# HIF-dependent Signaling Pathways in Cancer

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Abstract

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In the first part of this thesis we demonstrated increased expression of the Notch1 signaling pathway in ccRCC compared to normal kidney. To further evaluate the role of increased Notch1 signaling we conditionally deleted *Vhl* combined with NICD1 overexpression in the proximal tubules of the renal cortex. NICD was demonstrated to co-operate with *Vhl* loss to promote early signs of ccRCC tumorigenesis by inducing the presence of clusters of dysplastic cells with a clear cytoplasm. Next we demonstrated that hypoxia induced expression of the dopamine transporter SLC6A3 in normal renal epithelium but not in other tissues. We further demonstrated that ccRCC tumors uniquely harbor a functional uptake of dopamine though the SLC6A3 transporter, which constitute a possible target in the clinic.

In the second part of this thesis we showed that CD44 signaling, through CD44-ICD, modulate hypoxic and stem-like phenotypes of glioma stem cells by interacting with HIF-2α. Pharmacological inhibition of CD44 was demonstrated to reduce the hypoxic and stem like phenotypes and by contrast induced expression of differentiation markers. We finally showed that glioma-associated astrocytes promote stem-like phenotypes of glioma cells both in response to hypoxia and after radiation therapy. Such phenotypes were mainly mediated by direct cell-cell interactions and by changes in the ECM. All together these data highlight some of the molecular mechanisms explaining how tumor hypoxia and hypoxic signaling confer aggressive tumor growth.



Key words: Cancer, Hypoxia, ccRCC, glioma, CD44, Notch1, reactive astrocytes

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The circle(s) of research.



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# Content

# List of Papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. Simultaneous targeted activation of Notch1 and *Vhl*-disruption in the kidney proximal epithelial tubular cells in mice
**Elinn Johansson**, Birgitte Rönö, Martin Johansson, David Lindgren, Christina Möller, Håkan Axelson* and Emma Smith*
Scientific Reports 5;6:30739, 2016
*These authors contributed equally to the work

II. Overexpression of functional SLC6A3 in clear cell renal cell carcinoma
Jennifer Hansson, David Lindgren, Helen Nilsson, **Elinn Johansson**, Martin Johansson, Lena Gustavsson and Håkan Axelson
Clinical Cancer Research, 23 (8); 2105-15, 2016

III. CD44 interacts with HIF-2α to modulate the hypoxic phenotype of perinecrotic and perivascular glioma cells
**Elinn Johansson**, Elisa S. Grassi, Vasiliki Pantazopoulou, Bei Tong, David Lindgren, Tracy J. Berg, Elin J. Pietras, Håkan Axelson and Alexander Pietras
*In press, Cell Reports*

IV. Radiation induced changes in the tumor microenvironment
Tracy J. Berg, Vasiliki Pantazopoulou, **Elinn Johansson**, Kristoffer von Stedingk, Alexander Pietras
*Manuscript*

# Abbreviations

| | |
|---|---|
| ABCG2 | ATP binding Casette Subfamily G Member 2 |
| ADAM | A disintegrin and metalloprotease |
| αKG | α-ketoglutarate |
| ANK | Ankryn repeats domain |
| APH1 | Anterior pharynx-defective 1 |
| ARD | Ankyrin repeat domains |
| ARNT | Aryl hydrocarbon receptor nuclear |
| ATP | adenosine triphosphate |
| BAP1 | BRCA1 associated protein |
| BBB | Blood brain barrier |
| bFGF | basic fibroblast growth factor |
| bHLH | Basic Helix-Loop-Helix |
| CaIX | Carbonic anhydrase 9 |
| CAMII | Ca2+/calmodulin-depenent kinase II |
| cAMP | Cyclic adenosine monophosphate |
| CBP | CREB binding protein |
| ccRCC | Clear cell renal cell carcinoma |
| CDK | Cyclin dependent kinase |
| CDKN2A | cyclin-dependent kinase inhibitor 2A |
| CD44ICD | CD44 intracellular domain |
| chRCC | Chromofobe renal cell carcinoma |
| CNS | Central nervous system |
| CNTF | Cilliary neurothropic factor |
| CSL | CBF1 suppressor of hairless Lag1 |
| CT | Computer tomography |
| DLL | delta like |
| DNA | deoxyribonucleic acid |
| DSL | Delta/serat-Lag2 domain |
| ECM | Extra cellular matrix |
| EGFR | Epidermal growth factor receptor |

| | |
|---|---|
| EPAS1 | Endothelial PAS-1 |
| Epo | Erythropoietin |
| FIH | Factor inhibiting HIF-1α |
| FGF | Fibroblast growth factor |
| GABA | Gamma-aminobutyric acid |
| GAG | Glucose amino glycans |
| GBM | Glioblastoma multiforme |
| G-CIMP | Glioma-CpG island methylator phenotypa |
| GFAP | Glial fibrillary acidic protein |
| GPCR | G-protein coupled receptor |
| GSCs | Glioma stem cells |
| GSI | Gamma-secretase inhibitor |
| HA | Hyaluronan |
| HD | Heterodimerization domain |
| HDAC | Histone deacetylase |
| HES | Hairy-enhancer of split |
| HEY | Hairy/enchancer of spit-related with YRPW moti |
| 2HG | 2-hydroxyglutarate |
| HGF | Hepatocyte growth factor |
| HIF | Hypoxia inducible factor 1 alpha |
| HRE | Hypoxia responsive elements |
| IDH | Isocitrate dehydrogenase |
| ID1I | Inhibitor of DNA binding one |
| IL | Interleukin |
| IFN-α | Interferon-α |
| JAG | Jagged |
| JAK2 | Janus kinase 2 |
| JARID1C | Lysine (K)-specific demethylase 5C |
| kap-2 | kidney androgen promoter 2 |
| Ksp1.3 | Kinesin spindle protein |
| Kif3a | Kinesin family member 3A |
| LDHA | Lactate dehydrogenase |
| LOH | Loss of heterozygosity |
| LOX | Lysyl oxidase |
| Maml | Master mind-like-1 |
| MAPK | Mitogen activated protein kinases |
| MDR1 | Multi drug resistance protein 1 |

| | |
|---|---|
| MERTK | MER Proto-Oncogene tyrosine kinase |
| MGMT | O-6-methylguanine-DNA methyltransferase |
| MMP | Matrix metalloproteinases |
| MRI | Magnetic resonance imaging |
| mRCC | Metastatic renal cell carcinoma |
| mTOR | mammalian target of rapamycin |
| mTORC1 | mammalian target of rapamycin complex 1 |
| NEFL | neurofilament light popypeptide |
| NSCLC | Non-small cell lung cancer |
| NF1 | Neurofibromin 1 |
| NICD1 | Notch 1 intracellular domain |
| NLS | Nuclear localization signal |
| NPC | Neural progenitors |
| NTT | Neurotransmitter transporter |
| PAS | Per-Arnt-Sim |
| PBRM1 | BAF180 subunit of the SWI/SNIF chromatin-remodeling complex encoded by the polybromo 1 |
| PDGFB | platelet derived growth factor |
| PDGFR | platelet derived growth factor receptor |
| PDK1 | Phosphoinositide dependent kinase 1 |
| PD | Parkinson´s disease |
| PD1 | Programmed cell death protein 1 |
| PDL-1 | Programmed death-ligand 1 |
| Pax-8 | Paired box gene 8 |
| FDA | Food and Drug Administration |
| PDGF | Platelet derived growth factor |
| PDX | patient derived xenograft |
| PDZ | PSD-95/Dlg/Zo-1 |
| PEC | Parietal epithelial cells |
| PEN-2 | Presenilin enhancer 2 |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PEST | Proline/glutamic acid/serine/threonine degradation domain |
| PFKFB3 | phosphofructokinase |
| PHD | prolyl hydroxylase domain protein |
| PI3K | Phosphoinositide 3-kinase |
| PKM | Pyruvate kinase |
| PN | Partial nephrectomy |

| | |
|---|---|
| PTEN | Phosphatase and tensin homolog |
| pRCC | Papillary renal cell carcinoma |
| p300 | 300-kilodalton co-activator protein |
| RAM | RBPjκ association module domain |
| Rb1 | Retinoblastoma protein 1 |
| RN | Radical nephrectomy |
| RTK | Receptor tyrosine kinases |
| SCC | Squamous carcinomas |
| SCLC | Small cell lung cancer |
| SETD2 | Set domain containing 2 |
| SHH | sonic hedgehog |
| SLC | Solute carrier family |
| SPECT | Single photon emission computed tomography |
| STAT | Signal transducer and activator of transcription |
| SQSTM1 | Sequestome-1 |
| SYT1 | Synaptotagmin-1 |
| TACE | Tumor necrosis factor alpha converting enzyme |
| TAD | Trans activating domain |
| T-ALL | T-cell acute lymphoblastic leukemia |
| TAM | Tumor associated macrophage |
| TCEB1 | Transcription enlongation factor B |
| TFF | Tumor treating field |
| TGF | Transforming growth factor |
| THP | Tamm-Horsfall protein |
| TICs | Tumor initiating cells |
| TKI | Tyrosine kinase inhibitors |
| TMD | Transmembrane domain |
| TMN | Tumor metastasis node |
| TPA | 12-Otetradecanoylphorbol 12-acetate |
| TRACK | Transgenic mouse model of von Hippel-Lindau renal cancer |
| UPR | Unfolded protein response |
| UTX | Lysine (K)-specific demethylase 6A |
| VHL | Von Hippel- Lindau tumor suppressor gene |
| VEGF | Vascular endothelial growth factor |
| VWFC | Von Willenbrand factor type C |
| WHO | World Health Organization |

# Abstract

The cellular response to hypoxia, primarily orchestrated by hypoxia-inducible factors (HIFs; mainly HIF-1α and HIF-2α), is at the center of several signaling pathways conferring aggressive tumor behavior. HIFs may be crucially involved in tumor initiation, as seen in clear cell renal cell carcinoma, as well as maintenance of resistance cancer stem cell phenotypes, as observed in glioma.

In the first part of this thesis we demonstrated increased expression of the Notch1 signaling pathway in ccRCC compared to normal kidney. To further evaluate the role of increased Notch1 signaling we conditionally deleted *Vhl* combined with NICD1 overexpression in the proximal tubules of the renal cortex. NICD was demonstrated to co-operate with *Vhl* loss to promote early signs of ccRCC tumorigenesis by inducing the presence of clusters of dysplastic cells with a clear cytoplasm. Next we demonstrated that hypoxia induced expression of the dopamine transporter SLC6A3 in normal renal epithelium but not in other tissues. We further demonstrated that ccRCC tumors uniquely harbor a functional uptake of dopamine though the SLC6A3 transporter, which constitute a possible target in the clinic.

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# Chapter 1. Tumor development

## Overview

Malignant transformation of cells is a consequence of accumulation of genetic changes in the cellular DNA that perturbs a number of features related to cellular proliferation, differentiation and cell death (Hanahan and Weinberg 2000). While normal cells have a restricted growth potential, malignant cells acquire a limitless number of cell divisions along with the ability to invade normal tissues and metastasize, thus disrupting the normal organ function.

Furthermore tumor cells need to ensure adequate oxygen and energy supply to support their rapid expansion and thus induce formation of new blood vessels in a process called neoangiogenesis. Changes in energy metabolism of tumor cells has emerged as an important feature of cancer cells which has been shown to induce a metabolic shift towards anaerobic energy generation. The immune system has been assigned an important role in tumorigenesis, where the tumor cells may induce a tumor-promoting phenotype of the immune cells. Moreover, tumors reprogram the surrounding non-tumor derived stromal cells thus creating a tumor microenvironment, a so-called tumor niche, that promotes tumor growth (Hanahan and Weinberg 2011).

# Clonal evolution vs. Cancer stem cell hypothesis

The classical model for tumor development includes tumor development by clonal evolution i.e. that any cell, independently of differentiation status, has the potential to form a tumor due to acquired oncogenic mutations that provides traits of advantage including survival potential and the ability to metastasize. As a result most tumors display significant intratumoral heterogeneity and the cells within a single tumor may harbor very different genetic profiles and as a result display different sensitivity towards various therapies. Such phenomena can be exemplified in clear cell renal cell carcinoma (ccRCC) where multiple samplings of the same tumor thus imply parallel evolution of tumor-cell clones (Gerlinger, Horswell et al. 2014).

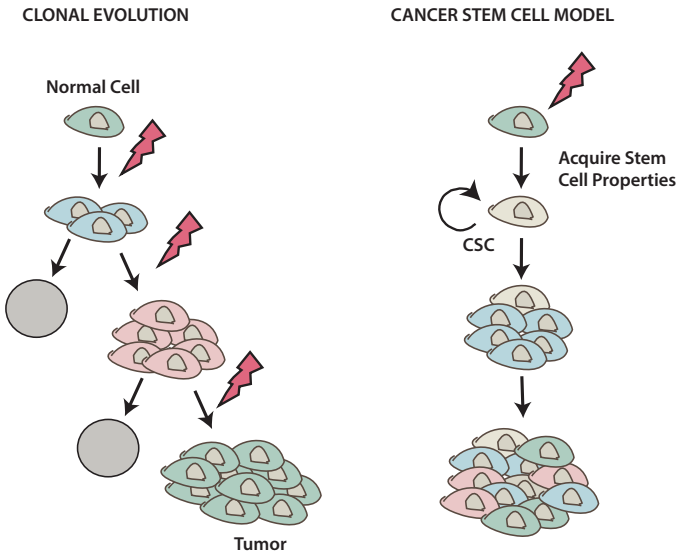


Figure1. Illustration of tumor development by clonal evolution, vs. the cancer stem cell model. The clonal evolution model proposes that any cell may form a tumor due to acquired mutations. The cancer stem cell model by contrast proposes that only a subset of cells that acquired stem cell properties have the ability to form a tumor.

The classical model has been challenged by the cancer stem cell model (Reviewed in (van Niekerk, Davids et al. 2017)), where it is believed that tumors develop from normal stem cell population. The model suggests that only a pool of cells with stem cell properties within each organ has the potential to form a tumor. The cancer stem cell hypothesis further proposes that this subset of cells possesses self-renewal capacity and displays multipotency along with therapeutic resistance,

whereas the main bulk of the tumor cells are suggested to be specified to a more proliferative phenotype. The presence of a cell population possessing such traits was first described in acute myeloid leukemia (Bhatia, Wang et al. 1997) and has since then been described in numerous solid tumors (Al-Hajj, Wicha et al. 2003, Bao, Wu et al. 2006, Ricci-Vitiani, Lombardi et al. 2007). Cancer stem cells constitute a minority of cells within the tumor, and has dependent on the tissue type been estimated to constitute 0.01-0.1% of the tumor population. Importantly, although cancer stem cells may resemble normal stem cells with regards to marker expression (such as CD133 and nestin in brain) it is debated whether cancer stem cells are derived from normal stem cell populations or rather dedifferentiated from more mature cell types (Mallard and Tiralongo 2017).

There is experimental evidence that cancer stem cells confer therapeutic resistance and it has therefore been a scientific goal to identify cellular markers that differentiate tumor initiating cells from "bulk" cells without tumor initiating properties. This goal has been pursued in leukemia as well as in solid tumors including glioma (Lee et al 2006). In the glioma study, cancer stem cells xenotransplanted into immune compromised mice form tumors containing the different cell types of the parental tumor. In addition studies have shown that stem cells may provide mechanisms of therapeutic resistance where only the tumor initiating cells survive treatment (Li et al 2008). An alternative approach to differentiate tumor stem cells from tumor bulk cells, is by expression of drug efflux pumps such as MDR1 (ABCB1) or ABCG2 (Cui, Zhang et al. 2015).

# Chapter 2. Tumor Hypoxia

## Overview

Oxygen is essential to generate a robust energy supply to maintain the viability and function of our complex body. Hypoxia can be described as the insufficient oxygen supply to any given tissue leading to impaired biological function. Reduced oxygen supply could have many causes such as low oxygen tension due to high altitude, reduced ability of oxygen transport by erythrocytes, or by reduced tissue perfusion. As tissues display different sensitivity to reduced oxygen tension it has proven difficult to set a specific oxygen level that defines hypoxia. For the *in vitro* experimental setting, however, the atmospheric oxygen pressure of 21% oxygen (160 mmHg) has been used to describe normoxia. Similarly, pathologically low oxygen tensions *in vitro,* occurs at 8-10 mmHg corresponding to around 1% oxygen and has been used to describe hypoxia. Although 21% oxygen is used on a regular basis in cell cultures it is often argued to be far from "physoxia". Physoxia can be defined as the oxygen levels found in normal peripheral tissues, and varies between tissue types, ranging from around 7.4% to 3% oxygen depending on the tissue. For *in vitro* studies 5% oxygen has been proposed to be a good estimation of physiological and end capillary oxygen tensions (Hockel and Vaupel 2001, McKeown 2014).

Tumor hypoxia is likely to be an early event in tumorigenesis as the tumors are rapidly expanding and outgrowing their vascular supply. Diffusion of oxygen is limited to 5-10 cell layers (100-150 μm) from the nearest capillary before it is completely metabolized, and as a result tumor cells of solid tumors residing at a longer distance from the nearest blood vessel, will experience hypoxia. In addition, expanding tumors often have an impaired blood flow due to insufficient development of new functional vessels (Figure 1.) (Carmeliet and Jain 2000).

Cells have the ability to adapt to low oxygen pressures, by activating a transcriptional program to change a wide range of cellular processes including cellular metabolism, protein translation, DNA repair, cell division, and induction of angiogenesis/neovascularization (Semenza 2003).

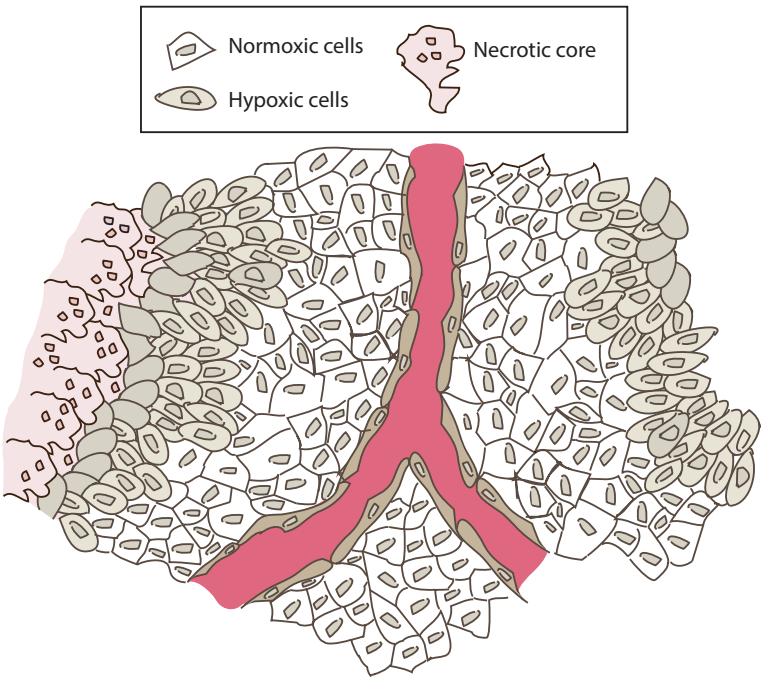


**Figure 2.** Illustration of a solid tumor with hypoxic cells and formation of necrotic cores as a result of that the tumor outgrows its vascular supply.

# Hypoxia-inducible factors

The initial discoveries of adaption to cellular hypoxia were uncovered in the 1990s when Semenza´s group noticed binding of a heterodimer of transcription factors, HIF-1α and HIF-β/ARNT 1/2 (aryl hydrocarbon receptor nuclear translocator)(Hirose, Morita et al. 1996, Pongratz, Antonsson et al. 1998) to the hypoxia-response element (HRE) 3´enhancer region of the blood hormone erythropoietin (Epo) when hepatocytes were grown under hypoxic oxygen conditions (Semenza and Wang 1992, Wang, Jiang et al. 1995). Soon after the initial finding that HIF-1α induced Epo, HIF-1α was shown to regulate more aspects of the hypoxic response, such as angiogenesis by inducing expression of vascular endothelial growth factor (VEGF), (Forsythe, Jiang et al. 1996) and metabolic reprogramming with increased expression of enzymes related to glycolysis (Firth, Ebert et al. 1994)(Reviewed in (Thompson 2016)).

Not long after the discovery of HIF-1α a second subunit, HIF-2α or endothelial PAS-1 (EPAS1) was cloned, and found to bind the same hypoxia-response elements (Tian, McKnight et al. 1997, Wiesener, Turley et al. 1998). In addition, there is a third, less studied, alpha subunit, HIF-3α, with one splice variant lacking the C-TAD domain. HIF-3α has been described to act as a negative regulator of hypoxic response, although its function is much less clarified (Makino, Cao et al. 2001, Maynard, Evans et al. 2007)(Reviewed in (Duan 2016)).

Adaptation to hypoxia seems important already in the initial stages of life. Indeed, lack of hypoxia inducible factors during embryogenesis has proven to be incompatible with life, since HIF-1α as well as most HIF-2α null mice display early embryonic lethality largely due to vascular malformations/abnormal neural fold formation and impaired catecholamine synthesis, respectively (Ryan, Lo et al. 1998, Patel and Simon 2008).

