechanism. The same results were also reproduced in human cell lines

(Yogev et al., 2010). Similar to the effect observed in kidneys with an *Fh1*$^{-/-}$ genotype, knocking out *Hif-1α* or *Hif-2*α only leads to DNA damage when it is knocked out in combination with *Fh1.* Furthermore, combining *Fh1* deletion with either Hif*1*-α allele does not increase levels of γH2AX. This indicates that Fh1 (independent of Hif-1α or Hif-2α) may have a protective function in renal epithelial cells. Interestingly, though at variance with our data, previous studies have shown that expression of *Hif-1α* prevents DNA damage repair by inhibiting DNA mismatch recognition mechanisms in the cell (Koshiji et al., 2005).

DNA damage is important in terms of tumourigenesis. Inhibition and repair of DNA damage response is important both in precancerous and cancerous lesions. In the precancerous state, the response to DNA damage is important in keeping the cell in senescence. In tissues which are neoplastic but not malignant, it is seen that DNA damage response mechanisms such as ataxia telangiectasia mutated (ATM), check point kinase 2 (Chk2) and p53 are active. However, inhibition of these molecules and the pathways in which they are involved in acts as a barrier to progression of a pre-malignant state to a malignant state (Bartkova et al., 2006, Bartkova et al., 2005, Halazonetis et al., 2008).

Studies suggest that the role of HIF-2α in DNA damage response contrasts with the role of HIF-1α. The relationship between HIF-2α and DNA damage has been studied in ccRCC and lung adenocarcinoma cell lines where it has been shown that the p53 protein, which is a tumour suppressor and is involved in the response to DNA damage (and cell cycle control), is activated when HIF-2α expression is inhibited. Reduction in HIF-2α expression is also associated with increases in ROS levels in the cells (Bertout et al., 2009). In addition, Biswas et al., (2010) analysed the consequences of HIF-2α expression in ccRCC 786-0 cells. The results show that HIF-2α expression increases DNA damage and ROS in addition to elevating *cyclin D1* expression and promoting cell growth (Biswas et al., 2010).

4.3. Proliferation and Cyst Formation

It has been reported that the cysts in *Fh1*$^{-/-}$ kidneys have increased proliferation but no change in apoptosis (Pollard et al., 2007). Conversely, it has been suggested that apoptosis is important in breaking down the parenchyma of the kidney so that the epithelium can proliferate to forms cysts (Wilson, 2004). Therefore, we investigated proliferation (using Ki67 as a proliferation marker) and apoptosis (using caspase-3 and hematoxilin- eosin as apoptosis markers) in the kidneys. Epithelial cells lining both micro and macro cysts in *Fh1*$^{-/-}$ kidneys show increased proliferation compared to the noncystic tubules. However, neither knocking out *Hif-1α* nor *Hif-2α* alone increases proliferation in the kidneys. On the other hand, *Fh1*$^{-/-}$/*Hif1α*$^{-/-}$ kidneys show an upregulation of proliferation in micro cysts and macro cysts compared to non-cystic tubules (comparable with the *Fh1*$^{-/-}$ kidneys). Micro cysts in *Fh1*$^{-/-}$/*Hif-2α*$^{-/-}$ kidneys also show a significant increase in proliferation. This increased proliferation rate is observed only in cysts from mice from all age groups. No significant difference was found when proliferation rates in the epithelial cells of the cysts were compared in *Fh1*$^{-/-}$ kidneys versus *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidneys, or *Fh1*$^{-/-}$ kidneys versus *Fh1*$^{-/-}$/*Hif-2α*$^{-/-}$ kidneys. These results indicate that *Fh1* is important in regulation of cell proliferation.

Absence of *Fh1* expression is associated with increased proliferation. There could be several explanations for this. As discussed in the introduction of this thesis, knocking out *Fh1* switches the metabolism of the cell from the Krebs cycle to glycolysis which gives a growth and survival advantage to the cell. This advantage might also contribute to an increased proliferation rate in the cell. However, the literature provides evidence contradicting a link between *Fh1* deficiency and increased cell proliferation. It is known that the pH of the cell decreases due to lactic acid production when the cell starts glycolysis. The increase in acidity of the cell is suggested to trigger mechanisms involved in enhancing apoptosis (Schmaltz et al., 1998). On other hand, more recent

studies suggest that increased acidity in endothelial and non-endothelial cell types prevents apoptosis. This anti-apoptotic effect is mediated through activation of Gas6/Axl signalling pathway (D'Arcangelo et al., 2002). This result supports the increased proliferation seen in our results. Therefore, it might be suggested that changes in the pH of *Fh1* deficient cells causes an increase in cell proliferation.