The HIF-α subunits are bHLH-PAS transcription factors with bHLH (basic Helix-Loop-Helix) and PAS (Per-Arnt-Sim) domains (PAS-A and PAS-B) required for DNA binding and for protein interactions to form heterodimeric complexes to oxygen insensitive, constitutively expressed HIF-β/ARNT1/2 subunit (also belonging to the bHLH-PAS family), respectively. In addition the HIF-α subunits contain two transactivating domains important for interactions with co-regulators, one C-terminal (C-TAD) and one N-terminal (N-TAD) that also overlaps with the oxygen dependent degradation domain (ODD) involved in HIF-α stability (Figure 2).

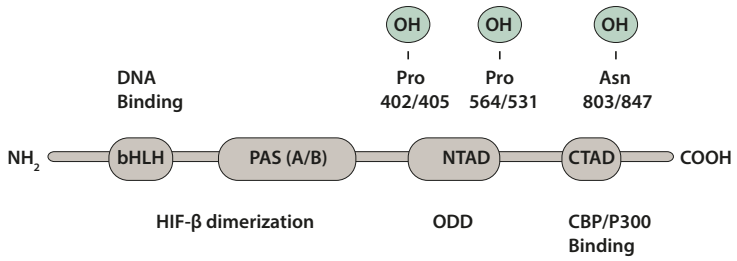


**Figure 3.** Schematic of the HIF-α subunits where bHLH and PAS domains important for DNA binding and HIF β/ARNT dimerization are indicated by boxes. Furthermore, the transactivating domains N-TAD (with the overlapping ODD domain) and C-TAD with the proline and asparagine hydroxylation sites are specified. The HIF-α subunits are hydroxylated by proly hydroxylases at conserved proline residues (HIF-1α Pro402 and Pro-564, HIF-2α at Pro-405 and Pro-531) and hydroxylated by asparagine hydroxylases at a conserved asparagine residue (HIF-1α Asn-803 and HIF-2α Asn-847).

## Oxygen-dependent regulation of the hypoxia inducible factors

Oxygen dependent modification of HIF-1α and HIF-2α proteins is mainly mediated at the post-translational level by prolyl hydroxylases PHD1 (EGLN2), PHD2 (EGLN1) and PHD3 (EGLN3) that mediate hydroxylation of highly conserved proline residues in the N-TAD domain. PHD mediated hydroxylation generates a high-affinity binding site for the von Hippel-Lindau (pVHL) ubiquitin ligase complex that, by ubiquitination, targets the oxygen-sensitive HIF-α subunits for subsequent proteasomal degradation (Huang, Gu et al. 1998, Ivan, Kondo et al. 2001, Kaelin and Ratcliffe 2008, Jokilehto and Jaakkola 2010, Ivan and Kaelin 2017).

The PHDs (except PHD1) have been described be up-regulated in response to hypoxia (conceivably to compensate for their reduced activity at low oxygen levels) and display differences in oxygen affinity, HIF-α subunit isoform- and proline residue preference. PHD2, has the lowest oxygen affinity and is thus generally considered the main oxygen sensor and shows preferred binding for HIF-1α. By contrast, PHD1 (induced by estrogen) and PHD3 (regulates apoptosis) have a preferred binding to HIF-2α. A fourth, much less characterized prolyl hydroxylase PHD4 (or P4H-TM) has been suggested to regulate erythropoiesis (Berra, Benizri et al. 2003, Appelhoff, Tian et al. 2004, Laitala, Aro et al. 2012, Ivan and Kaelin 2017).

The second category of HIF-α regulation is mediated by the factor inhibiting HIF-1α (FIH) (official symbol *FIH1AN*) and mediates asparagine hydroxylation at conserved asparagine residue (HIF-1α; Asn-803 and HIF-2α; Asn-847) located in the C-TAD domain. FIH mediated asparagine hydroxylation at these residues blocks subsequent association with the co-factors CREB binding protein (CBP) and the 300-kilodalton co-activator protein (p300). In contrast to proline hydroxylation by the PHDs, asparagine hydroxylation by FIH does not appear to regulate the stability of the HIF-α subunits but rather regulates their transcriptional activity, where asparagine hydroxylation prevents transcriptional activity (Huang, Gu et al. 1998, Lando, Peet et al. 2002, Sang, Fang et al. 2002, Semenza 2007). Such ability of FIH to regulate transcription of hypoxic response genes was demonstrated in overexpression/knockdown experiments of FIH where overexpression results in suppression of HRE target genes whereas knockdown of FIH induced transcription HRE target genes (Stolze, Tian et al. 2004, Dayan, Roux et al. 2006).

Prolyl hydroxylases and asparagine hydroxylases have in common that they contain a catalytic $Fe^{2+}$ in the active site and are oxygen dependent. Their enzymatic activity can thus be inhibited using iron chelates. They further use diatomic oxygen and α-ketoglutarate (αKG) to hydroxylate substrates by

decarboxylating αKG, thus generating succinate and $CO_2$ as waste products (Thompson 2016). Consequently, lack of oxygen prevents PHD and FIH mediated hydroxylation of HIF- α subunits and leads to rapid accumulation and dimerization to nuclear HIF-β followed by recruitment of a number of co-factors including CBP/p300 and subsequent activation of hypoxia responsive elements to induce expression of HIF target genes (Lando, Peet et al. 2002) (Reviewed in (Semenza 2007)).

Interestingly, FIH has been demonstrated to have higher affinity for oxygen than the proline hydroxylases. This was demonstrated for HIF-1α by use of hydroxylation-site-specific antibodies in renal cells exposed to an oxygen gradient. In this study hydroxylation at Asn-803 was achieved at lower oxygen tensions than at both sites for proline hydroxylation (Pro-402, Pro564) and FIH could thus be concluded to be enzymatically active at oxygen levels that are low enough to stabilize HIF-1α (Tian, Yeoh et al. 2011).

While PHD (especially PHD2) enzymes can be considered gatekeepers of transcriptional adaption to hypoxia, FIH is thought to have a more fine-tuning role of the hypoxic response. This can be exemplified by the fact that PHD2 null mice display a severe phenotype including embryonic lethality at E14.5 (notably PHD1 and PHD3 knockout mice develops normally). FIH null mice, however do not display such dramatic phenotype. Likewise FIH loss in mouse embryonic fibroblasts only causes a modest increase in activation of hypoxia responsive elements (Takeda, Ho et al. 2006, Zhang, Fu et al. 2010).

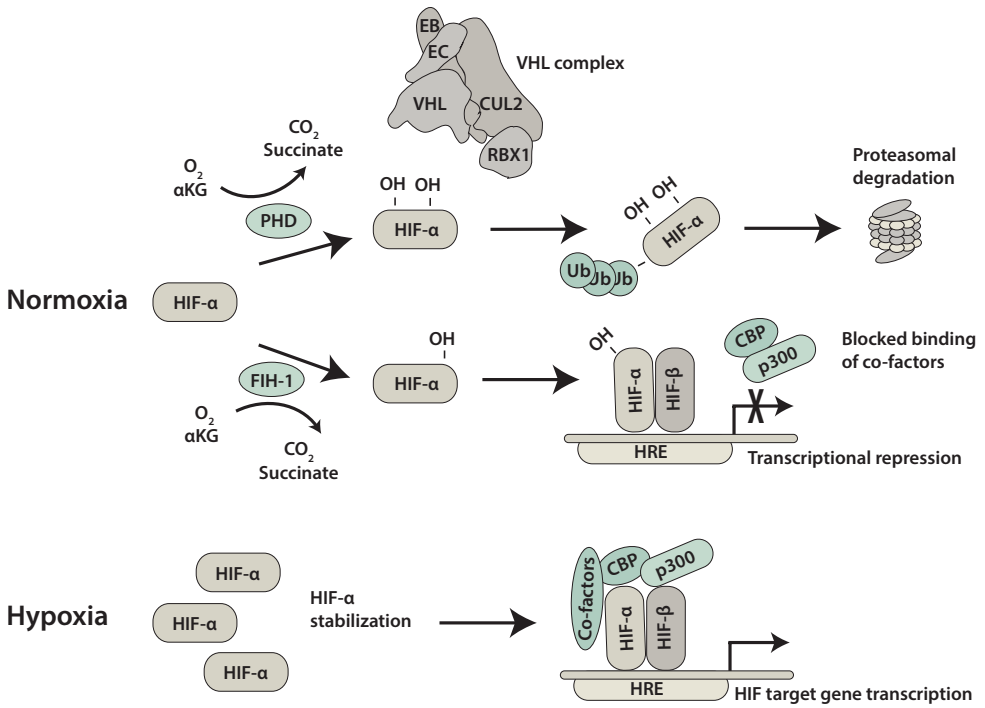


**Figure 4**. Regulation of HIF activity by PHD and FIH mediated hydroxylation of highly conserved proline and asparagine residues on the HIF- α subunits. For the enzymatic reactions $O_2$ and αKG are used as substrates and generate succinate and $CO_2$ as byproducts. PHD mediated proline hydroxylation leads to VHL mediated HIF- α proteolysis whereas FIH-1 mediated asparagine hydroxylation leads to blocked binding of co-factors and subsequent transcriptional repression. Lack of $O_2$ prevents PHD and FIH-1 activity and induces HIF-α stabilization and nuclear translocation followed by dimerization with HIF- β and co-factors such as CBP/p300 to activate HRE dependent transcription.

## HIF-α isoforms display differential sensitivity for FIH- mediated asparagine hydroxylation

Although FIH is able to target both HIF-α isoforms for asparagine hydroxylation, FIH has been described to be more prone to target HIF-1α than HIF-2α, which can be explained by several independent lines of experiments. By use of a panel of N-TAD/C-TAD mutated constructs Kaelin's group showed that the HIF-2α N-TAD portion could induce transcription of hypoxia responsive elements in the absence of the C-TAD portion. The same study further concluded that HIF-2α C-TAD is more insensitive to FIH-mediate hydroxylation than HIF-1α at normoxic oxygen tensions in ccRCC (Yan, Bartz et al. 2007).

Parts of the HIF-1α preference by FIH can further be explained by the fact that different features of the C-TAD domains of HIF-1α and HIF-2α confer differential ability for asparagine hydroxylation (at Asn-803/HIF-1α and Asn-847/HIF-2α). This can be explained in part by a single conserved amino acid within the C-TAD domain. This residue, just C-terminal of the asparagine hydroxylation site of HIF-2α is swapped compared to HIF-1α and seems to make HIF-2α less sensitive for asparagine hydroxylation by FIH. This was demonstrated by mutating this particular residue on HIF-1α (A804V), which partially increased HIF-1α activity without altering its protein levels, suggesting that HIF-1α A804 is important for interaction and hydroxylation by FIH. Likewise HIF-2α substitution of V848A to mimic the sequence of HIF-1α induced slight decreases in transcriptional activity of hypoxia responsive elements (Bracken, Fedele et al. 2006). Taken together the sequence difference of HIF-1α and HIF-2α may thus explain their differences in in affinity towards FIH (Koh and Powis 2012).

# Tumor hypoxia, HIFs, and tumor aggressiveness and stemness

A large number of studies have linked tumor hypoxia and expression of HIFs to de-differentiation/stemness as well as tumor aggressiveness (Reviewed in (Jogi, Ora et al. 2002, Semenza 2003, Holmquist-Mengelbier, Fredlund et al. 2006, Lofstedt, Fredlund et al. 2007, Ruan, Song et al. 2009, Semenza 2016)). Interestingly direct measurement of intratumoral oxygen pressure has demonstrated that oxygen pressure can predict survival (Hockel, Knoop et al. 1993). Similarly, hypoxic tumor regions, with oxygen levels below 10 mmHg, have been shown to become increasingly resistant to ionizing radiation (Jordan and Sonveaux 2012).

The link between hypoxia and tumor aggressiveness may in part be explained by the fact that aggressive, and thus fast growing solid tumors, develop hypoxic tumor regions due to rapid increase in tumor mass with formation of functional blood vessels lagging behind. Such tumors are likely to rapidly develop hypoxic tumor areas as a byproduct simply because they are intrinsically aggressive. There is, however, also evidence that dysregulated HIF signaling induces a phenotypic shift towards a more aggressive phenotype. Hypoxic tumor cells overexpress growth-promoting factors, most of them related to the PI3K/mTOR and MAPK pathways (Reviewed in (Semenza 2003)). Further, tumors are known to rapidly trigger a metabolic shift towards energy generation by increased glycolysis (Warburg 1956) and HIFs are thought to contribute to this increased glycolysis, for example by increasing the glucose uptake. Hypoxia has also been linked to genetic

instability by reduced DNA repair, which is associated with acquisition of additional mutations as result (Bristow and Hill 2008).

Hypoxia is one of the factors that govern stemness and differentiation during embryonic development (Tian, Hammer et al. 1998). Also adult stem cells commonly, but not always, reside in areas with low oxygen levels. Interestingly elimination of HIF function by HIF- β knock out was demonstrated to reduce the pool of hematopoietic progenitor cells in the embryonic yolk sac (Ramirez-Bergeron and Simon 2001)(Reviewed in(Keith and Simon 2007)).

In the tumor setting, HIFs have been shown to induce expression of the stem cell marker CD133 (Ohnishi, Maehara et al. 2013). Tumor hypoxia has further been linked to aggressive tumor growth with increased invasion and metastasis, thus conferring therapeutic resistance and poor patient outcome in several tumor forms such as breast cancer (Giatromanolaki, Sivridis et al. 2006, Helczynska, Larsson et al. 2008), neuroblastoma (Holmquist-Mengelbier, Fredlund et al. 2006), glioma (Li, Bao et al. 2009) and ccRCC (Mandriota, Turner et al. 2002).

## Differential regulation by HIF-1α and HIF-2α in cancer disease

Together the hypoxia inducible factors orchestrate the transcriptional response to low oxygen pressures and co-ordinate the response via alterations in gene expression of several hundred genes (Semenza 2017). While both HIF-1α and HIF-2α bind the same DNA motif, many of the transcriptional targets are distinctly regulated by either of the transcription factors. This may in part be explained by differences in HIF stabilization over time, and with varying oxygen availability (Holmquist-Mengelbier, Fredlund et al. 2006). HIF preference may be further discriminated at the promoter regions by diversity among co-factors, chromatin remodelers and other protein-protein interactions (Reviewed in (Ivan and Kaelin 2017)).

HIF-1α has commonly been described to be responsible for adaption of acute responses to hypoxia, and in solid tumors, for example, uniquely induces expression of components of the glycolytic pathway such as increased expression of phosphofructokinase (PFKFB3), phosphoinositide dependent kinase 1 (PDK1), lactate dehydrogenase (LDHA) or pyruvate kinase (PKM) thus promoting glucose to pyruvate conversion (Kim, Tchernyshyov et al. 2006). With increased production of glycolytic metabolites, such as lactate, it is crucial to maintain intracellular pH, which is regulated by HIF-1α by inducing expression of carbonic

anhydrase 9 (CaIX). HIF-1α further drives expression of genes that limits the cellular oxygen consumption, by suppressing expression of genes involved in mitochondrial respiration (Reviewed in (Ivan and Kaelin 2017, Samanta and Semenza 2017, Semenza 2017)).

By contrast, HIF-2α has been reported to regulate the prolonged effects of hypoxia, which includes processes such as erythropoiesis (by induction of EPO), cell cycle regulation, growth signals and up-regulation of enzymes of importance for invasion such as matrix modulating enzymes, including MMP2, MMP13, and LOX (to mention a few). (Reviewed in (Semenza 2003, Lofstedt, Fredlund et al. 2007))(Holmquist-Mengelbier, Fredlund et al. 2006, Cho and Kaelin 2016, Ivan and Kaelin 2017)).

HIF-1α and HIF-2α differentially regulate promoters of several growth-promoting signaling pathways including MYC and mTORC1 signaling. In the case of MYC signaling, HIF-1α and HIF-2α have been suggested to have opposing roles in ccRCC, where HIF-1α suppresses MYC-dependent transcription while HIF-2α induces MYC-dependent transcription thus promoting RCC growth (Gordan, Bertout et al. 2007, Dang, Kim et al. 2008). Nevertheless in MYC driven diseases such as neuroblastoma, HIF-1α and MYC have been shown to act in favor of tumor aggressive behavior by enhancing glycolysis related signaling (Keith, Johnson et al. 2011) Similarly, HIF-1α and HIF-2α have opposing roles on cell proliferation in respect to mTORC1 signaling, where HIF-1α acts in a growth inhibitory fashion whereas HIF-2α promotes tumor growth (Keith, Johnson et al. 2011).

## HIFs in ccRCC aggressiveness

Over the years researches have taken an interest in unraveling the relative contribution of HIF-1α verses HIF-2α in tumorgienesis. There have been conflicting studies, some suggesting a more viral role for HIF-1α, and others for HIF-2α (Reviewed in(Shen and Kaelin 2013)).

In ccRCC HIF-2α, rather than HIF-1α, has, been suggested to be the main driver for tumorigenesis for several reasons. (I) Knock-in of HIF-2α showed a growth advantage over HIF-1α knock-in in teratomas generated from embryonal stem cells. Furthermore, knock-in of ether HIF-α isoforms was associated with a higher quantity of undifferentiated cells, thus suggesting a link between HIFs and more stem-like cells (Covello, Simon et al. 2005). The same group further linked HIF-2α specifically, as a driver of the stem cell marker Oct-4 (Covello, Kehler et al. 2006). (II) Individuals with germ line mutations of *VHL* display low levels of HIF-2α in early renal pre-neoplastic lesions or cysts compared to higher HIF-2α levels in further developed dysplastic lesions. In contrast, HIF-1α is highly expressed in

early lesions thus suggesting a role for HIF-2α in more advanced lesions (Mandriota, Turner et al. 2002). (III) It is enough to express a PHD insensitive mutant version of HIF-2α (HIF-2α P531A) in pVHL re-constituted (WT8) cells to generate RCC tumors *in vivo* (Kondo, Klco et al. 2002). In contrast, a PHD insensitive mutant HIF-1α introduced in a VHL reconstituted RCC cell line failed to induce tumor growth *in vivo* (Maranchie, Vasselli et al. 2002). Likewise, elimination of HIF-2α in VHL deleted renal carcinoma cell lines has been shown to be enough to suppress tumorigenesis *in vivo* (Kondo, Kim et al. 2003, Zimmer, Doucette et al. 2004).

In addition, HIF-1α has been correlated with a more favorable prognosis (Lidgren, Hedberg et al. 2005). Furthermore, HIF-1α is often deleted or expressed as an aberrant isoform in clear cell renal cell carcinoma cell lines. Reintroduction of wild type HIF-1α protein is correlated with suppressed proliferation both *in vitro* and *in vivo* (Raval, Lau et al. 2005, Shen, Beroukhim et al. 2011)

In stark contrast, other studies have proposed HIF-1α to be more important for ccRCC pathogenesis than HIF-2α (Razorenova, Castellini et al. 2014). One such example includes the TRACK mouse model, where constitutive HIF-1α or HIF-2α was conditionally expressed in the proximal tubules of the renal cortex. This model associated HIF-1α rather than HIF-2α with early signs of ccRCC-like phenotypes such as cyst formation and distorted tubular structure. It is, however, worth mentioning that both HIF-α isoforms in this study were mutated at proline and asparagine residues and thus made insensitive to hydroxylation by PHD as well as FIH, although HIF-1α is likely to be targeted by FIH, and thus opening up for discussion about the relevance of this particular experimental set up (Fu, Wang et al. 2011, Fu, Wang et al. 2013).

Taken together, studies describe a predominant role for HIF-2α, rather than HIF-1α in driving ccRCC tumor growth (Kondo, Kim et al. 2003, Zimmer, Doucette et al. 2004)

## HIFs in GBM aggressiveness

Glioblastoma aggressiveness is partly conferred by presence of drug resistant glioma stem cells (GCSs) residing within perivascular (well oxygenated) and perinecrotic (hypoxic) tumor regions (Hambardzumyan and Bergers 2015).

Hypoxia has been linked with stemness in glioblastoma (Bar, Lin et al. 2010). However, the relative contribution of HIF-1α versus HIF-2α in glioblastoma is still somewhat unclear. Both HIF-α isoforms are expressed in glioblastoma where HIF-2α has been described to be expressed specifically in CD133 positive glioma stem cells (GSC). In contrast, HIF-1α is expressed primarily in the hypoxic GSC

population as well as in the tumor bulk (non-GSC) cells. Similarly, knock down of HIF-2α decreased survival of GSCs, whereas knockdown of HIF-1α decreased survival of GSCs as well as tumor bulk cells, suggesting that HIF-2α would be more specifically linked to stem cell marker expression than HIF-1α which is also expressed in non-GSCs (Li, Bao et al. 2009).

Some studies indicate a role for HIF-1α in inducing and maintaining the self-renewing capacity of GSCs (Soeda, Park et al. 2009, Bar, Lin et al. 2010) and even suggest a tumor suppressive role for HIF-2α (Acker, Diez-Juan et al. 2005).

By contrast, expression of a PHD insensitive mutant version of HIF-2α induced de-differentiation of non-GSCs towards a stem-like phenotype with expression of stem cell markers such as *NANOG*, *OCT4* and *SOX2* (Heddleston, Li et al. 2009). Furthermore, *HIF2A* expression has been correlated with less favorable patient outcome of glioblastoma patients, thus suggesting a role for HIF-2α in glioma stemness and aggressiveness (Li, Bao et al. 2009) Reviewed in (Semenza 2016).

# HIF –independent hypoxic regulation and transcription-independent roles of HIFs

Whilst the hypoxia inducible factors (HIFs) are well-described regulators of cellular adaption to hypoxia other mechanisms of hypoxic regulation have been proposed during recent years. Such mechanisms include the unfolded protein response (UPR) and autophagy (Corazzari, Gagliardi et al. 2017).

Although still a bit controversial, HIFs have further been suggested to have other transcription-independent roles unrelated to hypoxic regulation, including regulation of DNA replication (Hubbi, Kshitiz et al. 2013). More recent efforts have suggested HIF-2α to have a cytoplasmic role involving cap-dependent translation during hypoxic oxygen tensions; this however is still a bit controversial (Timpano and Uniacke 2016).

# Chapter 3. Notch signaling

## Overview

Notch signaling is an evolutionarily conserved pathway first described in *Drosophila melanogaster* and was named after the "notched" appearance of the wings of heterozygous *Notch* flies (Mohr 1919). The Notch gene was cloned in the 1980s (Artavanis-Tsakonas, Muskavitch et al. 1983) and the pathway has since then been extensively characterized and found to be important for a diverse set of cellular processes during embryonic development and adult life (Reviewed in (Zhang, Engler et al. 2017).

Notch signaling acts via direct cell-to-cell communications and is highly context and tissue dependent. Notch has a well-described role in determining cell fate during embryogenesis and is involved in regulating stemness and differentiation and thus maintaining homeostasis of adult tissues. One such classical example involves cell fate determination of stem cells located in the intestinal crypts. The intestine has a high cell turn-over dependent on asymmetric cell division from a pool of intestinal stem cells which divide into enterocytes with absorbing properties or enteroendocrine cells with secretory properties (including mucus-secreting goblet cells, antimicrobial peptide-secreting cells, and chemosensing tuft cells (Barker, van Es et al. 2007). Notch was shown to regulate this differentiation process by inducing differentiation into absorbing enterocytes. By contrast inhibition of the Notch pathway (for example after treatment with gamma-secretase inhibitors) results in expansion of the secretory cell types (Fre, Huyghe et al. 2005, van Es, van Gijn et al. 2005)(Reviewed in (Noah and Shroyer 2013)).

# Canonical Notch signaling pathway

In mammals canonical Notch signaling occurs by juxtacrine signaling from one of the five ligands (Jagged 1-2. Delta-like 1,3 and 4), to one of the four Notch receptors (Notch1-4). The Notch receptors are synthesized in a pro-form that is further processed by furin-like convertases (S1 cleavage) in the Golgi-apparatus leading to formation of the extra- and intracellular domains of the receptor (Logeat, Bessia et al. 1998). Additionally the Notch receptors are subjected to Fringe-mediated glycosylation, which is thought to specify affinity for the receptor-ligand activation (Moloney, Panin et al. 2000). Mature Notch receptors are transmembrane proteins active as heterodimers that are held together by non-covalent interactions between the N-terminal extracellular portion and C-terminal portion composed by the transmembrane and intracellular domains (Logeat, Bessia et al. 1998). The ligand-receptor interaction results in a conformational change, thus enabling binding and proteolysis by a series of enzymes. The first cleavage is mediated by a disintegrin and metalloproteases (ADAM) (S2 cleavage) and occurs in the extracellular space after the ligand has been targeted by ubiquitination following subsequent internalization by endocytosis. Ligand endocytosis is proposed to induce a conformational change of the Notch receptor heterodimer inducing dissociation of the Notch receptor heterodimer, thus allowing for modifications by ADAM proteases (Brou, Logeat et al. 2000, Parks, Klueg et al. 2000). Several members of the ADAM family, in particular ADAM10 and tumor necrosis factor- α -converting enzyme (ADAM17/TACE) have been reported to be responsible for the S2 cleavage in a context dependent manner (Bozkulak and Weinmaster 2009). The S2 cleavage results in release of the extracellular domain and allows two subsequent cleavages at the intracellular portion, mediated by the gamma-secretase complex (S3 cleavage at Val1744 and S4 cleavage at Ala1731-1732) (Schroeter, Kisslinger et al. 1998, Okochi, Steiner et al. 2002). The gamma-secretase complex is a heterotetrameric complex composed of the membrane bound catalytic subunit presenilin1/2 that interacts with nicastrin, PEN-2 and APH1. Importantly all four subunits are needed for the complex to stabilized and become enzymatically active (Li, Bohm et al. 2014). The S3-S4 cleavages induces release of Notch intracellular domain (NICD), which is translocated to the nucleus (Kopan, Schroeter et al. 1996) where NICD forms a complex with the DNA binding protein complex CBF1 suppressor of hairless Lag1 (CSL/CBF1) (or RBPjκ in mice) (Jarriault, Brou et al. 1995). In the absence of NICD, the CSL complex is bound to co-repressors such as the histone deacetylase (HDAC-1) (Kao, Ordentlich et al. 1998). Upon NICD binding the composition is changed towards association with co-factors including Master mind-like-1 (Maml) which induces a shift towards transcriptional activation. Classical target genes include *Hairy-enhancer of split* (HES) and *hairy/enchancer of spit-related with YRPW*

*motif* (HEY) (Jarriault, Brou et al. 1995), both belonging to the bHLH family of transcriptional factors (Illustrated in Fig 5)(Reviewed in (Kopan and Ilagan 2009, Kovall, Gebelein et al. 2017)).

Still, very few universal notch targets have been characterized, which might be explained by the fact that Notch signaling is highly context and tissue dependent. Additionally the ligand-receptor specificity adds on to the complexity of the pathway. Ligand-receptor specificity is partly determined by posttranslational modifications by glycosyltransferases (Moloney, Panin et al. 2000). Such modifications have been demonstrated to balance the equilibrium of Notch binding to the antagonizing ligands to regulate numerous physiological processes such as sprouting of tip and stalk cells during angiogenesis. Notch-DLL4 interaction inhibits sprouting, a process antagonized by Notch-Jagged1 pro-angiogenic signaling. Fringe-glycosylation of the Notch receptor was further shown to enhance Notch-DLL4 interaction and weaken the competing Notch-Jagged 1 interaction in regulating angiogenesis (Benedito, Roca et al. 2009).

Importantly, Notch signaling may be activated by numerous non-canonical mechanisms, for instance without cleavage of the Notch receptor or by activated Notch signaling independently of binding to CSL; for example by interactions with other signaling pathways such as the hypoxic pathway or though the TGF-β pathway (Sjolund, Bostrom et al. 2011, Landor and Lendahl 2017). In general terms non-canonical Notch signaling has been proposed to more tightly associate with potential pathological conditions including cancer, whereas canonical, CSL dependent Notch signaling is required for normal embryogenesis to occur and is thus more associated with normal physiological processes (Oka, Nakano et al. 1995). Furthermore soluble ligands may bind the receptors and are generally, but not always, considered to confer with inhibitory Notch signaling, since they act by sequestering the Notch receptors from its regular ligands residing on neighboring cells (Reviewed in (Falix, Aronson et al. 2012, Ayaz and Osborne 2014)).

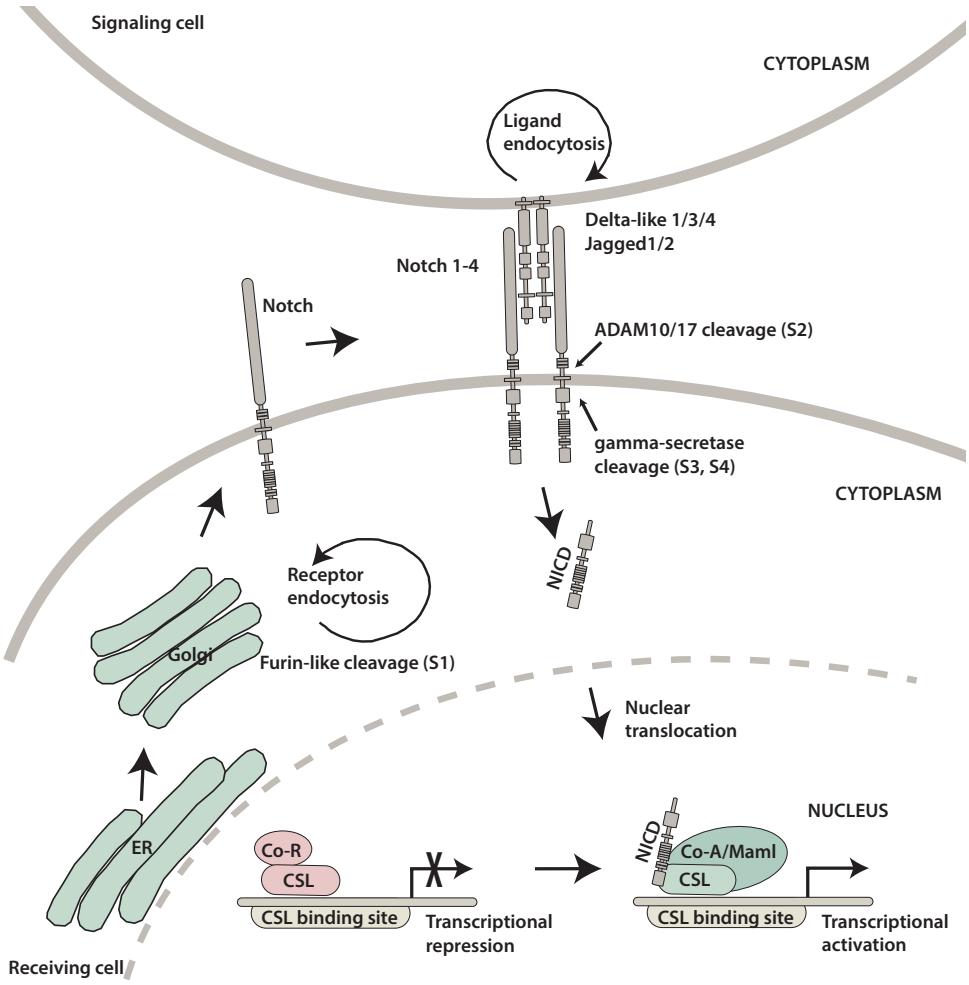


Figure 5. Illustration of the core Notch signaling pathway. Pro-Notch receptors are processed by furine-like convertases and are targeted at the cell suface. Mature heterodimeric receptors (Notch1-4) interact with membrane bound ligands (Delta-like1/3/4 and Jagged 1/2) on neigboring cells. The receptor-ligand interaction induces a confromational change of the receptors that enables proteolytic cleavage by ADAM10/17 and gamma-secretase following release of NICD that is subsequently subjected to nuclear translocation where it assocates with CSL and co-activators to activate transcription of taget genes.

## Notch pathway components

The mammalian Notch receptors are single-pass type I transmembrane receptors composed of an extracellular portion with varying numbers (29-36) of multiple EGF-like repeats that are important for ligand interactions, a negative regulatory region (NRR) composed of three cysteine-rich Lin12-Notch repeats (LNR) that prevent receptor activation in the absence of ligand (Greenwald and Seydoux

1990), and a heterodimerization domain (HD). Further, the Notch receptors contain a transmembrane domain (TMD), a juxtamembrane domain, RBPjκ association module domain (RAM), two nuclear localization domains (NLS), seven ankryn repeats domains (ANK) that coordinates assembly of the nuclear transcriptional complex including MAML (Wu, Aster et al. 2000), and a proline/glutamic acid/serine/threonine degradation domain (PEST) important for protein half life and which also overlaps with a transactivating domain (TAD) (Reviewed in (Niessen and Karsan 2007, Kopan and Ilagan 2009, Andersson, Sandberg et al. 2011, Kovall, Gebelein et al. 2017)).

Similar to the Notch receptors, the ligands Jagged1/2 and Delta-like 1/3/4 are single-pass type I transmembrane proteins. They belong to the DSL (delta/serate/Lag-2) family and in addition to their delta/serate-Lag2 domain (DSL) the Notch ligands are composed of an extracellular domain with 7-16 EGF-like repeats important for receptor interactions. The extracellular portion of the Jagged1/2 further contains a cysteine-rich domain involved in specificity of Notch receptor binding, and a von Willenbrand factor type C (VWFC) domain that is thought to be involved in ligand dimerization (Fleming 1998). The ligands further contain a, relatively short, intracellular domain where Jagged1/2 and DLL1/4 contains a PSD-95/Dlg/Zo-1 (PDZ) domain that is thought to interact with receptor tyrosine kinases (Hock, Bohme et al. 1998, Ascano, Beverly et al. 2003) (Reviewed in (Niessen and Karsan 2007, Andersson, Sandberg et al. 2011)) (Ligand -and receptor domains are depicted in Fig 6).

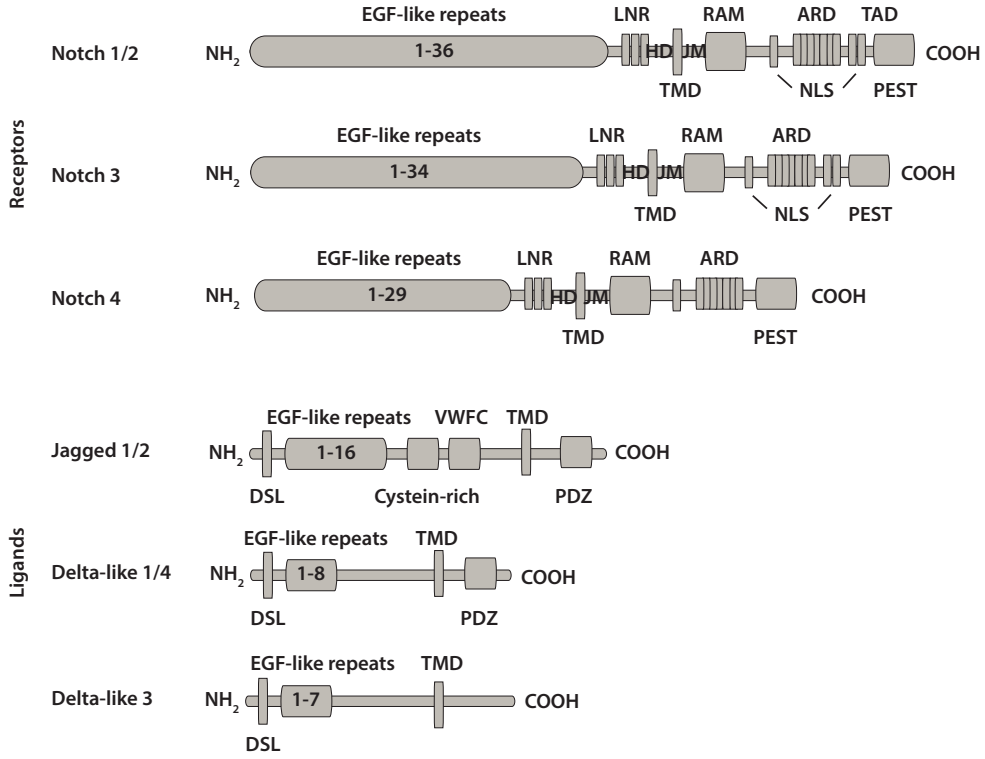


Figure 6. Schematic of the Notch receptors and ligands where protein domains are represented by boxes. The Notch receptors contain an extracellular domain with EGF-like repeats important for ligand interactions, Lin12-Notch repeat (LNR) and heterodimerization domain (HD) that together act as a negative regulatory domain. The receptors further contain a transmembrane domain (TM) which can be cleaved to generate an intracellular domain (NICD). The NICD is composed of several domains; a juxtamembrane portion (JM), rtp-associated molecule domain (RAM), ankryn repeats (ANK), two nuclear localization signals (NLS), transactivating domain (TAD) and a proline (P), glutamic acid (E), serine (S) and threonine (T) degradation domain (PEST). The Notch ligands contain Delta/Serrate/LAG-2 (DSL), EGF-like repeats, a cystein-rich domain, von Willenbrand factor type c (VWFC), transmembrane domain (TMD) and PDZ domains.

# Notch signaling in cancer and disease

Dysregulated Notch signaling is associated with a number of disorders, particularly a wide range of cancers, where Notch can act ether as an oncogene or tumor suppressor depending on the tissue and context.

The first report that the Notch pathway might play a role in tumor formation came from studies of T-cell acute lymphoblastic leukemia (T-ALL), where the Notch gene was shown to be rearranged by chromosomal translocation. Demonstrated by bone marrow transplantation experiments in mice, a truncated nuclear variant of Notch was assigned an oncogenic role in the development of T-ALL (Aster, Pear

et al. 1994, Pear, Aster et al. 1996). Since then around 60% of T-ALLs were found to harbor Notch1 mutations, the majority within the domains regulating dimerization (HD) and degradation (PEST) of the receptor, thus promoting ligand-independent signaling and enhanced receptor stability (Weng, Ferrando et al. 2004) with direct activation of potential oncogenic singling including cMYC, CyclinD3, and CDK4/6. The DLL-4-Notch1/3 axis represents a major mediator of physiological T-cell development (Ciofani and Zuniga-Pflucker 2005). Similarly, overexpressed DLL4-induced hyperactivated Notch singling was demonstrated to support development of T-ALL by regulating proliferation and apoptosis (Yan, Sarmiento et al. 2001). By contrast, inhibition of DLL-4 in T-ALL was associated with impaired tumor growth in T-ALL xenografts by promoting apoptosis (Minuzzo, Agnusdei et al. 2015) (Reviewed in (Braune and Lendahl 2016, Oliveira, Akkapeddi et al. 2017)).

Since the initial description of involvement of Notch in T-ALL, Notch signaling has been assigned an oncogenic role in a wide range of tumors such as breast cancer, lung cancer and prostate cancer (Braune and Lendahl 2016). The oncogenic role of Notch in breast cancer is to a high extent conferred by dysregulated Notch signaling rather than by mutations. For example, many breast tumors display elevated levels of the Notch ligand Jagged1. Notch has been assigned an oncogenic role in non-small cell lung cancer (NSCLC) where around 25% of the patients harbor *NOTCH1* mutations (Chen, De Marco et al. 2007, George, Lim et al. 2015). Notch further has an oncogenic role in prostate cancer (Santagata, Demichelis et al. 2004) where elevated levels of *NOTCH1* and *JAGGED1* correlated with impaired patient outcome (Deng, Ma et al. 2016) (Reviewed in(Ranganathan, Weaver et al. 2011, Braune and Lendahl 2016)).

By contrast, inactivating mutations of *NOTCH1* are commonly seen in patients with small cell lung cancer (SCLC) patients, thus suggesting a tumor suppressive role of Notch in this case. Evidence further supports a tumor suppressive role for Notch in squamous carcinomas (SCC) such as cutaneous SCC (Nicolas, Wolfer et al. 2003) and head -and neck SCC (Agrawal, Frederick et al. 2011). SCC commonly harbor inactivating or truncating mutations in the Notch receptors *NOTCH1*, *NOTCH2* and *NOTCH3* and express truncated versions of the Notch receptors, particularly lacking the C-terminal transactivation domain. The tumor suppressive role of Notch in SCC was further supported by evidence from mice with Notch deletion in the skin. Such mice were more prone to chemical-induced carcinogenesis (Nicolas, Wolfer et al. 2003) (Reviewed in (Braune and Lendahl 2016, Nowell and Radtke 2017).

Notch signaling is known to confer tumor development by several mechanisms, such as through mediation of tumor angiogenesis where blocking DLL-4 is associated with reduced tumor vessel formation in several tumors (Ridgway,

Zhang et al. 2006, Garcia and Kandel 2012). Notch has also been shown to induce epithelial to mesenchymal transition (EMT) by induction of *SNAIL* (Timmerman, Grego-Bessa et al. 2004). Furthermore, Notch signaling has been shown to support the maintenance of tumor initiating cells (TICs)(Rangel, Bertolette et al. 2016). Another example of the complex situation by which Notch signaling influences tumorigenesis relates to cellular energy homeostasis. In breast cancer it was demonstrated that high levels, as well as low levels, of Notch shifts the cellular metabolism towards an increased glycolysis. Interestingly, only tumors with hyper-activated as opposed to tumors with low levels of Notch signaling could shift between energy generation by oxidative phosphorylation and glycolysis, which conferred with more aggressive tumor behavior (Landor, Mutvei et al. 2011).

## Role of Notch in the kidney and renal cancers

Similar to other tissues, dynamic expression of several components of the Notch signaling pathway has been described to regulate cell fate decisions in the developing kidney. Briefly, the kidneys develop from the metanephric mesenchyme giving rise to glomerular podocytes, parietal epithelial cells (PECs), proximal tubular cells, loop-of-Henle and the distal tubular cells, whereas the collecting duct are derived from the uteric bud. Studies in have demonstrated that Notch singling is crucial for proper duct formation and segmentation of nephrons. For example cells committed to become proximal tubule epithelium express high levels of Notch1/2 (Cheng, Miner et al. 2003) where as precursors of distal tubular cells express high levels of Jagged1. Similarly, reduced canonical Notch signaling by conditional heterozygous deletion of RBPjκ in metanephric progenitors largely prevented formation of the proximal tubular compartment (Bonegio, Beck et al. 2011). Furthermore Notch signaling is important for proper vascularization of the kidneys (Reviewed in(Bonegio and Susztak 2012)).

During the recent years a role for Notch signaling in ccRCC tumorigenesis has evolved and several studies have linked components of the Notch singling pathway to worse clinical outcome. One study proposed high levels of Jagged-1 ligand to be a prognostic factor for ccRCC and further linked Jagged-1 expression to reduced overall survival (Wu, Xu et al. 2011).

Further evidences of involvement of Notch signaling, was provided previously by our laboratory, where non-canonical Notch-TGF-β signaling was shown to confer aggressive tumor behavior. Also, inhibition of Notch singling resulted in reduced tumor growth both *in vitro* and *in vivo* (Sjolund, Johansson et al. 2008, Sjolund, Bostrom et al. 2011).