Quantification of proliferation in the epithelial cells of the cystic tubules also shows that knocking out *Hif-1α* and *Fh1* together increases proliferation significantly when compared to non-cystic tubules, whereas this effect is not seen when *Hif-1α* alone is knocked out (no cysts are seen with this genotype). If absence of *Hif-1α* expression in the cell causes increased proliferation, it could be argued that overexpressing *Hif-1α* will prevent the cell from proliferating or induce apoptosis. Literature provides contradicting data about the role of *Hif-1α* in proliferation and apoptosis. Within a population of cells such as in the epithelial lining of the cysts a balance will exist between proliferation and apoptosis. Detecting subtle changes in this balance might be difficult without dual labelling of markers for both these pathways in individual cells. To date such an analysis has not been performed; but if conducted, might help to reconcile some of the conflicting reports relating to the role if HIF-1 in proliferation. Examples of some of this controversy are outlined below. The relationship between *Hif-1α* and apoptosis has been studied in pancreatic cancer cell lines where it has been shown that overexpression of HIF-1α inhibits apoptosis and also allows the cells to survive under decreased glucose levels (Akakura et al., 2001). In addition, it has been shown that apoptosis due to HIF-1α expression takes place when p53 performs its tumour suppressor function in solid tumours. However, in cells where overexpression of HIF-1α is induced, but p53 is mutated and therefore is not functional, cells have reduced apoptosis and show increased proliferation (Graeber et al., 1996, Hopfl et al., 2004). HIF-1α is also known to activate Phosphatidylinositol 3-Kinase/Akt pathway. This inhibits apoptosis, allows cell survival and induces cell proliferation

(Alvarez-Tejado et al., 2001). Increased expression of glucose transporter 1 (SLC2A1/GLUT1) due to hypoxia induced stabilization of HIF-1α is also shown to have an important role in inhibiting apoptosis and allowing cell survival. It is implied that expression of glucose transporter-1 allows the cell to survive under acidic and nutrient deprived conditions in hypoxia and therefore has an anti-apoptotic role (Lin et al., 2000). In HIF-1α $^{-/-}$ B cells, proliferation is significantly increased. Further investigations in these cells show that knocking out HIF-1α causes an upregulation in *cyclin dependent kinase inhibitor 1a (CDKN1a), cyclin dependent kinase inhibitor 1a* (*CDKN1b), and cyclin dependent kinase 2 (CDK2).* Therefore, it has been suggested that knocking out *HIF-1α* causes increased cell proliferation by up regulating cell cycle regulators and causing cell cycle arrest (Goda et al., 2003).

Our results suggest that there is no effect on proliferation either by knocking out *Hif-1α* or *Hif-2α* and *Fh1* together. However, there are data reported in the literature supporting a role for *Hif-2α* in proliferation. It has been shown that the effect of HIF-2α expression in human pulmonary artery fibroblasts leads to an increase in proliferation possibly explained by a consequent upregulation in autocrine release of growth factors such as insulin-like growth factor, fibroblast growth factor and endothelin-1 (Eul et al., 2006). In a different study, analysis of ccRCC samples showed that the group which expressed HIF-2α showed significant upregulation in the proliferation marker Ki67and study, downregulated γH2AX expression. These results indicate that *HIF-2α* is important in promoting proliferation and has a protective effect against DNA damage. This increase in proliferation is suggested to take place due to *c-Myc* activation as a consequence of *HIF-2α* expression. In addition, when ccRCC expressing both HIF-1α and HIF-2α are studied, it is seen that Akt/mTOR and ERK/MAPK signalling pathways, which have important roles in impairing the DNA damage response mechanism, are activated (Gordan et al., 2008). Analysis in HCT116 colorectal cancer, A549 lung cancer and U87MG glioblastoma cell lines also show increased *Hif-2α.* It was observed

that, in these cell lines, cell autonomous cell proliferation takes place. This upregulation in proliferation is caused by activation of the receptor tyrosine kinase signalling and phosphorylation of EGFR and (insulin like growth factor receptor) IGFR through activation of Myc (Franovic et al., 2009).