Dysregulated angiogenesis is a common feature in ccRCC tumorigenesis. Two recent studies demonstrated a role for one of the main angiogenesis regulators DLL-4. In the studies DLL-4 was demonstrated to be negatively regulated by a microRNA, miR-30a, which was down-regulated in ccRCC specimens along with increased DLL-4 in endothelial cells. DLL-4 was further proposed to, through the DLL-4-Notch-Hey1-MMP9 axis, promote ccRCC metastasis by cell-cell communication of endothelial -and tumor cells (Huang, Ma et al. 2013, Huang, Ma et al. 2014). Similarly, an independent study linked DLL-4 expression to adverse patient outcome (Wang, Yu et al. 2014).

## Role of Notch in the normal brain and gliomas

During embryogenesis a population of neural progenitors (NPCs) in the neural tube give rise to adult brain structures such as the cerebral cortex. This process requires asymmetric cell division of non cycling NPCs along with migration and differentiation of prospective neurons (McConnell 1995). During neural development Notch maintains neuronal stem cells in an un-differentiated state by transcription of Hes1 and Hes5. (Reviewed in(Zhang, Engler et al. 2017)). In general terms active Notch signaling maintains stemness of NPCs and shifts differentiation towards the glial linage, rather than the neural linage (Teodorczyk and Schmidt 2014). Knock-down of Notch components in developing mice, have underlined the importance of Notch signaling during neural development. Indeed, mutation of *Notch1 or RBPjκ* was associated with embryonic lethality due to improper spatio-temporal migration and differentiation of neurons (de la Pompa, Wakeham et al. 1997).

In the cancer setting glioma stem cells (GSCs) are well-established contributors of the aggressive growth and therapeutical resistance of gliomas (Singh, Hawkins et al. 2004, Chen, Li et al. 2012). Notch signaling has been implicated as an important player in maintaining these GSCs by regulating cellular differentiation and for example by activating the nestin promoter *in vitro* (Shih and Holland 2006). This theory was further supported by studies using glioma cultures grown in stem-cell media. Cells grown in stem-cell conditions up-regulated several members of the Notch pathway, including Notch1/3 and DLL1/3, as compared to cultures grown in serum-containing media, thus further linking Notch signaling to more stem-like tumor cells (Lee, Kotliarova et al. 2006). Another study further linked high Notch levels to increased capacity to form neuro-spheres (Gunther, Schmidt et al. 2008), an assay that is widely used to identify stem cells based on self-renewal from a single cell (Pastrana, Silva-Vargas et al. 2011). Notch 1 have also been associated with glioma vascularization, were forced expression of NICD1 in tumor xerographs induced more vascular tumors. NICD1 expression

did, however not associate with more aggressive tumor growth (Guichet, Guelfi et al. 2015).

The role of Notch has not yet reached a consensus with regards to glioma growth and progression, but Notch has been assigned an oncogenic as well as a tumor-suppressive role. On one hand NICD staining is lower in high grade tumors (grade IV) than in low grade gliomas (grade I-III) (Cheung, Corley et al. 2006) and high levels of Notch signaling has been associated with better patient outcome in high grade gliomas, suggesting that Notch expression may associate with a favorable patient outcome (Phillips, Kharbanda et al. 2006). A recent study demonstrating accelerated growth of PDGFB-driven murine gliomas when subjected to genetic inactivation of *RPBjκ*, *Notch1* or *Notch2*, further supports a tumor suppressive role of Notch in gliomas (Giachino, Boulay et al. 2015).

On the other hand some studies have associated Notch expression with glioma progression and predictor of poor patient outcome. (Xu, Yu et al. 2009, Li, Cui et al. 2011). Similar to ccRCC, Notch signaling has emerged as a potential therapeutical target also for treating gliomas. Administration of GSIs have been shown to repress tumor growth if CD133 positive GSCs (Fan, Khaki et al. 2010) and further showed a delayed recurrence of engrafted gliomas when GSI was used in combination with chemotherapy (Temozolomide) (Gilbert, Daou et al. 2010). The double-edged role of Notch, being associated both with favorable and unfavorable outcome, prompts for identification right subgroup of glioma patients when it would be appropriate to use Notch inhibition. One such subgroup might be glioma patients with high expression of proneural genes, which are more likely to benefit from GSI treatment (Saito, Fu et al. 2014).

## Targeting the Notch signaling pathway

Considering the oncogenic role of Notch, researchers have been evaluating the effect of Notch inhibition by administrating gamma-secretase inhibitors (GSIs). The GSIs have shown promising results on tumor progression associated with decreased metastatic spread in several pre-clinical models (Wei, Walls et al. 2010, Yabuuchi, Pai et al. 2013). Further, a phase I clinical trial of the GSI inhibitor PF-03084014 demonstrated promising response in several solid tumors (Messersmith, Shapiro et al. 2015). Administration of GSIs are however associated with side effects. The dose limiting toxicity for GSIs is caused by expansion of secretory goblet cells in the intestine, thus giving rise to sever diarrhea. Such phenotype could however be circumvented by introducing a treatment holiday and thus restoring interstitial homeostasis (Sjolund, Johansson et al. 2008), it however remains unclear whether such treatment holidays would impair the GSIs therapeutic effect. Another disadvantage of GSIs are that they are targeting the

gamma-secretase enzyme, which importantly has more than 90 substrates except for the Notch receptors, and the effect of GSIs are consequently fairly unspecific. The Notch receptors have the advantage of being surface receptors, which potentially could be targeted using monoclonal antibodies. Such antibodies, targeting Notch1, Notch2/3, are currently being developed and tested both pre-clinically and in the clinic. The Notch antibodies are however, similarly to GSIs, associated to dose limiting toxicity largely due to disrupted intestinal and skin homeostasis (Reviewed in (Andersson and Lendahl 2014, Takebe, Nguyen et al. 2014)). Another strategy is to target tumor angiogenesis, by directing monoclonal antibodies towards DLL4. Anti-DLL4 treatment by Enoticumab demonstrates clinical efficacy associated with impaired tumor vasculature however along with severe side effects and an increased risk for developing VEGF-induced hemangiomas (Yan 2011). Other means of targeting Notch signaling includes for example monoclonal antibodies targeting the gamma-secretase complex (nicastrin), soluble decoy receptors that would interfere with ligand-receptor interactions or by targeting. In addition a recent effort involves development of disease specific protease-activated monoclonal antibodies ("probodies") targeting Notch ligands (Reviewed in (Takebe, Miele et al. 2015)).

Taking into account that Notch also posses a tumor suppressive role, there are situations where Notch inhibition would be highly inappropriate and it would be important to anticipate in which situations they could be used safely. Similarly, use of GSI inhibitors in Alzheimer's disease have been considered inappropriate due to the severe side effects partly due to reduced cognitive function and in part due to an increase incidence of skin cancers (Penninkilampi, Brothers et al. 2016).

## Crosstalk of Notch- and hypoxic signaling pathways

Interestingly Notch and HIF signaling pathways cooperates to regulate many physiological processes, such as angiogenesis; HIFs by inducing expression of angiogentic factors such as VEGF, and Notch signaling by regulating tip-and stalk cells during vessel sprouting (Lendahl, Lee et al. 2009). In the cancer setting several studies suggests that hypoxic signaling collaborates with Notch signaling to maintain cancer stem cells (Wang, Liu et al. 2011, Villa, Chiu et al. 2014).

It has become clear that the Notch pathway and hypoxic signaling pathways crosstalk; both directly and indirectly through several proposed mechanisms (Reviewed in (Landor and Lendahl 2017)). This crosstalk is, however, yet to be fully unraveled.

The initial discoveries that Notch might interact with HIFs were done when Notch target genes (Notch1 and *HES1*) were found to be up-regulated in response to hypoxia (Jogi, Ora et al. 2002). Since the initial discovery that hypoxia and HIFs

can influence Notch signaling several molecular mechanisms has been proposed. Firstly, both HIF-α isoforms have been suggested to affect Notch by direct binding to the receptors and activate Notch target gene transcription. In support of this mechanism, HIF-1α was demonstrated to bind NICD1 as well as Notch responsive elements and activate downstream signaling (Hey2) (Gustafsson, Zheng et al. 2005). Similarly, HIF-2α was demonstrated to bind NICD1 (within the RAM domain) (Chen, Houshmand et al. 2010). Both HIF-α isoforms induce both HRE and CSL binding elements and activate DLL-4, Hey1, Hey2 during hypoxia (Reviewed in (Lendahl, Lee et al. 2009). In addition HIF-1α was demonstrated to bind the promoter regions of Notch target genes directly (Wang, Liu et al. 2011).

Alternatively, another study suggests that hypoxia may enhance Notch signaling by up-regulating the activity of the gamma-secretase complex, where HIF-1α were proposed to directly associate with and favor gamma-secretase complex stabilization (Villa, Chiu et al. 2014).

On the other hand, components of the Notch signaling pathway were also shown to influence hypoxic signaling. Demonstrated by reporter assays, NICD (as well as the HIF-α subunits) was shown to activate both hypoxic and Notch reporters, where the presence of NICD was reported to reduce HIF1-α hydroxylation resulting in HIF1-α stabilization along with increased activation of HREs (Coleman, McDonough et al. 2007, Zheng, Linke et al. 2008).

A second mechanism of HIF-Notch crosstalk involved hydroxylation mediated by FIH, which is not limited to asparagine hydroxylation of HIF-α subunits but can also hydroxylate asparaginyl residues of proteins with ankyrin repeat domains (ARD), including the Notch receptors (NICD1 Asn-1945 and Asn-2012, with higher affinity for site 1). ARD proteins was demonstrated to compete with HIFs for FIH binding and hydroxylation (Coleman, McDonough et al. 2007). FIH was shown to bind Notch (1-4) with highest affinity for Notch2 and was further observed to hydroxylate Notch 1-3, but not Notch-4 (Wilkins, Hyvarinen et al. 2009). Interestingly, FIH has more than 250 fold higher affinity for the Notch receptors than HIF1-α and increased Notch is therefore suggested to mediate an increased activation of hypoxia responsive elements by sequestering FIH and prevents its asparagine hydroxylation of HIF-α (Coleman, McDonough et al. 2007). FIH further interacts with the E3 ubiquitin liagses Mindbomb 1/2 (Mib1/2) that regulates stability of the Notch ligands (Tseng, Zhang et al. 2014).

# Chapter 4. Renal Cancer

## Overview

Renal cancers comprise a diverse group of solid tumors accounting for around 3-4% of the yearly cancer incidence (Siegel, Miller et al. 2017) where the renal cell carcinomas (RCC), originating from the tubular epithelium of the kidney comprises the vast majority (around 90%) of all renal tumors (Patard, Leray et al. 2005). This corresponds to a yearly RCC incidence of nearly 300 000 individuals worldwide and is further associated with more than 100 000 deaths yearly. The incidence is approximately twice as high in men compared to women (Ferlay, Shin et al. 2010).

Generally, RCC patients are diagnosed among the elderly population, with a mean age of 64 years at the time of diagnosis. Also, genetic mouse models of ccRCC present with renal pathology only after long latency (Shuch, Vourganti et al. 2014, Gu, Cohn et al. 2017, Harlander, Schonenberger et al. 2017).

The RCC incidence is higher in industrialized countries than developing countries. Similarly established risk-factors are largely associated with "western lifestyle factors" such as an increased prevalence of hypertension, obesity and diabetes, thus affecting the level of oxygenation of several tissues, inducing obesity-induced inflammation and affecting the endocrine milieu through insulin resistance, respectively. Further smoking increases the lifetime risk of developing kidney cancer, presumably due to the chronic tissue hypoxia that may appear along with regular tissue exposure to carbon monoxide and due to long-term development of chronic obstructive pulmonary disease. Finally, genetic conditions, pre-existing kidney diseases and the fact that we are getting older appear to confer increased RCC incidence (Chow, Dong et al. 2010, Ljungberg, Campbell et al. 2011, McGuire and Fitzpatrick 2011).

At least 16 different forms of RCC have been reported (Ng, Rajandram et al. 2014) and classification is based on histological features and genetic profile where the RCCs are commonly sub-grouped into clear cell RCC (ccRCC), papillary RCC (pRCC), chromofobe RCC (chRCC), collecting duct RCC and oncocytomas, respectively (Kovacs, Akhtar et al. 1997).

# Clear cell renal cell carcinoma (ccRCC)

Clear cell renal cell carcinoma (ccRCC) is the most common form of cancer in the kidneys, accounting for more than 75% of all renal cancers (Linehan 2012). RCCs are believed to arise from distinct cell types along the nephron where several lines of evidence indicate that the ccRCCs originates from the proximal tubular epithelium of the renal cortex. Such evidence includes expression of proximal tubular markers such as CD10, multidrug resistance protein 2 and villin. Further support of proximal tubular origin of ccRCCs may be provided by transcriptional clustering analysis demonstrating clustering of ccRCC specimens to micro dissected proximal tubules, rather than other tubular compartments (Delahunt and Eble 2005, Prasad, Narra et al. 2007, Davis, Ricketts et al. 2014, Lindgren, Eriksson et al. 2017) (Reviewed in (Frew and Moch 2015)).

Histologically ccRCCs are characterized by cells with a clear cytoplasm, due to cytoplasmic accumulation of lipids and glycogen, which is washed out during staining procedures (dehydration-paraffinembedding). ccRCC tumors are further very well vascularized, express high levels of VEGF and are typically organized by nests of cells with this clear phenotype surrounded by vascularized stroma. (Reviewed in (Lopez 2013)).

## The genomic landscape of ccRCC

ccRCC is characterized by bialellic inactivation of the *von Hippel-Lindau* (*VHL)* tumor suppressor gene which resides at chromosome 3p25. Bialelic inactivation of VHL occurs in 70-90% of cases, the remaining 10-30% have wild type *VHL*. Importantly around 8% of the *VHL* wild type tumors harbor inactivation of *TCEB1* encoding enlongin C, which is another component of the pVHL complex and thus highlighting the importance of a dysfunctional pVHL complex for ccRCC tumor progression (Sato, Yoshizato et al. 2013).

A small subset of ccRCC patients (around 2-3%) harbors germ line mutation of *VHL,* which cause the hereditary “von Hippel-Lindau syndrome” (Latif, Tory et al. 1993). Patients with the von Hippel-Lindau syndrome have genetic inactivation of the *VHL* gene and inherit one defective *VHL* allele, but pathology occurs only after somatic inactivation of the second allele (Stolle, Glenn et al. 1998). Individuals with the von Hippel-Lindau syndrome are strongly predisposed to develop ccRCC but also have an increased risk of developing tumors in several other parts of the body including retina, cerebellar and spinal cord tumors (hemangioblastomas), and neuro-endocrine tumors of the adrenal medulla (pheochromocytomas) (Reviewed in (Kaelin 2002, Cho and Kaelin 2016).

The majority of ccRCC cases however occur due to spontaneous somatic mutations (around 80% of cases), deletions or hypermethylations (around 7% of cases) (Herman, Latif et al. 1994) rather than germ-line mutations. As outlined elsewhere in the thesis, HIFs are important for ccRCC tumorigenesis. Restoring pVHL in ccRCC prevents tumor formation in immunocompromised mice, this however can be overridden by introducing HIF variants insensitive to VHL-mediated degradation, indicating that the HIFs are critical in ccRCC tumorigenesis (Kondo, Klco et al. 2002) (Reviewed in (Cho and Kaelin 2016) (2013).

Beyond *VHL* inactivation the genomic landscape of ccRCC displays pronounced heterogeneity. Recent exome-sequencing efforts have however, revealed that many of the recurrent mutations occurs in genes involved in maintaining chromatin states or chromatin modifying genes alternatively regulates response to redox stress/DNA damage. Such genes include the BAF180 subunit of the SWI/SNIF chromatin-remodeling complex encoded by the polybromo 1 (*PBRM1*) gene, which is mutated in 40% of ccRCC (Varela, Tarpey et al. 2011) and the nuclear deubiquitanse BRCA1 associated protein *(BAP1)* is mutated in 14% of ccRCC (Pena-Llopis, Vega-Rubin-de-Celis et al. 2012). Interestingly although *PBRM1* and *BAP1* appears to regulate largely different transcription programs, mutations of *PBRM1* appears mutually excusive from *BAP1* mutations. *BAP1* mutation is further associated with more aggressive disease than *PBRM1* mutated tumors. Further histone H3 lysine 36 trimethylase (H3K36Me3) encoded by the Set domain containing 2 (*SETD2*) gene, is mutated in 10-15% of ccRCC (Dalgliesh, Furge et al. 2010). Other demetylases are mutated at lower frequency of ccRCC and includes the, lysine (K)-specific demethylase 6A (UTX) and lysine (K)-specific demethylase 5C (JARID1C) (Reviewed in (Cho and Kaelin 2016, Schodel, Grampp et al. 2016)).

The rational behind how chromatin-modifying genes may cooperate with *VHL* loss and the subsequent hypoxic activation to promote ccRCC tumorigenesis have been addressed in several studies. First, *in vitro* knock down of *PBRM1* in *VHL* defective cells was associated with increased proliferation (Varela, Tarpey et al. 2011). Second, active transcription of hypoxia responsive elements appears dependent of an accessible chromatin configuration, which was supported by the chromatin immunoprecipitation sequencing experiments (CHIPseq), demonstrating that only a small fraction of the potential HRE binding are indeed bound by the hypoxia inducible factors (Schodel, Oikonomopoulos et al. 2011). HRE activation further appears dependent on CpG methylation status (Wenger, Kvietikova et al. 1998)(Reviewed in (Piva, Santoni et al. 2015, Schodel, Grampp et al. 2016)).

Common copy-number changes include loss of 3p (harboring *VHL*, *PBRM1*, *BAP1* and *SETD2*). Other common copy-number changes include loss of 14q (harboring

*HIF1A*), which has been linked to poor prognosis. Also around 70% of ccRCC harbor gain of chromosome 5q, associated with higher levels of p62 (*SQSTM1*), which was demonstrated to regulate degradation of proteins by autophagy and was further shown to interact with mTOR signaling.

Exome sequencing have further revealed that around 15% of the ccRCC samples harbored recurrent somatic mutations in the PI3K/AKT/mTOR pathway (*PI3KCA*, *PTEN*, *MTOR)* and around 10% of ccRCC samples in the *TP53* (Reviewed in (Creighton, Morgan et al. 2013, Frew and Moch 2015, Chen, Zhang et al. 2016).

## The Von Hippel Lindau tumor suppressor gene

The von Hippel-Lindau tumor protein (pVHL) acts as an E3 ubiquitin ligase that target specific proteins for polyubiquitylation and consists of an α-domain responsible for binding to other members of the ubiquitin ligase complex, and a β-domain that acts as the substrate docking site. pVHL forms a complex with enlongin B, enlongin C (Duan, Pause et al. 1995) and cullin-2 (Pause, Lee et al. 1997). Furthermore Rbx1 has also been found to associate with pVHL-enlongin-CUL2 complexes (Kamura, Koepp et al. 1999) (Reviewed in (Kaelin 2002, Cho and Kaelin 2016).

In ccRCC hypoxia-inducible genes are activated in the presence of oxygen following pVHL inactivation, which might be somewhat surprising considering that regulation of co-activator recruitment by C-TAD asparigine hydroxylation remains largely unaffected in cells lacking pVHL (Sang, Fang et al. 2002) and one would thus expect C-TAD hydroxylation to prevent transcriptional activity by the HIFs. Yet cells lacking pVHL do not degrade the HIF- α subunits irrespective of changes in oxygen (Iliopoulos, Levy et al. 1996, Maxwell, Wiesener et al. 1999). However, HIF-1α was shown to activate hypoxia target genes in the absence of the C-TAD domain (Gothie, Richard et al. 2000, Wykoff, Pugh et al. 2000), which might suggest that the N-TAD and C-TAD domains differentially control activation hypoxia target genes and that a panel of HIF target genes may be activated despite of FIH mediated hydroxylation. As described elsewhere in this thesis, FIH has been described to have a preference for HIF-1α over HIF-2α and thus leaving HIF-2α (but not HIF-1α) transcriptionally active in VHL defective ccRCC. Such notion would offer an explanation to why ccRCC tumorigenesis correlate with hypoxic transcription driven by HIF-2α (Reviewed in(Cho and Kaelin 2016)).

Importantly, pVHL have been demonstrated to regulate several cellular processes apart from regulation of HIF- α stability that may contribute to tumor initiation independently of HIF. Such processes for example include regulation of microtubule stability (Hergovich, Lisztwan et al. 2003) and maintenance of

primary cilium (Thoma, Frew et al. 2007). Loss of pVHL may also confer suppression of p53 activation since pVHL regulate p53 stabilization by ubiquitination of Mdm2. Furthermore, pVHL has been demonstrated to regulate assembly and secretion of extracellular matrix components such as fibronectin (Ohh, Yauch et al. 1998) and collagen IV(Kurban, Duplan et al. 2008) (Reviewed in (Frew and Moch 2015)).

## Clinical manifestation and prognosis

Localized RCC is associated with clinical symptoms including hematuria, flank pain and a palpable abdominal mass, whereas metastatic symptoms for example include bone pain, unexplained fever, lung nodules or wasting syndromes. Patients with localized disease have a very good prognosis with a 5-year survival of around 90%, where the patients are generally cured by surgical resection of the affected kidney. However, due to the relatively vague clinical symptoms some tumors (around 30%) (Cohen and McGovern 2005) at a late stage of disease when the cancer already metastasized to lung, liver, bone, skin, brain and/or other soft tissues. At this point the 5-year survival is significantly reduced to around 10%, and is associated with a median survival of around 18 months (Reviewed in (Escudier, Eisen et al. 2012)). Also, some 25% of patients with localized disease at diagnosis later develop metastasis and hence very poor prognosis (Cohen and McGovern 2005).

The imaging modalities used for diagnosis commonly includes an initial ultrasonography followed by computer tomography (CT) to assess the degree of local invasiveness, involvement of lymph nodes and metastasis. Less frequently, magnetic resonance imaging (MRI) may be used to confirm diagnosis and confirm involvement of venous tumor thrombus. Kidney function and is further assessed by serum creatinine, hemoglobin, lactate dehydrogenase measurements. In addition, tests including leukocyte and platelet counts are taken to score prognosis. Before systemic treatment, diagnosis by imaging is commonly complemented by a histopathological diagnosis by generation of a renal tumor core biopsy or, when available, a nephrectomy specimen (Reviewed in (Escudier, Eisen et al. 2012)).

Considering the heterogeneous nature of RCC researchers and clinicians have been addressing different means of stratifying patients to predict prognosis and personalize treatment. The ULCA integrated staging system (UISS) is widely used to predict prognosis of RCC patients. This system gives a combined assessment of T stage, Furman´s grade and the ECOG prognostic indicator. Briefly, T stage defined by tumor metastasis node (TMN), grades localization of the primary tumor (T) from a small organ-confined tumor (T1) to a tumor that invades tissue beyond Gerota´s fascia or renal gland (T4). Further regional lymph node involvement (N)

is graded from no involvement (N0) to metastasis in more than one regional lymph node (N2). Finally absence (M0) or presence (M1) of distant metastasis is graded. Based on the TNM grading the tumor is staged I-IV, where stage IV patients are associated with the least favorable outcome. The Fuhrman nuclear grading system focus on nuclear appearance and grades nuclei from 1-4 based on size, irregularity and nucelolar visibility in tumor nuclei (Fuhrman, Lasky et al. 1982). Finally, the Eastern Cooperative Oncology Group (ECOG) performance status assess the patient´s general well being (Reviewed in (Escudier, Eisen et al. 2012)).

## Treatment

Renal cell carcinomas are generally refractory to treatment with radiation and common cytostatic therapeutics. Patients with localized disease thus typically undergo surgical treatment by partial nephrectomy (PN). Patients with more aggressive disease (T2-T4), and further tumors with venous thrombus, instead undergo radical nephrectomy (RN). Care must be taken when the patient is above 75 years are operated on, as they display an increased risk of surgery-associated morbidity. For such cases active surveillance is an alterative. Although not curative, patients with metastatic RCC (mRCC) may still be subjected to surgery by removal of the primary tumor(s) and when possible removal of metastasis, where particularly patients only presenting with lung metastasis displays a survival benefit from surgical removal of metastasis (de Riese, Goldenberg et al. 1991) (Reviewed in (Ljungberg, Bensalah et al. 2015)).

Classically mRCC patients have further been subjected to systemic treatments including the immune system stimulators interleukin 2 (IL-2) and interferon-α (IFN-α). Complete response using these cytokine therapies do occur, but are extremely uncommon, however around 20% and up to 15% of the patients demonstrate a partial response towards IL-2 and IFN-α, respectively (Reviewed in (Rini, McDermott et al. 2007, Ljungberg, Bensalah et al. 2015, Takyar, Diaz et al. 2016).

During recent years numerous targeted therapies have been developed. In general terms such targeted therapies focus on inhibiting the processes that are hyperactivated in response to *VHL* loss, in particular tumor angiogenesis, which is augmented du to the secretion of vascular endothelial growth factor (VEGF). VEGF is targeted ether by using monoclonal antibodies such as *bevacizumab* or by using the tyrosine kinase inhibitors (TKI) such as *sunitinib* that targets VEGF, PDGFR and cKit, or *sorafenib* that targets Raf1, b-Raf. VEGFR2, PDGR and c-Kit. Other targeted therapies target both tumor proliferation and angiogenesis by targeting the mammalian target of rapamycin (mTOR) (PI3K/Akt/mTOR). *temsirolimus and everolimus*. However, around 20-30% patients display intrinsic

resistance towards VEGF therapies and further resistance almost invariably develop over time (Philips and Atkins 2014).

As, long-term survival benefits are generally not seen using the anti angiogenic therapies alterative approaches to treat mRCCs are much needed. During recent years researchers have explored new immunotherapeutic strategies for mRCC. Such strategies include the identification of immune modulatory pathways that protect host cells and tumor cells from destruction by activated immune system and include the PD1 receptor is expressed on T, B and NK cells and the ligand PDL-1 that may be expressed on tumor cells and antigen presenting cells. The PDL-1/PD1 interaction confers inhibitory signaling of T-cell effector functions. Recently immune checkpoint inhibitors towards programmed death 1 (PD-1) (*nivolumab* or MK-37445) and its ligand (PD-L1) (MPDL3280A) was developed. ccRCC is considered a highly immunogenic tumor with high infiltration of lymphocytes, however only around 20% of the infiltrating cytotoxic T cells recognize ccRCC tumor cells, thus proposing that the ccRCC harbor mechanisms to escapes the immune system (Creighton, Morgan et al. 2013, Combe, de Guillebon et al. 2015). Notably, up to half of the ccRCC tumors have aberrant expression of PD-L1 thus providing a rational for evaluation of immunotherapies in ccRCC (Leite, Reis et al. 2015). Data so far demonstrate promising anti tumor effects in about one third of ccRCC patients along with tolerable side effects. PD-L1 responders have further been linked to high expression of PD-L1 in tumor cells, tumor micro environment or immune cells (McDermott, Sosman et al. 2016) (Reviewed in (Philips and Atkins 2014, Weinstock and McDermott 2015)).

Recent efforts have aimed at direct targeting of the hypoxia inducible factors. Historically therapeutic targeting of transcription factors (such as the hypoxia inducible factors) has proven difficult (Koehler 2010), however in 2009 Bruick and Gardner identified a hydrophobic pocket within the PAS-B domain of HIF-2α and were further able to design an allosteric inhibitor to prevent dimerization of HIF-2α to the HIF-β subunit (Scheuermann, Tomchick et al. 2009, Scheuermann, Li et al. 2013). Not long after the initial discovery of the hydrophobic pocket of HIF-2α, two structurally similar allosteric inhibitors, PT2399 and PT2385 (the latter with better pharmacological properties), were designed and found to specifically inhibit HIF-2α/ HIF-β dimerization along with HIF-2α specific target genes, whereas they did not affect HIF-1α/HIF-β dimerization nor affected HIF-1α target genes. Both inhibitors were found to have very good anti tumor effects *in vivo*, where PT2399 treatment was demonstrated to extended survival in nude mice with ccRCC xenografts. PT2399 was able to suppress growth in 56% of the tumors in a patient derived xenograft (PDX) model of ccRCC. PT2399 was however associated with resistance development in a subset of PDX tumors, which generally had lower levels of HIF-2α thus suggesting a variable degree of HIF-2α dependence of ccRCC tumors. These observations prompt for identification of

biomarkers to predict treatment-response in the clinic. Long- term treatment with PT2399 was further associated with HIF-2α/HIF-β mutations that would allow for dimerization in the presence of PT2399. Importantly several of the tumors that were unresponsive to *sunitinib* treatment responded well to PT2399, thus opening up for direct HIF targeting when targeting of downstream effects of HIF activity is associated with resistance. PT2385 entered a Phase I clinical trial which demonstrated promising results so far (Chen, Hill et al. 2016, Cho, Du et al. 2016, Wallace, Rizzi et al. 2016) (Reviewed in(Cho and Kaelin 2016)).

# Genetic mouse models of ccRCC

A large number of studies have been aimed at establishing genetically engineered mouse models of clear cell renal cell carcinoma. In concordance with the nearly obligatory *VHL* loss among ccRCC patients, most genetic models target the *Vhl* gene directly, or alternatively by mimicking the resulting pseuohypoxic phenotype by expressing non-degradable versions of the HIF-α subunits (Reviewed in (Kapitsinou and Haase 2008, Frew and Moch 2015)).

The first study to model *VHL* loss was published during the late 1990s and showed that mice harboring a general *Vhl* deletion (by homologous recombination) displayed embryonic lethality due to vascular abnormalities in the placenta, whereas *Vhl* heterozygous mice did not display any signs of renal pathology during embryogenesis or postnatal life (Gnarra, Ward et al. 1997). Similarly, mice carrying human *β-actin* to drive heterozygous mosaic deletion of *Vhl,* failed to induce renal tumorigenesis and displayed no apparent affect on normal embryogenesis (Ma, Tessarollo et al. 2003), thus suggesting that one wild type *Vhl* allele is enough to support normal development. This notion fits well with the renal phenotype of individuals with the von Hippel-Lindau disease, where genotyping shows that both *VHL* alleles are inactivated in renal cysts that progress into more advanced lesions (Walther, Lubensky et al. 1995, Mandriota, Turner et al. 2002).

To avoid the embryonic lethality seen in the general *Vhl* null mice, several groups have generated different strains of mice with conditional deletion of *Vhl* using nephron segment-specific promoters by utilizing the Cre/lox recombination technique (Gu, Marth et al. 1994). Several studies deleted *Vhl* in the ascending loop-of-Henle using *Thp-Cre* (Schley, Klanke et al. 2011) or collectively in ascending loop of-Henle, distal tubules and collecting duct using *Ksp1.3-Cre* (Shao, Somlo et al. 2002, Frew, Thoma et al. 2008) but the mice did, however not display any significant renal pathology. *Vhl* deletion in the proximal tubules, using the *Pepck-Cre* was on the other side associated with a low frequency of renal cyst formation (Rankin, Tomaszewski et al. 2006). In addition, two more recent studies utilized embryonal promotes. The first study used *Six-2* to drive expression of Cre recombinase in glomerular and tubular cells already from early embryogenesis. When *Vhl* deletion was assessed alone using the *Six-2* cre-driver, it similarly to *PEPCK*-Cre mice, gave rise to formation of renal cysts (Wang, Gu et al. 2014). By contrast, *Vhl* deletion in renal tubular cells using a second *Pax-8* cre-driver, (expressed later during embryonic development), was associated with HIF stabilization but did not induce any renal pathology (Mathia, Paliege et al. 2013). Thus, mice with conditional *Vhl* deletion in various parts of the nephron may, at the most, display renal tubular dysplasia but do not develop renal cancer.

Several studies have further targeted the hypoxia inducible factors, ether alone or in combination with *Vhl* loss. Two studies investigated the role of *Vhl* and HIF in hemangiomas, which is commonly seen in individuals with the von *Hippel Lindau syndrome*. Deletion of *Arnt* in *Vhl* deficient mice was demonstrated to suppress formation of vascular hepatic tumors (hemangiomas) whereas *Hif-1* deletion alone could not indicating that hemangioma formation is more dependent on dysregulated HIF than ccRCC, where *Hif2a* seems able to compensate for the loss of *Hif1a* (Rankin, Higgins et al. 2005, Rankin, Rha et al. 2008).

Again, these results should be compared to the von Hippel-Lindau syndrome patients, as these individuals display hundreds of dysplastic renal cysts that only occasionally develop into full-blown renal carcinoma (Walther, Lubensky et al. 1995), it becomes increasingly apparent that *Vhl* loss nor HIF-α stabilization alone provokes tumorigenesis and is clear that additional oncogenic events are required for tumor formation (Walther, Lubensky et al. 1995, Montani, Heinimann et al. 2010). In support of this theory other studies utilizing stable expression of *Hif1a or Hif2a* gives rise to a partial renal phenotype such as renal fibrosis phenotype along with renal cyst formation (Schietke, Hackenbeck et al. 2012) or is associated with abnormal glycogen accumulation and presence of distorted tubules (Fu, Wang et al. 2011, Fu, Wang et al. 2013) but does not fully mimic human ccRCC. On the other hand deletion of *Hif1a* and *Hif2a* in distal tubules largely rescued the phenotype of renal cysts and neoplastic nodules in *Vhl*/*Trp53* deleted mice, suggesting that both Hif-α isoforms harbors pro-tumorigenic properties in early cyst formation (Albers, Rajski et al. 2013).

One study addressed HIF independent functions of pVHL, including its ability to stabilize microtubules. By co-deleting *Vhl* together with the kinesin family member 3A *(Kif3a)* they demonstrated disruption of primary cilia along with increased frequency of renal cysts, thus suggesting that HIF-independent functions pVHL may explain parts of the cystic phenotype seen in von-Hippel-Lindau patients (Lehmann, Vicari et al. 2015). *Pten* loss has further been demonstrated to cooperate with *Vhl* loss to reduce ciliated renal cells, suggesting that genes that regulate primary cilia are of importance for early renal tumor pathology deletion (Frew, Thoma et al. 2008). Numerous efforts have targeted recurrent mutations in ccRCC including combined *Vhl/Pten* deletion (Frew, Thoma et al. 2008), combined *Vhl*/*Trp53* deletion (Albers, Rajski et al. 2013) and combined *Vhl*, *Trp53* and *Rb1*deletion (Harlander, Schonenberger et al. 2017) (Reviewed in (Dart 2017, Schmidt and Linehan 2017) where the first study demonstrated increased renal cyst formation and two last developed different degrees of neoplastic renal lesions. The model using combined *Vhl*, *Trp53* and *Rb1* deletion generated a relatively early onset of a spectrum of lesions ranging from simple cysts to neoplastic lesions with many of the features of human ccRCC. This model was

further demonstrated to mimic human disease with regards to partial response towards targeted therapies (*sunitinib*, *everolimus* and *acriflavine*).

It has been argued that human von Hippel-Lindau patients (harboring *VHL* heterozygous cysts) are more prone to develop ccRCC than *Vhl* heterozygous mice due to differences in positioning of genes on chromosomes. In humans, *VHL* loss is often a consequence of 3p loss, also leading to loss of *PBRM1*, *BAP1* and *SETD2* (where *PBRM1* and *BAP1* mutation is mutually exclusive), which all reside at the same chromosome arm 3p. By contrast, mouse *Vhl* is located at chromosome 6, whereas *Pbrm1* and *Bap1* are located on chromosome 14. Consequently, loss of heterozygosity (LOH) in humans would leave only one intact copy of 3p whereas LOH of chromosome 6 in mice would leave both copies of chromosome 14 intact. In support if such theory combined inactivation of *Vhl*/Bap1 under the embryonal Six-2 promoter was found to induce renal carcinogenesis (Wang, Gu et al. 2014). The usage of this model may be somewhat limited considering that the homozygous deletion of the *Vhl/Bap1* alleles results in early lethality. This notion prompted for finding a more suitable cre-driver. Two proximal promoters failed to induce renal pathology, however mice carrying the embryonal promoter *Pax-8-Cre* recapitulated renal pathologies ranging from simple renal cysts, low grade ccRCC tumors (using *Vhl/Pbrm1*) and high grade ccRCC (using *Vhl/Bap1*) (Gu, Cohn et al. 2017).

**Table 1. Summary of genetic mouse models of ccRCC and hemangioma. The promoters and their expression are listed along with, mutated alleles, renal phenotype and study. Tamm-Horsfall protein (*Thp*), 129 bp of the Ksp-cadherin (Cadherin 16) *(Ksp1.3)*, phosphoenolpyruvate carboxykinase (*Pepck*), Homeobox protein Six2 *(Six2)*, gamma-glutamyl transpepsidase *(GGT)*, Paired box gene 8 *(Pax8)*, sodium/glucose co-transporter 2 (Sglt2).**

| Promoter | Promoter expression | Mutated alleles | Phenotype | Study |
|---|---|---|---|---|
| ***Homologous recombination*** | General KO | $Vhl^{+/-}$ | $VHL^{-/-}$ embryonic lethality | (Gnarra, Ward et al. 1997) |
| | | $Vhl^{+/-}$ | $VHL^{+/-}$ normal development/no renal pathology | |
| **Human *β-ACTIN-Cre*** | Mosaic pattern in multiple organs | $Vhl^{fl/fl}$ & $Vhl^{fl/+}$ | No renal pathology | (Ma, Tessarollo et al. 2003) |
| ***Thp-Cre*** | Ascending loop-of-Henle, early distal tubules | $Vhl^{fl/fl}$ | No renal pathology | (Schley, Klanke et al. 2011) |
| ***Ksp1.3-Cre*** | Ascending loop-of-Henle, early distal tubules and collecting duct | $Vhl^{fl/fl}$ | No renal pathology | (Frew, Thoma et al. 2008) |
| ***Pepck-Cre*** | Proximal tubule, hepatocytes | $Vhl^{fl/fl}/PTEN^{fl/fl}$ | Renal cysts | (Rankin, Tomaszewski et al. 2006) |
| | | $Vhl^{fl/fl}$ | Renal cysts | |
| | | $Vhl^{fl/fl}Hif1$ | Renal cysts | |
| | | $Vhl^{fl/fl}Arnt$ | No renal cysts | |
| ***Six-2-Cre* (embryonal)** | Broad embryonal expression in glomeruli, and proximal tubule | $Vhl^{fl/fl}$ | Renal cysts | (Wang, Gu et al. 2014) |
| | | $Vhl^{fl/fl}/Bap1^{fl/+}$ | Spectrum of renal abnormalities; cysts, diluted tubules, small neoplastic nodules (some cells with clear cytoplasm). Lethal due to renal failure at 8 months | |
| | | $Vhl^{fl/fl}/Bap1^{fl/fl}$ | Allele lethal before 4 weeks of age | |
| ***Albumin-Cre or Pepck-Cr*** | Hepatocytes or proximal tubules and hepatocytes | $Vhl^{fl/fl}$ | Hemangiomas | (Rankin, Higgins et al. 2005) |
| | | $Vhl^{fl/fl}/Arnt^{fl/fl}$ | No liver pathology | |
| | | $Vhl^{fl/fl}/Hif1^{fl/fl}$ | Hemangiomas | |
| ***Pepck-Cre*** | Proximal tubule, hepatocytes | $Vhl^{fl/fl}/Hif1^{fl/fl}$ | Hemangiomas | (Rankin, Rha et al. 2008) |
| | | $Vhl^{fl/fl}/Hif2^{fl/fl}$ | No liver pathology | |
| ***GGT-Cre*** | Proximal tubule | Transgenic expression of HIF-1-3M (Human PHD and FIH insensitive HIF-1α) | Renal cyst formation, Disorganized proximal tubules | (Fu, Wang et al. 2011) |
| ***GGT-Cre*** | Proximal tubule | Transgenic expression of HIF-2-3M (Human PHD and FIH insensitive HIF-2α) | Glycogen accumulation | (Fu, Wang et al. 2013) |

**Table 1. Continued.**

| Promoter | Promoter expression | Mutated alleles | Phenotype | Study |
|---|---|---|---|---|
| ***Ksp1.3-Cre*** | Ascending loop-of-Henle, early distal tubules and collecting duct | *Vhl*$^{fl/fl}$/*Trp53*$^{fl/fl}$ | Renal cyst, disorganized tubules, neoplastic nodules (some cells with clear cytoplasm) | (Albers, Rajski et al. 2013) |
| | | *Vhl*$^{fl/fl}$/*Trp53*$^{fl/fl}$/*Hif1a*$^{fl/fl}$ | No renal pathology | |
| | | *Vhl*$^{fl/fl}$/*Trp53*$^{fl/fl}$/*Hif2a*$^{fl/fl}$ | Renal cysts | |
| ***Ksp1.3-Cre*** | Ascending loop-of-Henle, early distal tubules and collecting duct | Transgenic expression of Hif-2-HA (Mouse PHD and FIH insensitive Hif-2α) | Renal fibrosis and renal cysts | (Schietke, Hackenbeck et al. 2012) |
| ***Pax-8-stTA/LC1*** **(tetracycline inducible)** | Adult expression in renal tubules inclusive collecting ducts, hepatocytes | *Vhl*$^{fl/fl}$ | No renal pathology | (Mathia, Paliege et al. 2013) |
| ***Ksp1.3-Cre***$^{ERT2}$ **(inducible)** | Ascending loop-of-Henle, early distal tubules and collecting duct | *Vhl*$^{fl/fl}$/*Kif3a*$^{fl/fl}$ | Accelerated cystic burden compared to Kif3a$^{fl/fl}$ | (Lehmann, Vicari et al. 2015) |
| | | *Hif1a*$^{fl/fl}$/*Kif3a*$^{fl/fl}$ | Non-accelerated cystic burden compared to Kif3a$^{fl/fl}$ | |
| ***Sglt2*** | Proximal tubule | *Vhl*$^{fl/fl}$/*Bap1*$^{fl/fl}$ or *Vhl*$^{fl/fl}$/*Pbrm1*$^{fl/fl}$ | No renal pathology | (Gu, Cohn et al. 2017) |
| ***Villin*** | Proximal tubule | *Vhl*$^{fl/fl}$/*Bap1*$^{fl/fl}$ or *Vhl*$^{fl/fl}$/*Pbrm1*$^{fl/fl}$ | No renal pathology | |
| ***Pax-8-Cre*** **(embryonal)** | Embryonic expression (later than Six-2): mesonephros, metanephros, nephric duct, uteric bud. Broad adult expression in renal tubules including collecting ducts, hepatocytes. | *Vhl*$^{fl/fl}$/*Pbrm1*$^{fl/fl}$ | Lethal at 3 months, small ccRCC like tumors with, nuclear atypia, simple cysts, cytoplasmic clearing | |
| | | *Vhl*$^{fl/fl}$/*Pbrm1*$^{fl/+}$ | Lethal at 14.5 months | |
| | | *Vhl*$^{fl/fl}$/*Bap1*$^{fl/fl}$ | Lethal at14.5 months. Lesions ranging from simple cysts to ccRCC lesions at 10-11 months | |
| | | *Vhl*$^{fl/fl}$/*Bap1*$^{fl/+}$ | Lesions ranging from simple cysts to ccRCC lesions | |
| ***Ksp1.3-Cre***$^{ERT2}$ **(inducible)** | Ascending loop-of-Henle, early distal tubules and collecting duct | *Vhl*$^{fl/fl}$/*Trp53*$^{fl/ff}$/*Rb1*$^{fl/fl}$ | Renal cyst formation, dysplastic cysts and neoplastic lesions | (Harlander, Schonenberger et al. 2017) |

# The dopamine transporter SLC6A3

Most cells maintain a similar composition of ions including high intracellular levels of $K^+$, and low levels of $Na^+$ and $Ca^{2+}$ compared to the extracellular space. To maintain such cellular homeostasis the cells use ion pumps such as the $Na^+/K^+$ ATPase pump, which is widely expressed in the cellular plasma membranes. In addition energy is stored in the ionic gradients and facilitated secondary transport of molecules such as, other ions, sugars, amino acids and neurotransmitters. The presence of ion pumps like the $Na^+/K^+$ ATPase is crucial to maintain physiological processes such as maintaining the sodium gradient that is utilized for filtering the blood from waste products and reabsorb nutrients (Clausen, Hilbers et al. 2017).