After the phenotypic analysis of the kidneys, gene expression analysis was carried out on kidneys taken from mice aged 17 weeks or younger. The reason for choosing this time point was to detect the primary mechanism involved in cyst formation and avoid detecting any secondary changes in the aetiology of the cysts. Microarray and RT-Q-PCR analyses on the *Fh1*$^{-/-}$, *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ and wild type kidneys provide important information which supports what has been discussed so far to explain the possible mechanisms causing the observed cystic phenotype observed. Such analyses are important in order to identify genes as potential targets for clinical intervention. Genes encoding metabolic enzymes such as *Ldha, Pdk1, Pkm2, Hk2, CA9, Eno1* and *Eno2* are upregulated in *Fh1*$^{-/-}$ kidneys. The roles of these enzymes have been described previously. However, to summarize briefly, overexpression of these enzymes allow the cell to enter to glycolysis and promotes the Warburg effect in the cell. The Warburg effect favours cell survival which can explain the increased proliferation in the cystic tubules. An upregulation of *Glut1* (*Slc2a1*) is also seen when *Fh1* is knocked out. As explained earlier, this is also important in explaining the increased proliferation. *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys also show changes in the expression levels of genes that are involved in metabolism and the regulation of metabolism such as Glutathione transferase enzymes. These enzymes are involved in glutathione metabolism, which is perturbed in many diseases such as cancer, inflammatory diseases and coronary artery disease (Exner et al., 2000). The end product of the glutathione metabolism is glutathione. Glutathione is found in reduced (GSH) and oxidised (GSSG) forms in the cell and is an important regulator of redox reactions. Increases in the level of glutathione are associated with increased proliferation,

initiation of inflammatory responses and redox reactions. Glutathione metabolism is also associated with glutamine metabolism. Glutamine, which is an important amino acid for protein synthesis, acts as a precursor for glutathione. It plays a role in amino acid transamination through its interactions with α-ketoglutarate. Glutamine has a critical role in purine and pyrimidine synthesis which is vital for the viability of the cell. In addition, glutamine is also very important in the regulation of immune responses. It regulates interferon, interleukin and cytokine secretion in addition to natural killer cell production and proliferation and apoptosis of lymphocytes (Roth et al., 2002). One of the main regulators of glutamine metabolism in the cell is *Myc*. As discussed previously in the results section, our microarray and RT-Q-PCR results also show that *Myc* is upregulated in *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidneys. Glutamate, which is produced from glutamine by glutaminase enzyme, is used for ATP production, anabolism and increasing cell proliferation. Increased expression of *Myc* causes downregulation of miR23a and miR23b which normally prevent glutaminase expression. Therefore, an increase in *Myc* expression allows glutamine metabolism to be activated (Gao et al., 2009). Upregulation of glutathione transferases and molecules involved in immune responses in *Fh1*$^{-/-}$/*Hif-1α*$^{-/-}$ kidneys can be explained by the upregulation of *Myc* in these kidneys. As indicated earlier, upregulation of these mechanisms in the cell increase proliferation and DNA damage which are mediated through changes in expression of tumour suppressor or certain signalling pathways. This logic can be used to explain the upregulation of p53, WNT and MAPK signalling pathways in *Fh1*$^{-/-}$/*Hif1α*$^{-/-}$ kidneys. Over expression of *Ccnd1, Cdkna2b* and *Cdkn3* in these kidneys, which alter the cell cycle, also explains the increased proliferation.

Increased expression of immune response genes is also seen in the double knockout kidneys. This suggests that *Hif-1α* is important in regulation of immune responses. Literature shows evidence that *Hif-1α* expression increases immune responses. It is especially important in activating the innate immune response,

activation of the Toll like receptor–NFκB signalling and secretion of cytokines (Nizet and Johnson, 2009, Kim et al., 2010, Fang et al., 2009). However, the results obtained from our experiments indicate the opposite since knocking out *Hif-1α* causes an increase in the expression of immune response genes.