SLC6A3 is a transporter protein belonging to the large family of solute carriers (SLC), which is composed of around 400 different members. The SLC transporters are mainly driven by transmembrane ion gradients.

The SLC6 subfamily generally contains 12 transmembrane (TM) domains with N and C termini located intracellularly. They transport small amino acids such as creatine and taurine, or neurotransmitters such as norepinephrine, GABA, seretonin and dopamine. While some transport proteins may be driven by passive diffusion along a concentration gradient or by active, ATP dependent, transport against a concentration gradient the SLC6 family mediate substrate transport by secondary active transport. Such transports are facilitated by the concentration gradients generated by other (primary) active transporters to move their substrate against their own gradient together with co-transport of $Na^+$ or $K^+$ along their electrochemical gradient. The co-transport can be moved in the same direction (symporters) or in the opposite direction (antiporters) from the substrate.

SLC6A3 (also named DAT) belongs to the neurotransmitter transporter (NTT) subgroup of SLC6 transporter proteins, and mediate substrate transport by symport of one $Cl^-$ and two $Na^+$ from the extra cellular space (Hediger, Clemencon et al. 2013, Pramod, Foster et al. 2013).

The substrate of SLC6A3 is dopamine. Dopamine is released into the synaptic cleft of dopaminergic neurons where it acts by binding one of five dopamine receptors D1-D5 in the post-synaptic neuron. Based on coupling to G-protein coupled receptors (GPCR) dopamine release may confer excitatory or inhibitory signals in dependent on the receptor. The dopamine receptors are sub-divided into two families; the D1-like family (containing D1 and D5) and the D2-like family (containing D2, D3 and D4). D1-like dopamine receptors generally confer stimulatory signaling by GPCR mediated stimulation of adenylyl cyclase, which results in subsequent increased levels of cyclic adenosine monophosphate (cAMP). By contrast the D2-like dopamine receptors confer inhibitory signaling,

since the receptors are couple to inhibitory GPCRs that inhibit adenylyl cyclase and cAMP production (Kebabian and Calne 1979, Beaulieu, Espinoza et al. 2015).

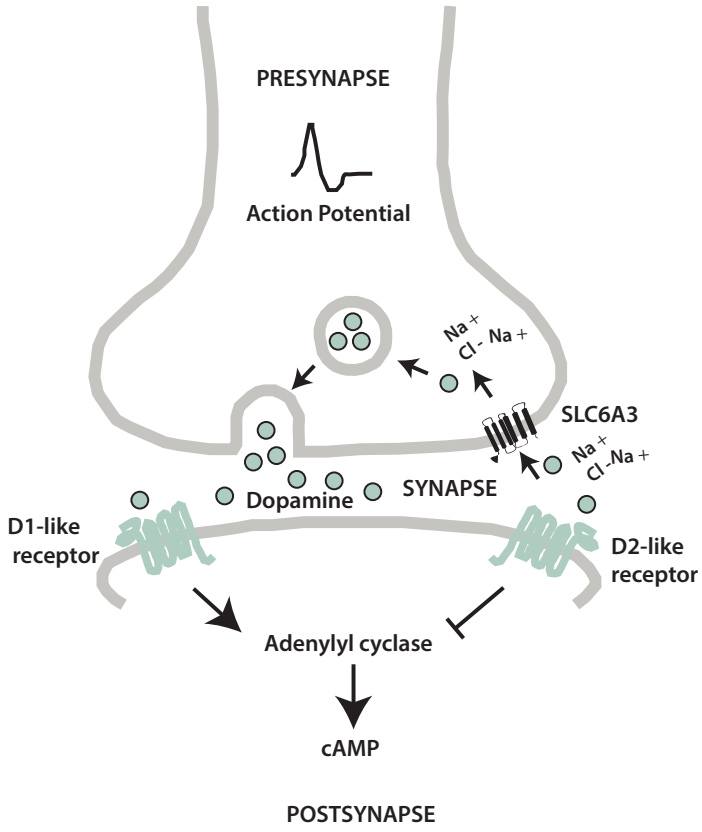


Figure 7. Schematic of dopaminergic signaling in the synaptic cleft. An action potential mediates release of dopamine from the presynaptic cell following binding to dopamine receptors. The dopamine are grouped into D1-like receptors mediating stimulatory signaling by adenylyl cyclase and cAPMP, whereas binding to D2-like receptors mediate inhibitory signal. Dopamine is recycled into the pre synaptic cell through transport by SLC6A4/DAT together with co transport of sodium and chloride ions. SLC6A3 is expressed in the membranes of dopaminergic neurons in brain regions, mainly within the substantia nigra pars compacta with projections to several brain regions including the ventral tegmental area and striatum and nucleus accumbens. SLC6A3 rapidly mediates dopamine transport from the extra cellular space to the cytosol of the pre-synaptic neuron and thus controls the duration of the dopamine signal and further mediates recycling of dopamine into the dopaminergic cell.

One major function of dopaminergic transmission in the brain is to control voluntary movements. Additionally, dopaminergic exerts modulatory effects on human reward, sleep, attention, behavior and cognitive function. Dysregulated dopaminergic signaling by elevated dopamine levels or by degeneration of the dopaminergic neurons have been associated to several neurological disorders displaying motor deficiencies such as Huntington´s disease and Parkinson´s

disease (PD), respectively. Further dysregulated dopaminergic signaling causes cognitive disorders and impaired memory. Mutations in SLC6A3 have been reported to reduce the levels of SLC6A3 alternatively reduce its biding affinity to of dopamine. Such mutations has been linked to syndromes that give rise to symptoms similar to PD (Blackstone 2009). Similarly SLC6A3 null mice display alterations in presynaptic dopamine homeostasis associated with neurological symptoms including hyperactivity (Gainetdinov 2008)(Reviewed in(Lohr, Masoud et al. 2017)).

The dopaminergic signaling components are well established targets for pharmacological disruption. Classical examples include dopaminergic drugs such as cocaine and amphetamine. Cocaine was demonstrated to act by blocking SLC6A3 transporter resulting in elevated levels of extracellular dopamine. Amphetamine by contrast acts as a substrate of SLC6A3 and exerts its effect by competing with dopamine to enter dopaminergic cells. Intracellular amphetamine further disrupts the proton gradient required for storage of dopamine in vesicles. As a consequence dopamine leaks out into the cytoplasm of the presynaptic neuron. The combined effects of amphetamine i.e. cytoplasmic pre-synaptic dopamine leakage along with competitive uptake of dopamine by amphetamine, has been demonstrated to induce reversed dopamine transport by SLC6A3 by Ca2+/calmodulin-depenent kinase II (CAMII) mediate phosphosylation of the SLC6A3 transporter. Phosphorylation induces a conformational change of SLC6A3 that shifts the direction of dopamine efflux towards the synaptic cleft rather than transport to the pre-synaptic neuron. Such transport disrupts dopaminergic signaling by increasing the levels of extracellular dopamine (Reviewed in(Lohr, Masoud et al. 2017)).

Quantitative measure of striatal SLC6A3 levels has been used for imaging purposes to verify diagnosis of the Parkinson´s disease. Several radiolabelled cocaine analogues $^{123}$I-β-CIT and $^{123}$I-Ioflupane (DaTSCAN) have been developed and enables quantitative imaging if SLC6A3 by single photon emission computed tomography (SPECT)(Tissingh, Bergmans et al. 1998, Dickson, Braak et al. 2009).

Importantly dopaminergic singling has been demonstrated to regulate physiological process also in non-neural cells including the cells of the kidneys where expressions of all five dopamine receptors have been experimentally verified (Carey 2001). Dopamine was demonstrated to regulate redox balance and blood pressure by regulating reabsorption and secretion of $Na^+$. However, since transport of dopamine in the kidney was shown to be unaffected by cocaine analogues, dopamine is likely to exert its effect independently of SLC6A3 (Soares-Da-Silva, Serrao et al. 1998).

# Chapter 5. Glioma

## Overview

Brain tumors consist of a diverse group of tumors that are classified based on morphological and histological features. Notably, secondary brain tumors caused by metastasis are the most frequent tumors of the brain, however malignant gliomas account for most of primary brain tumor among adults whereas medulloblastoma account for the most primary tumor among children (Reviewed in (Huse and Holland 2010)). Glioblastoma multiforme (GBM) constitutes the most common subtype of malignant glioma with a yearly incidence of around 13 000 cases in the United States (Ostrom, Gittleman et al. 2015).

Historically brain tumors within the central nervous system (CNS) were proposed to originate from glial or neuronal precursor cells and brain tumors were thus classified by morphological origin (Bailey P 1926). The current modern classification by World Health Organization (WHO) is based on the initial classification but also divide tumors based on histological features and further grades the tumors from grade I-IV based on biological behavior (Louis, Perry et al. 2016). Briefly, gliomas are sub-divided into astrocytomas, oligodentrogliomas and mixed oligoastrocytic gliomas, where presence of nuclear atypia, increased proliferation, pseudopalisading necrotic regions and regions with microvascular proliferation classifies the tumor with a higher grade. Low grade tumors may further evolve into tumors with higher grade and called secondary glioblastomas (Reviewed in (Huse and Holland 2010)).

## The genomic landscape of glioblastomas

Primary glioblastomas are associated with activating mutations of genetic amplifications of the epidermal growth factor receptor (EGFR), where EGFRvIII deletion giving rise to a constitutively active form of EGFR is found in 20-30% of glioblastomas. Interestingly around half of the EGFRvIII deleted tumors harbor amplification of the EGFR gene. Notably mice with forced expression of *Egfr* or

*EgfrvIII* alone does not form GBM-like tumors, suggesting that EGFR expression may be a later event in GBM tumorigenesis (Ozawa, Riester et al. 2014).

Around 60-90% of oligodendroglial tumors harbor 1p19q co-deletion, which is associated with a better response to chemotherapeutic treatment. Another independent marker associated with a favorable prognosis incudes heterozygous mutation of isocitrate dehydrogenase 1/2 (*IDH1/2*). During physiologic settings IDH proteins catalyzes an enzymatic reaction converting isocitrate to α-ketoglutarate (αKG), however in the cancer setting IDH harbors a point mutation in catalytical site thus mediating the alternative processing of αKG to the oncometabolite 2-hydroxyglutarate (2HG). The oncogenic role of 2HG in still not fully understood, but patients displaying accumulation of 2HG are associated with increased expression of HIF-1α and has been associated to earlier tumor onset but with better treatment response compared to wild type IDH tumors (Dang, White et al. 2009, Sanson, Marie et al. 2009, Zhao, Lin et al. 2009).

Signaling pathways largely affected by mutation deletion or amplification in GBM involves receptor tyrosine kinase signaling pathways mediated through PI3K-AKT-mTOR and Ras-MAPK signaling. Importantly, although mutations of individual genes are relative rare within these pathways the network itself altered in some way in nearly all cases (88%) of glioma. Elevated signaling further commonly occurs through platelet derived growth factor receptor-α (PDGFR-α) and PDGFB, PI3K-AKT-mTOR and Ras-MAPK and by loss of the negative regulator PTEN. Neurofibromin 1 (*NF1*), which negatively regulates Ras is recurrently suppressed in glioma patients and was further demonstrated to contribute to tumor formation in mice (Zhu, Guignard et al. 2005). Heterozygous loss of *Pten* was further demonstrated to accelerate the formation of high-grade glioma tumorigenesis (Kwon, Zhao et al. 2008)

Considering the frequent dysregulation of the Rb1 and p53 networks ether by inactivating mutations in *RB1* and *TP53* (which is the most frequent mutation in GBM*)* or by activating mutations of *CDK4/CDK6* and *MDM2/MDM4* cell cycle progression, these networks may be suggested to constitute an important mechanism for glioma progression. Recurrent inactivating mutations or deletions in the *CDKN2A* locus encoding *INK4A* and *ARF*, which are positive regulators of PB and p53, further highlights the role of dysregulated cell cycle in glioma pathogenesis. The importance of dysregulated cell cycle regulation can be confirmed using genetic mouse models of glioma where inactivation of p53 as well as Rb is enough to drive formation of high grade murine gliomas. Secondary glioblastomas evolving from low grade glioblastomas have further been associated to *TP53* mutations (Louis 1994) Reviewed in (Huse and Holland 2010, Verhaak, Hoadley et al. 2010).

# Glioblastoma Subtypes

Sequencing have sub-grouped gliomas based on transcriptional profiling, into classical, mesenchymal, pronerual and neural subtypes, which essentially can be defined by genetic alterations in *EGFR*, *PDGFR*, *IDH1/2*, or *NF1*, respectively (Verhaak, Hoadley et al. 2010).

The classical subtype is largely defined by activating amplification of *EGFR*. Furthermore amplification of Chromosome 7 (harboring *EGFR*) combined with loss of chromosome 10 (harboring *PTEN*) occurs virtually all classical GBM tumors. Moreover classical GBM tumors have often lack of *TP53* and further often display deletion of *CDKN2A* encoding $p16^{INK4A}$ and $p14^{ARF}$ or deletions of components of the *RB* pathway. The mesenchymal subtype is defined by loss of *NF1* often in combination with loss of *PTEN*. The mesenchymal subtype display elevated expression of mesenchymal and astrocytic markers such as *CD44, MERTK* and *MET*. Moreover the mesenchymal subtype is associated with high degree of tumor necrosis and immune infiltration associated with high expression of several components of the NFκB signaling pathway. The proneural subtype makes up around 25% of all glioblastomas and can be further sub-classed into IDH1 mutated tumors associated with hypermethylated DNA, which is referred to the glioma CpG island methylator phenotype (G-CIMP) and non IDH1 mutated tumors. While *PDGRFA* amplification almost never coincide with G-CIMP tumors, proneural (non-G-CIMP) tumors are characterized by over expression PDGFR and further commonly associated with loss of p53. Notably, secondary GBM often fall into the proneural subtype which further generally affect younger individuals. By contrast the neural subtype tend to associate with expression of neuron markers such as *NEFL*, *GABARA1*, *SYT1* and *SLC12A5* (Reviewed in (Verhaak, Hoadley et al. 2010, Brennan, Verhaak et al. 2013)).

Although the transcriptional profiles of the molecular subgroups look very different no significant correlations to differences in clinical outcome have been demonstrated based on subgroup. One exception is the proneural G-CIMP tumors, which have been linked to a favorable outcome (Brennan, Verhaak et al. 2013). Single-cell sequencing further demonstrated major glioma subgroup heterogeneity already at the intra tumoral level (Patel, Tirosh et al. 2014).

## Clinical manifestation and prognosis

Glioblastoma patients may display virtually any neurological symptoms dependent of tumor location and size and may for example include symptoms like nausea, vomiting, persistent headaches, personality change, memory loss, seizures, changes in speech or changes in the ability to think and learn. In majority of cases the symptoms can be explained due to rapid development of elevated intracranial pressure along with compression and infiltration of the normal surrounding tissue (Iacob and Dinca 2009). The presence of a brain tumor is determined by imaging by computer tomography (CT) and magnetic resonance imaging (MRI). Diagnosis by imaging may be assisted by a histological diagnosis through obtaining a core biopsy. Despite conventional therapy including surgical resection, radiation therapy and chemotherapy glioblastomas remains among the most treatment resistant tumors and are associated with a median survival of less than 15 months (Stupp, Mason et al. 2005).

Exposure to ionizing radiation is the only established risk factor for development of glioblastoma (Wen and Kesari 2008). While most gliomas are sporadic, a small subset (around 5%) can be linked to rare familial syndromes such as neurofibromatosis (Farrell and Plotkin 2007).

Since very few molecular markers have been found to significantly affect patient outcome, the prognosis among glioblastoma patients largely depends on tumor size and location along with patient age and general well being (indexed by Karnosky Performance Scale). Silencing of the O-6-methylguanine-DNA methyltransferase (*MGMT*), which acts by repairing DNA damage, was however demonstrated to predict sensitivity towards Temozolomide and O-6-methylation. (Bleau, Huse et al. 2009).

## Treatment

Glioblastomas generally display rapid recurrence to standard therapy, which include treatment by de-bulking the tumor though surgical resection. Since glioblastomas are highly infiltrative, aggressive and fast growing tumors that lacks a clear margin, a complete tumor resection commonly not feasible. Indeed, preservation of neurological function post-surgery have been demonstrated to influence patient survival and has been considered equally important to tumor removal (Krivosheya, Prabhu et al. 2016). Despite existence of multiple tools to assist surgery including multiple preoperative and intraoperative imaging techniques and functional mapping, surgery alone does not improve overall

survival by more than 3-6 months. Surgery is commonly followed by adjuvant radiotherapy (RT), commonly administrated by total dose of 60 Gy fractioned by 2 Gy over 30 days, and may prolong overall survival to around 12 months. The RT may be further combined with adjuvant chemotherapy, where administration of the alkylating agent Temozolomide have demonstrated a significant survival benefit associated with an overall survival of around 15 months. Primary glioblastomas are associated to symptoms such as seizures, elevated intracranial pressure, and deep vein thrombosis and embolisms and to improve quality of life the standard therapies listed above are commonly combined with anticonvulsant drugs, corticosteroids and anticoagulant drugs (Stupp, Mason et al. 2005)(Reviewed in (Bianco, Bastiancich et al. 2017)).

The poor treatment response of glioblastoma patients to standard therapies clearly underlines the need for additional treatment regimes. A novel FDA approved treatment modality includes the use of tumor treating fields (TFFs). Briefly, the technique is non invasive and may be used in addition to RT. TFFs acts by low intensity electric fields that targets mitotically active cells for apoptosis by disrupting formation of the mitotic spindle. Clinical trials have demonstrated improved quality of life and the technique is thus suggested as a forth treatment regimen for glioblastoma patients (Taillibert, Le Rhun et al. 2015).

The vast majority of glioblastomas harbors aberrant activity of EGFR thus rationalizing evaluation of anti-EGFR therapies. Several small molecule inhibitors *erotinib* and *gefitinib* have been used in clinical trials, however without significant effects of treatment response. At present the anti-EGFR inhibitor (*erlotinib*) are being evaluated in glioblastoma patients harboring EGFRvIII mutation. Similar to patients with metastatic ccRCC, anti-angiogenic therapies including bevacizumab are being evaluated for glioblastoma patients. Results so far demonstrated no effect on overall survival but slightly prolonged progression free survival. Furthermore, glioma tumor cells, similar to ccRCC cells, express enhanced levels of the immune checkpoint inhibitors molecules (PD1 and PDL-1) and monoclonal antibodies are thus being evaluated as potential therapeutic targets for glioblastoma patients. Indeed, there are in several ongoing phase II and phase III clinical trials evaluating the effects of immune modulators, mainly PD-L1, combined with radiation, Temozolomide or anti-angiogenic therapies (Yeo and Charest 2017) Reviewed in (Bianco, Bastiancich et al. 2017) .

# Glioma stem cell niches

Several studies have addressed the presence of treatment resistant glioma stem cells (GSC) where GSCs have been described to express stem cells markers such as CD133, nestin and CD44, possess multipotent properties and further have the ability to self-renew and initiate tumor formation in xenograft models (Singh, Clarke et al. 2003, Singh, Hawkins et al. 2004). GSCs have been described to reside within specialized environments or niches that generate factors to support their maintenance. Much like stem cells of the normal brain (Riquelme, Drapeau et al. 2008), GSCs has been associated to well vascularized tumor regions and thus exists in a perivascular niche. Such notion was demonstrated in a xenograft model where the GSCs expanded along with increased number of endothelial cells (Calabrese, Poppleton et al. 2007). CD133 positive GSCs was demonstrated to secrete high levels of VEGF thus further supporting the interaction with blood vessels (Bao, Wu et al. 2006). The blood brain barrier (BBB), composed of endothelial cells, pericytes and astrocytes, forms a so-called neurovascular unit important conferring specific transport of molecules along with low permeability (Abbott 2013). During gliomagenesis disrupted BBB allows passage of circulating immune cells (such as monocytes and neutrophils), which was shown to secrete additional angiogenic factors and moreover acts in an immune suppressive manner, thus supporting microvascular hyperplasia and proliferation (Reviewed in (Huse and Holland 2010) and (Li, Wang et al. 2009, Hambardzumyan and Bergers 2015)).