There are several other important differentially expressed genes in the kidneys which can be used to explain the cystic phenotype such as *Gata3,* which codes for an important transcription factor. *Gata3* expression is increased in both *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys. Upregulation of *Gata3* is seen in tumourigenesis and is associated with increased proliferation. Mutations in *Gata3* also cause abnormal kidney development through disruption of the epithelial-mesenchymal interactions during development (Engelsen et al., 2008, Grote et al., 2008). Therefore, *Gata3* could potentially be one of the contributors to the increased proliferation observed in cystic kidneys. The notch signalling pathway is also upregulated in *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys compared to wild type kidneys. The notch pathway is an important pathway which is involved in a variety of mechanisms including proliferation and in different diseases including cancer (Takahashi et al., 2010, Tien et al., 2009). In addition, it is also important in translating a hypoxia insult to epithelial-mesenchymal transition (EMT). EMT causes alterations in cellular interactions and arrangements of the extracellular matrix. Subsequently, it increases cellular motility and is associated with malignant transition and metastasis (Sahlgren et al., 2008, Radisky, 2005). EMT is also important in renal cyst development. Increased TGF expression plays a role in EMT in renal cysts (Chea and Lee, 2009). When gene expression in *Fh1*$^{-/-}$/*Hif1α*$^{-/-}$ kidneys is compared to the gene expression pattern seen in *Fh1*$^{-/-}$ kidneys, it is seen that many genes which are involved in cell structure and cytoskeleton such as *keratin, actin, laminin, fibronectin* and *transforming growth factors* are upregulated in *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$ kidneys. This shows that these genes are upregulated as consequence of knocking out *Hif-1α.* Over expression of these genes can be used to explain the exacerbation of the

cystic phenotype and increased proliferation seen in the double knockout kidneys. In addition, the results discussed above imply that increased expression of *Notch* and *TGF* cause EMT in the kidneys. As a result of this, genes which are involved in cell structure are upregulated and the tubules are transformed into a cystic structural model. Since EMT is involved in invasion and malignant transition, the cystic phenotype of the kidneys can also be hypothesized to be a malignant transition state.

To summarize, we have seen increased proliferation and DNA damage mainly in the *Fh1*$^{-/-}$ kidneys. Since no significant difference was seen between *Fh1*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif-2α*$^{-/-}$, the focus of the study was on the relationship between *Fh1* and *Hif-1α.* Our data suggests that exacerbation of the cystic phenotype takes place as a consequence of the activation of target genes which in turn activate metabolic pathways, the cell cycle and genes involved in cell anatomy. The synergistic effects of the actions of all these genes might lead to morphological changes in the epithelial cells such as EMT, which could also suggest that the cystic phenotype is potentially an oncogenic transition state. Future studies on the pathways discussed above might be informative.

5. FUTURE WORK

In this project, kidneys with *Fh1*$^{-/-}$, *Hif-1α*$^{-/-}$, *Hif-2α*$^{-/-}$, *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$, *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$ and wild type genotypes were investigated to understand the role of *Fh1, Hif-1α* and *Hif-2α* genes in renal cyst development and their interaction with each other. In order to have an even a better understanding of these genes and their function and interactions with each other in the kidney, a triple knockout (*Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$*/Hif-2α*$^{-/-}$*)* mouse model could be generated. Similar to the analyses performed in this project, kidney sections from the triple knockout model could be analysed for proliferation, DNA damage and apoptosis levels using Ki67, γH2AX and caspase-3 markers respectively. In addition, dual staining of the tissue with these markers might afford more information about subtle changes in the cells. A 2D approach is limited. For example, some apparent micro cysts might be part of macro cysts. Therefore, future analyses should include 3D reconstructions of the kidney, for example *in vivo* imaging. In the 3D construction, specific markers should be used to be able to identify different nephron segments.

Primary cilia are another potential contributor to the renal cyst development which should be investigated in the *Fh1*$^{-/-}$, *Hif-1α*$^{-/-}$, *Hif-2α*$^{-/-}$, *Fh1*$^{-/-}$*/Hif-1α*$^{-/-}$, *Fh1*$^{-/-}$*/Hif-2α*$^{-/-}$, wild type and triple knockout kidneys. Primary cilia are microtubular organelles of the cell which lack ribosomes. Therefore, protein is taken to the cilia and transported along the axoneme of the cilia. This is called the intraflagellar transport which plays an important role in the interaction between the cell and the extracellular matrix. Abnormalities in the primary cilia are associated with different diseases such as renal cystic diseases, brain aneurysms and liver disease (Yoder, 2007). Primary cilia are involved in important renal functions such as solute reabsorption and maintaining the interaction between urinary ligands and receptors on the nephronic membrane. Mutations in several proteins that are a part of the primary cilia play a critical role in renal cyst development. Cystin, inversin, polycystin-1 and polycystin-2 are the most important examples of these proteins (Rodat-Despoix and Delmas, 2009). Mutations in

polycystin-1 and polycystin-2 lead to ADPKD. Mutations in fibrocystin protein which is also located in the primary cilia cause another renal cystic disease called nephronophthisis (NPHP). Cilia are also important in regulating cell polarity and therefore cell cycle. Normal functioning cilia mediate entry to G0 phase of the cell cycle. However, mutations in cilia prevent it from functioning normally and affect the polarity of the cell. Consequently, the proliferation rate of the cell is increased. In addition, dysfunctional cilia also activate the noncanonical Wnt signalling pathway, which is involved in several diseases including different types of cancer (Simons and Walz, 2006). Recent studies also show a disruption in primary cilia function in VHL cancers and cysts. The data implies that VHL mediates this effect on primary cilia through HIF-1α expression (Esteban et al., 2006).

According to the miRNA assays performed, it is seen that miR210 is differentially expressed in the cystic kidneys. Considering our results and the important data available in the literature which suggest an important role of the miRNAs in hypoxic response, metabolism and cancer, a function for miRNAS in triple knockout kidneys and the *Fh1*$^{-/-}$, *Hif1α*$^{-/-}$, *Hif2α*$^{-/-}$, *Fh1*$^{-/-}$/*Hif1α*$^{-/-}$ and *Fh1*$^{-/-}$/*Hif2α*$^{-/-}$ kidneys could be investigated in more detail. One way of carrying out this investigation would be by performing microRNA sequencing experiments. Furthermore, specific candidate miRNAs can also be identified with guidance from literature and the expression levels of these miRNAs could be quantified in the kidneys by using miRNA assays. For instance, miR-9 is one such miRNAs that downregulates E-Cadherin expression and causes EMT. miR-9 is regulated by Myc and it has been shown to have an important role in proliferation and the cell cycle. miR-9 is also associated with metastasis. Since our data provide strong evidence supporting the regulatory role of Myc in the mechanism of cyst formation, focusing on a Myc regulated miRNA might generate important data (Ma et al., 2010, Khew-Goodall and Goodall, 2010). Other miRNAs which are regulated by Myc and associated with tumourigenesis are miR-19 and let-7.

Understanding the role of these miRNAs will allow for their targeting for therapeutic purposes (Olive et al., 2009, Li et al., 2009a).

One of the limitations in our study, mainly in the RT-Q-PCR and microarray analyses, is that the tissue samples used consisted of both wild type and genetically manipulated cells since the RNA was extracted from whole kidneys. Therefore, the results we see do not purely reflect the gene expression levels and mechanisms in the pathological cell alone. One way of overcoming this problem and getting more precise results is using laser-capture microdissection. This technique allows us to identify specific *in vivo* cell populations and isolate them in their own microenvironment. Laser-capture microdissection of tissue and subsequent RT-Q-PCR analysis or in-situ hybridization will also be used to investigate tissue-specific expression patterns of genes identified in the microarray analysis. One technical advantage of using the laser-capture method is that both fixed and frozen tissues could be processed using laser-capture microdissection. Once the cell population of interest is identified and isolated, the equipment is capable of performing complex and very informative analyses on the cells such as RT-Q-PCR, identification of interactions in the microenvironment of the cells and genomic profiling. One of the main limitations of laser-capture microdissection is in identifying the cell population correctly (Espina et al., 2006, Pollard et al., 2007).

Although a severe cystic phenotype is seen in the kidneys studied in this project, the mechanism which leads to cyst formation is still not clear. Throughout the thesis, the role of several genes and pathways involved in proliferation, DNA damage, cell cycle, cell structure and metabolic pathways has been speculated. However, none of these statements are conclusive. For example, even although no obvious effects were seen between the different genotypes, when proliferation and damage were analysed on their own with Ki67 and γH2AX, co-staining with these markers and Hif-1α or Fh1 may show differences. In addition, studying *Fh1, Hif-1α* and *Hif-2α* in other cystic models could provide further explanation about their role in cyst development.

Further experiments, as explained in the future work section of this thesis, need to be carried out to have a better understanding of the mechanisms involved in the cystic phenotype observed in this study. Based on the data we have produced and the data available in the literature, an obvious next step to follow this study would be to generate a triple knockout mouse model and also to investigate further the role of primary cilia.

The observation that the cystic phenotype in *Fh1*$^{-/-}$ kidneys is exacerbated by *Hif-1α* deletion is surprising, yet of great interest. Given the multiple murine models of human cystic kidney diseases, it would be of great interest to investigate whether the apparent protective role of *Hif-1α* is tissue specific.

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