There is experimental evidence of interactions GSC-niche interactions on several levels by direct cell -to cell communications as well as by autocrine or paracrine singling. Several factors and signaling pathways have been shown to maintain the GSCs including *OCT4* (induced by HIF-2α) (Covello, Kehler et al. 2006), *OLIG2, BMI1* and Notch signaling (induced by NO from endothelial cells) (Charles, Ozawa et al. 2010) and in order to effectively target the GSCs it would be essential to inhibit such interactions to the surrounding microenvirormnet. Moreover, other micrenvironmental factors mediated by a variety of non-neoplastic, stomal cells including endothelial cells, pericytes, immune cells, glial cells appear to support the existence of GCSs. For example astrocytes were demonstrated to support a stem-like phenotype by secreting of SHH (Becher, Hambardzumyan et al. 2008). Further, maintenance of GSCs has further been associated with secreted factors such as IL-6 (Hossain, Gumin et al. 2015) and IL8 (Infanger, Cho et al. 2013). Stemness may further be mediated by matrix proteins such as laminins (Ma, Lim et al. 2016) and type 1 collagen (Motegi, Kamoshima et al. 2014), where ECM stiffness is regulated by enzymes like lysyl oxidase (LOX), which crosslinks the collagens (Handorf, Zhou et al. 2015).

In addition to the perivascular niche GSCs have been demonstrated to enrich in the hypoxic (perinecrotic) niche (Alcantara Llaguno, Chen et al. 2009). In support of a second GSC niche glioblastoma biopsies have shown that GSCs enrich to both vascular and perinecrotic regions (Seidel, Garvalov et al. 2010). The hypoxic tumor niche associated with non-functional vasculature along with necrotic cores surrounded by a row of palisading tumor cells displaying a typical appearance with enlongated nuclei. Mechanistically necrosis appears to arise du to collapsed thrombosed vessels. Similar to the perivascular areas the necrotic regions secrete inflammatory and that support the recruitment of immune cells to remove necrotic cells and debri, however being shifted immune suppressive and pro-angiogenic role of immune cells like TAMs and neutrophils (Reviewed in (Huse and Holland 2010) and (Li, Wang et al. 2009, Hambardzumyan and Bergers 2015)).

Not surprisingly, the hypoxic GSC niche is associated with low oxygen tension along with expression of hypoxia inducible factors (HIF-1α and HIF-2α), where HIF expression was demonstrated by co-expression of hypoxic genes with stem cell markers. Notably the HIF-2α is expressed also at physiological oxygen tensions in the perivascular GSC niche thus underlining the role of hypoxic signaling for maintenance of GSCs independently of oxygen tension (Li, Bao et al. 2009).

Finally it is common to see signs of microvascular proliferation in close proximity to necrotic cores, which in part can be explained by secretion of angiogenic factors from hypoxic tumor cells (Zagzag, Lukyanov et al. 2006) and immune cells (Du, Lu et al. 2008). Moreover GSCs have been suggested to trans-differentiate into endothelial cells, which could offer a partial explanation of the high density of blood vessels (Ricci-Vitiani, Pallini et al. 2010, Wang, Chadalavada et al. 2010, Soda, Marumoto et al. 2011).

## Radiation and the glioma-associated astrocytes

All stromal and niche-derived cells may contribute to the GSC tumor niche, including astrocytes. Importantly, astrocytes can become reactive in response to injuries such as traumatic or ischemic lesions caused by epilepsy or stroke. Further brain and spinal cord infection and radiation may case a reactive phenotype in astrocytes. Induction of reactive gliosis have been linked to a number of molecules including transforming growth factor alpha (TGFα), oncostatin M and cilliary neurothropic factor (CNTF). Moreover interleukins such as IL-6 have been shown to induce reactive gliosis trough JAK2/STAT3 signaling (Sriram, Benkovic et al. 2004). Notably, tumor associated astrocytes exhibit such reactive phenotype, which is characterized by hypertrophic astrocyte-cell-processes and up-regulation

of intermediate filament proteins particularly by increased expression of glial fibrillary acidic protein (GFAP) but also vimentin and nestin (Reviewed in (Placone, Quinones-Hinojosa et al. 2016)).

In the physiological setting activated astrocytes helps to repair the injured brain and produces a glial scar. In the cancer setting reactive astrocytes however may support tumor growth and metastasis by secretion of interleukins and growth factors such as IL-6, STAT3, TGF-B, bFGF, EGF, PDGF to support proliferation, or activate metalloproteases (MMPs) to promote invasion and finally secrete interleukins such as IL-10 to prevent tumor cells from the immune system (particularly T and NK cells). Reactive astrocytes may act in a chemoprotective manner by sequestering calcium, which prevents apoptosis by tumors cells induced by chemotherapy (Katz, Amankulor et al. 2012)(Reviewed in (Placone, Quinones-Hinojosa et al. 2016)).

Radiation is an effective mode of killing tumor cells but is however also associated with post-radiation side effects like radiation-induced necrosis and immune infiltration, where the increased presence of macrophages are known to promote invasion associates tumors with a higher grade and worse clinical outcome (Li and Graeber 2012). Moreover the vast majority of recurring tumors reappear as resistant lesions ether in near proximity or completely overlapping with the site of the initial tumor (Hess, Schaaf et al. 1994) supporting the idea that treatments may change the tumor niche to support regrowth and metastasis of the tumor. Such notion may be supported by studies in breast cancer xenografts where pre-treated mammary glands supported the ability of the tumor to metastasize (Bouchard, Bouvette et al. 2013).

# CD44 signaling

CD44 is expressed among stem cell populations including normal stem cells and tumor stem cells in many tumor entities including glioma (Anido, Saez-Borderias et al. 2010).

**Gene structure**

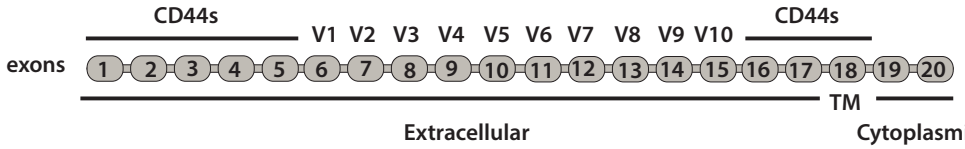


Figure 8. Schematic of CD44 gene structure. CD44 have 20 exons comprising the extracellular (exon 1-17), transmembrane (exon18) and cytoplasmic (exons 19-20) domains, respectively. Exons 6-15 are variant exons, which are encoded by alternative splicing.

CD44 functions as a receptor for several extra cellular matrix (ECM) components particularly hyaluronan (HA), but CD44 may also bind a number of other components of the ECM including collagens, fibronectin, osteopontin, fibrin and serglycin. The binding partner preference is highly regulated by post-translational modifications of CD44. CD44 is a type 1 transmembrane glycoprotein composed of three major domains; an extracellular domain, a transmembrane domain, and an intracellular domain.

The extracellular domain further contains the N-terminal globular domain and stem membrane-proximal region which are the domains that confer heterogeneity by alternative splicing (Tolg, Hofmann et al. 1993). CD44 contains 20 exons where exon 6-16 are represent the variant exons (v1-10) and may be included or excised in several combinations giving rise to numerous splice variants of CD44. Moreover, CD44 may be post-translationally modified by N- and –O glycosylations and further be linked to chondroitin or heparin sulfates (GAG chains) in the extracellular domain (Orian-Rousseau and Sleeman 2014). The multiple variants of CD44 supports the multifaceted role of CD44 to regulate a wide panel of cellular processes such as cell adhesion, proliferation, migration and apoptosis in an isoform and ligand specific manner (Reviewed in (Zoller 2011)).

The standard form of CD44 (CD44s) is widely expressed does not contain any of the variant exons in the extracellular domain. Variants of CD44 have however been implicated during pathological conditions such as cancer. Several splice variants have been implicated in cancer progression, particularly one variant expressing exon 6 (CD44v6) that is overexpressed in several cancer forms (Ishida 2000, Misra, Hascall et al. 2009). Moreover, particular isoforms of CD44 were shown to collaborate with receptor tyrosine kinases (RTKs), for example CD44v6 was demonstrated to control activation HGF-cMet signaling (Orian-Rousseau, Chen et al. 2002). CD44 isoforms with GAG chains (particularly CD44v3) have been demonstrated to bind several growth factors (FGF-2 and VEGF) (Jones, Tussey et al. 2000).

Similar to Notch, CD44 cleavage may be targeted by membrane-type 1 matrix metalloproteinase (MT1-MMP) including ADAM10/17 (Kajita, Itoh et al. 2001, Nakamura, Suenaga et al. 2004). Experiments in a glioma cell line further showed that 12-Otetradecanoylphorbol 12-acetate (TPA)-induced activation of CD44 induces redistribution of the gamma-secretase component presenilin1 to co-localize with CD44 in ruffing areas of the cellular membrane. Supporting that the CD44 proteolysis was mediated by the gamma-secretase complex specifically, the same study treated glioma cells with GSIs, which blocked CD44 cleavage all together. The gamma-secretase-mediated proteolysis was demonstrated to result in ectodomain shedding and release of the intracellular portion into the cytoplasm (Okamoto, Kawano et al. 2001, Murakami, Okamoto et al. 2003).

The intracellular portion (CD44ICD) has been demonstrated to interact with components of the cytoskeleton such as ezrin, radixin and moesin (ERM) proteins (Tsukita, Oishi et al. 1994). Further CD44ICD may interact with Src family of kinases and Rho GFPases (Reviewed in (Dzwonek and Wilczynski 2015). Several studies reported the intracellular portion of CD44 contains a nuclear localization signal supporting nuclear translocation and subsequent role in transcriptional modulation (Kajita, Itoh et al. 2001, Lee, Wang et al. 2009). Cleaved CD44ICD has further been linked to stemness by activating stemness factors such as *NANOG*, *OCT4* and *SOX2* (Pietras, Katz et al. 2014, Cho, Lee et al. 2015). It was demonstrated that binding of osteopontin mediates generation of CD44ICD in glioma and further was shown to activate transcription dependent on CBP/p300 (Pietras, Katz et al. 2014).

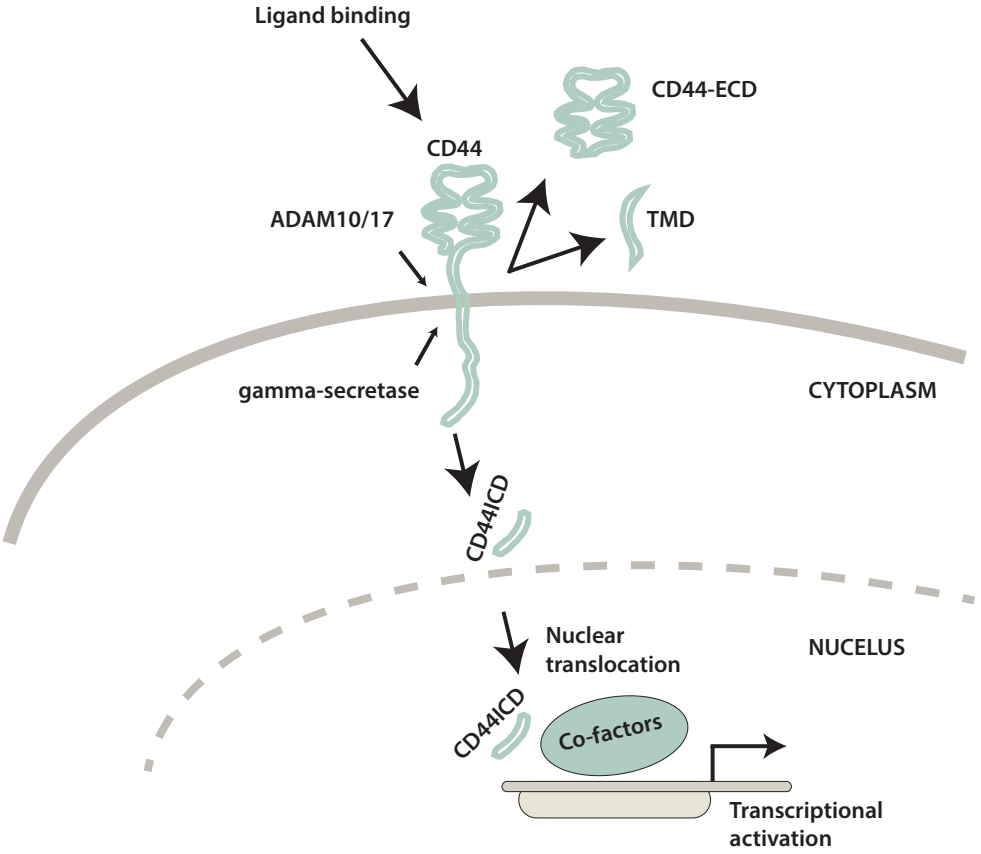


Figure 9. Schematic over CD44ICD signaling. Interaction by ligands such as HA or OPN mediate a series of proteolytic cleavages mediated by ADAM10/17 and gamma-secretase and results in shedding of the ectodomain and transmembrane domain. CD44 intracellular domain is released into the cytoplasm following nuclear translocation where it acts by modulating transcriptional regulation.

## CD44 in normal brain and Glioblastoma

CD44s is the predominant isoform expressed in normal adult brain where CD44 has been linked to neuron development, response to injury and has further been demonstrated to regulate plasticity of the synapses during formation of memories. In the developing brain CD44 has been used as a marker for astrocyte progenitor cells and in the adult brain CD44 is expressed in CNS stem cells and glial cells whereas neurons are generally CD44 negative. CD44 appears important for memory formation which is supported by the fact that CD44 null mice display impaired memory, along with impaired sensorimotor functions, compared to their wild type counterparts (Raber, Olsen et al. 2014) (Reviewed in (Dzwonek and Wilczynski 2015)). In glioblastoma multiforme (GBM) CD44 expression has been tightly linked to glioma stem cells (GSC), stemness and drug resistance. Elevated CD44 expression has been linked specifically to the mesenchymal subtype of GBM (Verhaak, Hoadley et al. 2010). One might be surprised by the high levels of CD44 in the mesenchymal GBM, considering that CD44 was described to mark GCS, which are thought to only constitute a minor fraction of the tumor cells. In mesenchymal GBM by contrast the vast majority of cells are positive for CD44. A partial explanation may be that CD44 expression has been linked to expression of mesenchymal genes such as TWIST, SLUG and SNAIL (Xu, Tian et al. 2015) suggesting a partial overlap of proteins that marks stem with proteins that mark mesenchymal genes. CD44 may thus not mark only GSCs in this particular GBM subtype.

When examining glioblastoma samples CD44 further localized to the perivascular niche and expression of CD44 has further been linked to increased expression of stem cell markers such as inhibitor of DNA binding one (*ID1I*) (Anido, Saez-Borderias et al. 2010, Pietras, Katz et al. 2014). CD44 also appears to regulate proliferation of glioblastoma. The EGFR pathway has been described to converge to promote proliferation of GBM; EGFR by increasing mRNA levels of CD44 and CD44 in turn by up-regulating the downstream EGFR mediators, Akt and Erk (Reviewed in (Mooney, Choy et al. 2016)).

Numerous studies have addressed expression of CD44 variants in GBM, however results point to that the standard CD44 is predominantly expressed in GBM samples (Kaaijk, Troost et al. 1995, Nagasaka, Tanabe et al. 1995, Ranuncolo, Ladeda et al. 2002, Xu, Stamenkovic et al. 2010). With regards to variant expression CD44v6 was found absent in GBM samples, but a subgroup of high grade gliomas were found to express CD44v5 (Kaaijk, Troost et al. 1995). Moreover, CD44 expression has been linked to high grade tumors and further associated CD44 expression with a poor prognosis in glioma (Yoshida, Matsuda et al. 2012, Pietras, Katz et al. 2014, Guadagno, Borrelli et al. 2016).

Targeting the standard-isoform of CD44 by use of a monoclonal antibody was shown to reduce migration of a glioblasoma cell line (Yoshida, Matsuda et al. 2012) and further prevented metastasis in rat glioblastoma (Breyer, Hussein et al. 2000) thus underlining CD44 as a potential therapeutical target in glioblastoma (Reviewed in (Mooney, Choy et al. 2016).

# The present investigation

## Paper I: Simultaneous targeted activation of Notch1 and *Vhl*-disruption in the kidney proximal epithelial tubular cells in mice

### Overall Aims

The general aim of paper I was to determine the contribution of Notch signaling in ccRCC by investigating whether Notch signaling could induce ccRCC like tumors in V*hl* null proximal tubular epithelial cells *in vivo*.

### Summary

In this paper, we studied the level of Notch 1 signaling activity in ccRCC, firstly by utilizing an antibody directed against the intracellular part of cleaved human Notch 1 in a TMA, and secondly by analyzing the transcriptional levels of Notch 1 and its target genes in the TCGA data set. As expected, we found increased activity of Notch1 singling in ccRCC samples compared to its normal counterparts, thus supporting a role for NICD1 in ccRCC tumorigenesis.

To further study the contribution of Notch 1 signaling *in vivo*, we utilized the cre/lox technique to generate mice with ectopic expression of Notch intracellular domain 1 (NICD) combined with *Vhl* deletion. The combined Notch over expression/*Vhl* deletion was restricted to the proximal tubules of the renal cortex by using the proximal-specific, androgen inducible, kap-2 promoter to drive the expression of a cre recombinase.

Combined NICD1 overexpression/*Vhl* deletion induced expression of the hypoxic target gene CaIX and was associated with elevated mRNA levels of Notch. Phenotypically these mice only developed one overt tumor, but however more frequently displayed early signs of ccRCC pathology, including nests of dysplastic cells with a clear cytoplasm, where hyperactivated Notch was demonstrated to enhance such phenotypes. Based on stainings, the “clear cell” phenotype was

caused by accumulation of triglycerides and other neural lipids, rather than by increased deposition of glycogen. We concluded that Notch1 signaling contributes to some aspects of ccRCC tumorigenesis by enhancing the formation of dysplastic cells, along with the lipid accumulation seen in *Vhl* negative proximal tubular cells *in vivo*.

## Discussion

As detailed in "Role of Notch in the kidney and renal cancers" section, the background to this study was a number of findings indicating augmented Notch signaling in human ccRCC (Sjolund, Johansson et al. 2008, Sjolund, Bostrom et al. 2011, Wu, Xu et al. 2011). In concoradance with previous litterature, our data also indicated that Notch signaling is frequently enchanced in human ccRCC. It should be pointed out that mutations in the Notch recepters themselves are rare in ccRCC (Creighton, Morgan et al. 2013) thus indicating that the elevated expression of *NOTCH1* (and *HEY1* and *HEY2),* would be largely independet on structural alterations of *NOTCH1*. One might speculate that the elevated Notch signaling in ccRCC is related to the frequent inactivation of genes involved in chromatin modulation. Thus, altered epigenetic regulation of Notch signaling components might lead to the dysregulated Notch signaling seen in ccRCC. Supporting this theory, Susztak and colleuges recenctly performed an integrated analysis evaluating changes at the transcriptomic level, but also adressing methylation and copy number changes in a panel of 13 primay ccRCC sampels and microdissected healty tissue (Bhagat, Zou et al. 2017). Their studies revealed enrichemnt for differential methylation in the bidning sites at the Notch downstream tagets HES and HEY families. They further reveled epigenetic alterations (hypomethylations) in the Notch ligands *JAG1* and *JAG2* (Hu, Mohtat et al. 2014, Bhagat, Zou et al. 2017).

One hallmark of ccRCC is the presence of cells with a clear cytoplasm as a consequence of accumulation of various lipids. We observed such cytoplasmic lipid accumulation in the NICD1 overexpressing and *Vhl* deleted mice (NICD1/*Vhl*) mice, but not when *Vhl* was deleted on its own. These observations suggest that NICD1 may contribute to the "clear cell" phenotype seen in ccRCC. Altered Notch signaling have been demonstrated to shift cellular metabolism towards increased glycolysis (Landor, Mutvei et al. 2011). Interestingly, the Notch pathway has further been linked to aidopogenesis in other cellular systems such as in hepatic cells, where Notch1 gain of function was demonstrated to cause fatty liver phenotype (Pajvani, Qiang et al. 2013). Notably, hyperactivated Notch does not confer adiopogenesis in every cell type. Indeed, a recent mouse model overexpressing NICD1 (combined with *Vhl* loss) mainly in the distal tubular compartment, rather than the poximal tubular comparment was associated with

renal cyst formation, and presence of dysplastic cells but did not display presence of ”clear cells” (Bhagat, Zou et al. 2017), thus underlining the importance of targeting the right population of cells.
As previously mentioned, our findings indicate that *Vhl* deletion alone is insufficient to cause the clear cell phenotype seen in ccRCC. In line with our data, *Vhl* loss in xenografted mouse embryonic fibroblasts demonstrated a growth disadvantage, rather than advantage, compared to wild type (Frew and Moch 2015). Similarly, other mouse models with conditional deletion of *Vhl* alone, does not report such phenotype (summarized in table 1). Somewhat surprisingly, a mouse model expressing a non-degradable version of HIF-1α (mimics part of the *Vhl* loss*)* displayed cytoplasmic lipid accumulation similar to our study (Fu, Wang et al. 2011). Such phenotype was however not seen when non-degradable HIF-2α was expressed in the same mice (Fu, Yang et al. 2013). These results may in part be explained by that the oxygen insensitive HIF-1α construct regulates the hypoxic response differentially from wild type HIF-1α. Importantly the HIF-1α used in this study was made insensitive to PHD as well as FIH mediated hydroxylation (HIF1-3M), which not fully mimic the effects of pVHL loss of function in ccRCC. While pVHL loss prevents proteasomal degradation of HIF-1α it does not affect the function of FIH asparagine hydroxylation. Additionally, ccRCC tumors may experience low oxygen pressure from time to time, but have been described as well vascularized tumors. HIF-1α (considering the FIH-1 preference for HIF-1α) is thus very likely to be targeted for asparagine hydroxylation (thus mediating transcriptional repression) most of the time in ccRCC patients but, not in the HIF1-3M overexpressing mice. At the same time, this would not explain the lack of phenotype in the HIF-2α mice, and additional factors must affect the phenotype in this experimental model system.

At the time when we were combining NICD overexpression to *Vhl* deletion in the proximal tubules several attempts had been made to combine *Vhl* loss with in ccRCC-associated oncogenes or tumor suppressor genes. Such models did however not display any significant signs of renal neoplasms (summarized in table 1). One potential explanation may be that only a distinct subset of renal epithelial cells are susceptible to *Vhl*-mediated transformation, and one might speculate that many model systems failed in targeting the proper cell type. Indeed many studies used Cre drivers targeting the distal tubules and collecting ducts, alternatively utilized early embryonic (broad) cre drivers. As outlined in the “Clear cell renal cell carcinoma” section the marker expression of ccRCC largely overlaps with markers of the proximal tubular compartment, and ccRCC is thus proposed to originate from the proximal tubular cells (Lindgren, Eriksson et al. 2017). These observations strongly argue for a model system aimed at achieving conditional targeting in the proximal tubules rather than other tubular compartments. In our study we choose the kap-2 promoter, which have been shown to have an

expression limited to the proximal tubules of the renal cortex (Li, Zhou et al. 2008). An additional advantage of the kap2-icre driver compared to previous drivers is that the system is induced by androgen administration, which gives us the possible to induce expression at any given time point. As most cases of ccRCC are caused by sporadic mutations and is likely to develop during adult life, we reasoned that kap2-icre induction in adult mice (around 8 weeks) would be a good strategy to mimic the human disease progression.

In our study, the combined NICD overexpression and *Vhl* deletion in the proximal tubular cells gave rise to some aspects of ccRCC pathology, including the presence of dysplastic cells with a clear cell phenotype, but the model did not fully recapitulate human ccRCC. This opens up for the possibility that additional genetic alterations cooperate with *Vhl* loss and NICD1 expression in to induce tumor formation. Another explanation may be that only a certain subset of less differentiated cells may be transformed to induce ccRCC tumors (Lindgren, Bostrom et al. 2011). The possibility remains, that such population has not yet been targeted in mice. There is also a possibility that such progenitor population exists in humans, but perhaps not in mice (Hansson, Ericsson et al. 2016). Arguing against such theory two newly developed models combining *Vhl* loss in combination with inactivation of *Bap1* or *Pbrm1* succeeded to develop ccRCC-like lesions (Gu, Cohn et al. 2017). This models utilized an embryonic promoter (Pax-8) expressed in most tubular compartment perhaps not completely reflecting human ccRCC, but these models highlight the importance of the frequent 3p deletion (harboring *VHL*, *PBRM1*, *BAP1* and *SETD2*) in human ccRCC tumorgienesis.

# Paper II: Overexpression of functional SLC6A3 in clear cell renal cell carcinoma

## Overall Aims

The general aim of paper II was to explore the expression of transporter proteins in RCC compared to normal kidney, and potentially to discover novel biomarkers or therapeutic targets for renal cancer.

## Summary

In this paper we used the TCGA database to perform an unbiased exploration of transporter proteins in various cancer types. We found that the dopamine transporter *SLC6A3* was specifically expressed in ccRCC as opposed to other tumor types and normal kidney, which barely expressed any levels of *SCL6A3*. By using [$^{3}$H]-dopamine along with a specific SLC6A3 inhibitor (GBR-12909), we demonstrated that ccRCC cells actively transport dopamine specifically through the SLC6A3 transporter. We further found a link between SLC6A3 expression and hypoxia, where SLC6A3 expression could be induced in normal kidney cells grown under hypoxic conditions. Importantly, hypoxic SLC6A3 induction was not as pronounced in other epithelial cells suggesting that hypoxia induced *SLC6A3* expression is specific for kidney epithelial cells. Finally, knock down of both HIF-α isoforms showed that *SLC6A3* expression was induced specifically by HIF2-α.

## Discussion

Transporter proteins are an important group of proteins to study with regards to cancer. Indeed, the expression of several members of the ABC transporters (including ABCG1 and ABCG2) has been linked to drug efflux, which in turn has been linked drug resistance in several tumor types. Similarly, several members of the SLC family including SLC4A7 (Fletcher, Johnson et al. 2011) and SLC14A1 (Rafnar, Vermeulen et al. 2011) have been implicated in cancer.

In this paper we looked at the expression of 442 genes encoding SLC and ABC transporter proteins and as expected found that normal kidney samples (along with liver) displayed expression of more transporter proteins than other normal tissues. The kidney is an organ that control reabsorption of ions and other nutrients, and thus expresses a multitude of transporter proteins.

In the hierarchical clustering analysis the RCC tumors often clustered together with its corresponding normal tissue proposing that tumors maintain their transporter expression found in the normal tissue. Similarly, the ccRCC samples harbored low expression of several transporter proteins specifically expressed in the distal tubules, thus further supporting that ccRCC does not have a distal origin but rather originates from the proximal tubular cells.

When comparing normal kidney samples to kidney cancer samples we found several transporters that were expressed in normal tissue but that were lost in the corresponding tumor, which may be an effect of dedifferentiation during tumorigenesis. Quite surprisingly, found that the dopamine transporter SLC6A3 was upregulated specifically in ccRCC while other RCC types only expressed minute levels of *SLC6A3*. In the physiological setting SLC6A3 is mostly linked to dopaminergic neurons, where SLC6A3 acts as a dopamine re-uptake transporter. Dopamine is has however also a function in normal kidney to regulate processes like sodium homeostasis and vasodilatation. Dopamine is likely to bind to the dopamine receptors, which are all (D1-D5) expressed in the kidney. Dopamine is however unlikely to be recycled into the tubular cells (at least by means of SLC6A3 transport) as normal renal tubular cells largely lack expression of SLC6A3.

One still unresolved question is why ccRCC tumors express the dopamine transporter. While we demonstrated that *SLC6A3* is induced by HIF2-α and thus may be a result from the pseudohypoxic profile caused by *VHL* loss, the exact function of SLC6A3 still remains open for interpretation. One possible explanation may be that the increased *SLC6A3* would be a passenger event without any specific link to ccRCC progression. The increased *SLC6A3* expression may be caused by the differential regulation of chromatin state caused by deletion of genes on chromosome 3p (pseudohypoxia caused by VHL loss in combination deletion of chromatin modulators). Assuming that *SLC6A3* is a direct target of HIF2-α (it might however be a secondary target), pseudohypoxia combined the changed chromatin would potentially allow for binding of HIF2-α in HRE element in the promoter region of *SLC6A3* in a cell type specific manner.

Alternatively ccRCC tumors would need dopamine for the same reasons as normal renal cells, for example to control redox balance. The majority of the classical serum-grown ccRCC cell lines expressed rather low levels of *SLC6A3* compared to primary material thus suggesting that SLC6A3 is needed *in vivo,* but not so much when cultured as monolayer cultures. One might speculate that the pH buffered culture media itself results in down regulation of pathways involved in redox balance (Soares-Da-Silva, Serrao et al. 1998). Moreover, since many cancers are associated with an altered metabolism one possibility may be that

dopamine is used as a building block, or rather is needed as an alterative pathway to generate ATP, such notion still remains to be investigated.

A recent publication demonstrated that the levels of *SCL6A3* were lower in ccRCC, compared to normal proximal tubular cells (Schrodter, Braun et al. 2016). However, our studies argue against such notion, and by contrast demonstrate increased expression of *functional* SLC6A3 protein in ccRCC primary material compared to normal renal tissue, as measured by [$^3$H]-dopamine assays.

The unique expression of SLC6A3 in ccRCC along with the fact that SLC6A3 has functional uptake of dopamine opens up for translational possibilities, including the use of SLC6A3 as diagnostic or as a therapeutic target for ccRCC patients. One possibility may be to therapeutically target SLC6A3 using dopamine analogues. One challenge of metastatic RCC is to find biomarkes with potential prognostic value. To date no biomarkers have reached the clinic and the prognosis still largely relies on the ULCA integrated staging system. The levels of SLC6A3 are already assessed, by imaging of dopaminergic neurons, to aid diagnosis of Parkinson's disease. This opens up for the possibility to use a similar strategy to diagnose patients with metastatic ccRCC. Similarly the imaging of SLC6A3 could potentially be used to follow tumor burden of metastatic ccRCC patients to guide the choice of treatment.

# Paper III: CD44 interacts with HIF-2α to modulate the hypoxic phenotype of perinecrotic and perivascular glioma cells

## Overall Aims

The overall aim of paper III was to investigate the role of CD44 in hypoxia signaling in glioma, and potentially to unravel the mechanism underlying CD44ICD dependent regulation of hypoxic and pseudohypoxic phenotypes in glioma.

## Summary

In this paper we show that CD44 cleavage, is enhanced at hypoxia in human and murine glioma cells. By using three structurally different pharmacological inhibitors of ADAM10/17, we demonstrated that inhibition of CD44 cleavage inhibits the hypoxic response. Specifically, hypoxia mediated by stabilization of HIF-2α, but not HIF-1α, was reduced in response blocked CD44 cleavage, and CD44ICD was further shown to interact with HIF-2α. By using a panel of assays measuring different aspects of stemness including, sphere formation, side population, and quantification of stem cell markers, inhibition of CD44 cleavage was shown to reduce hypoxia-induced stem cell characteristics. By contrast, inhibition of CD44 cleavage induced expression of differentiation markers GFAP and Tuj1. Using the TCGA data set we found a positive correlation of CD44 and the enzymes responsible for CD44ICD generation with a hypoxic gene signature.

Interestingly CD44ICD could enhance HIF target activation both at 1% oxygen and 5% oxygen corresponding to hypoxic and perivascular oxygen tensions, respectively. In glioblastoma, GSCs are described to reside within the perivascular and the hypoxic tumor niches. Similarly in murine proneural GBM, CD44 was located both in the perivascular GSCs expressing HIF-2α, and in the hypoxic GSCs co-expressing both HIF-1α and HIF-2α, indicating that CD44ICD can interact with HIF-2α to maintain stemness in both the perivascular and hypoxic GSC niches.

## Discussion

The background to these studies was a number of findings regarding CD44, which was found to be expressed in the perivascular niche of proneural GBM (Pietras,

Katz et al. 2014). Historically, CD44 is perhaps most known as the receptor for hyralonan, but during recent years CD44 has been demonstrated to have major intracellular functions in the cytoplasm where the c-terminal portion of CD44 has been demonstrated to interact with cytoskeletal components as well as several kinases. Furthermore CD44ICD contains a NLS signal and has been proposed to be important also for regulation at the transcriptional level (Reviewed in (Zoller 2011)).

Both expression of CD44 and tumor hypoxia associated with expression of HIFs, particularly HIF-2α, has been linked to more stem like phenotypes on their own (Li, Bao et al. 2009, Anido, Saez-Borderias et al. 2010). A couple of years ago, Pietras et al. further suggested that CD44 and HIF-2α were able to co-operate to maintain stemness (Pietras, Katz et al. 2014). While their studies demonstrated that CD44ICD was able to enhance activation of hypoxia responsive elements dependent on CBP/p300, when hypoxia was driven by HIF-2α, the exact mechanism by which CD44/ HIF-2α co-operate to maintain GSC stemness still remained to be unraveled.

Activation of the hypoxic response is mainly regulated by stabilization of the HIF-α subunits. Two classes of oxygen-sensing enzymes; the PHDs and FIH, which mediate hydroxylation N-TAD and C-TAD domains, respectively, regulate HIF-α stability in an oxygen dependent manner. PHD and FIH lose their ability to target the HIF-α subunits for hydroxylation when in cells experiencing hypoxia, thus resulting in stabilization, binding to, and transcriptional activation of hypoxia responsive elements. Notably, in addition to being expressed in response to hypoxia, HIF-2α is somewhat surprisingly expressed in the glioma tumor regions with (presumably) the highest levels of oxygen, the perivascular niche (Li, Bao et al. 2009). Similarly, our data indicate that perivascular glioma cells express HIF-2α but not HIF-1α. As outlined in the "HIF-α isoform displays differential sensitivity for FIH-mediated asparagine hydroxylation" section, FIH has significantly higher affinity for HIF-1α than HIF-2α. The presence of HIF-2α in perivascular well oxygenated tumor regions can thus be partly explained if one takes into account that FIH-1 has higher sensitivity for HIF-1α than HIF-2α and that FIH-1 has higher oxygen affinity compared to the PHDs, and thus maintains sufficient activity to hydroxylate HIF-1α but not HIF-2α at intermediate oxygen levels. While our studies did not address the impact of FIH hydroxylation on HIF stability directly, it has been previously suggested that FIH mediate asparagine hydroxylation affects the transcriptional activity of HIFs rather than their protein stability (Lando, Peet et al. 2002, Sang, Fang et al. 2002).

Oxygen pressure alone, is however not able to explain the HIF-2α stabilization seen in perivascular cells. Indeed, HIF-2α stainings of murine PN GBM demonstrated HIF-2α expression overlapped with expression of the stem cell

marker CD44. By contrast CD44 negative tumor bulk cells lacked HIF-2α staining, suggesting differential regulation of HIF-2α in GCS vs tumor bulk cells. By co-immunoprecipitation experiments, our present studies demonstrate that CD44ICD interact with PHD insensitive HIF-2α, but not with PHD insensitive HIF-1α by protein-protein interactions. By contrast, CD44ICD can interact with a version of HIF-1α that has been made insensitive to C-TAD hydroxylation mediated by FIH (in addition to PHD mediated N-TAD proline hydroxylation), thus centralizing the role of this particular hydroxylation site. In line with such notion, our data indicates that knock down of FIH (thus preventing HIF-1α C-TAD hydroxylation) potentiates interaction of CD44ICD and HIF-1α in addition to HIF-2α. GSCs have been described to reside in two separate tumor niches; the perinecrotic (hypoxic) tumor niche and in the perivascular (well vascularized) niche. Our data adds on to the notion that GSCs may represent two distinct glioma stem cell populations (at least with regards to HIF-1α expression). Both populations express CD44 and HIF-2α that maintain their stem like state. The hypoxic also stabilize HIF-1α and activate HIF-1α target genes, which may be speculated to make the hypoxic GSCs phenotypically different from the perivascular GSCs. Similarly the two GSC populations would be exposed to different stromal input due to their difference in localization (Hambardzumyan and Bergers 2015). Beyond differences in HIF-1α stabilization, our studies have not addressed other possible phenotypic differences of hypoxic and perivascular GSCs.

Our understanding of the CD44ICD induced HIF-2α stabilization could have several clinical implications where HIF-2α stabilization may be targeted indirectly though CD44. One strategy would be to use CD44 inhibition as an adjuvant treatment to sensitize GCSs to radiation therapy or Temozolomide treatment. In theory there are several possible ways of targeting the CD44 receptor. One approach would be to inhibit the oseopontin-CD44 interaction ether by using blocking antibodies or possibly by using small molecule inhibitors to compete with OPN binding. Another approach would be to target the proteolytic cleaveges mediated by ADAM10/17 (S2) or the gamma-secretase (S3)) that generates the CD44ICD.

Our studies focused on targeting the ADAM10/17 cleavage by pharmacological inhibition *in vitro*. Such inhibition was demonstrated to reduce expression of genes induced by the hypoxia responsive elements and was further shown to reduce stemness along with increased expression of markers of differentiation. As tumor stemness has been directly correlated with treatment resistance, a less stem-like GSC population may be more sensitive to standard therapy.

Despite promising effects *in vitro* it is hard to anticipate the effects of ADAM inhibition *in vivo*. ADAM seems important during embryonic development since

ADAM10 null mice die at E9.5 and ADAM17 null mice display perinatal lethality (Reviewed in (Weber and Saftig 2012). There are some concerns about using GSIs *in vivo*, sine they are associated with dose limiting adverse effects largely due to intestinal goblet cell expansion. One might have similar concerns about ADAM inhibitors as both ADAM10 and 17 are expressed in the intestine and are involved in regulating cellular homeostasis. Such concerns may however be circumvented by introducing intermittent treatment regimes and thus allowing the intestinal homeostasis to normalize (Reviewed in (Jones, Rustagi et al. 2016).

Another concern may be the unspecific nature of ADAM inhibition. In humans 22 ADAMs have been identified, where 12 have demonstrated proteolytic activity (ADAM8,9,19,12,15,17,19,20,21,28,30 and 33) (Reviewed in (Jones, Rustagi et al. 2016). ADAMs further regulate intramembranous proteolysis of other substrates than CD44 including Notch and further induces liberation of soluble ligands such as IL-6, TNFα, thus regulating processes such as inflammation (Reviewed in (Weber and Saftig 2012). Adding on to the complexity ADAM proteins are described to harbor dual roles. When membrane bound ADAM10 acts as a metalloprotease. Interestingly the ADAMs themselves are targets for intramembrane proteolysis and releases an intracellular domain. For example ADAM10 is targeted for intramembranous proteolysis by ADAM9 and ADAM15, followed by subsequent processing by the gamma-secretase. Some ADAMs are thus suggested to regulate transcriptional activity, which may lead to unexpected effects of ADAM inhibition *in vivo* (Tousseyn, Thathiah et al. 2009). One study addressed ADAM10/17 inhibition in orthotropic glioma xenografts by comparing an ADAM inhibitor (INCB3619) with GSI treatment (DAPT). When delivered by local nanoparticles the ADAM inhibitor was demonstrated to prolong survival compared to GSI treated and control mice (Floyd, Kefas et al. 2012).

At present there are very few targeted therapies for tumors in the brain, which may be speculated to be due to BBB as an obstacle for permeability of most such therapies. Consequently one needs to consider BBB permeability when designing new treatments for GBM. The GSIs are likely to cross the BBB considering that GSIs can be used in the brain to prevent gamma-secretase-mediated proteolysis of amyloid precursor protein (APP) into β amyloid (Aβ) and thus prevents formation of amyloid plaques, seen in Alzeimer´s disease (Barten, Meredith et al. 2006). Local treatment at the time of surgery, for example using nanoparticle methodology would be one way to circumvent problems with drug permeability and would further reduce the risk of adverse effects seen with systemic ADAM inhibition (Floyd, Kefas et al. 2012).

# Paper IV: Radiation induced changes in the tumor microenvironment

## Overall Aims

In paper IV the overall aim was to explore the contribution of astrocytes to the perivascular microenvironment and the effect of irradiation on perivascular astrocytes and potentially unravel mechanism(s) by which the astrocytes induce and maintain radioresistance.

## Summary

In this paper we demonstrated that astrocytes become reactive in response to radiation and further studied whether the irradiated astrocytes affected the phenotype of glioma cells. By co-culturing astrocytes with glioma cells we demonstrated that the glioma cells got more stem-like and displayed reduced sensitivity towards radiation. Similarly, culture of glioma cells on matrix derived from irradiated astrocytes acquired a more stem like phenotype as when compared to glioma cells grown on matrix from untreated astrocytes. To assess the contribution of soluble factors, 3D cultured glioma cells were grown in media from irradiated astrocytes, but our data demonstrated that such soluble factors did not induce a more stem like phenotype in glioma cells.

RNA sequencing of irradiated and control astrocytes revealed changed expression of genes involved in oligodendrocyte differentiation, cell cycle and glioma cell plasticity. Finally, a signature of the top 100 up-regulated genes in response to radiation associated with worse clinical outcome among glioblastoma patients, thus suggesting that reactive astrocytes confer aggressive behavior of glioma tumorigenesis.

## Discussion

Glioblastomas display significant tumor cell heterogeneity with regards to tumor cell differentiation and stemness, where the presence of such mixed tumor cell populations has been associated to varying sensitivity towards radiation. Increased radioresistance has been linked to a small subset of cells positive for stem cell markers such as CD133. Several intrinsic molecular pathways have been identified to regulate radio resistance of GCS. CD133 positive cells for example appears

better at repairing radiation-induced DNA damage compared to CD133 negative cells (Bao, Wu et al. 2006, Liu, Yuan et al. 2006).

Administration of radiation therapy has been demonstrated to significantly prolong survival for glioblastoma patients, where a common dosing scheme includes administration of a total dose of 60 Gy divided into 30 fractions. Doses beyond that point have been experimentally evaluated but were found to associate with higher toxicity (Morris and Kimple 2009). The toxicity may be speculated to be partially explained by the invasive growth pattern of glioblastomas that results in tumor cells intermingling with normal brain tissue and that radiation therapy interferes with normal brain functions.

A second layer of glioblastoma heterogeneity is provided by the fact that tumor cells are intermingled with stromal cells including various glial cells, endothelial cells, pericytes, astrocytes, microglia, and other infiltrating immune cells. Some parts of the microenvironment have been demonstrated to supports glioma growth, for example TAMs promote invasiveness though the TGFβ -MMP9 axis (Ye, Xu et al. 2012). GSCs have been demonstrated to reside in certain niches, such as the perivascular niche, where the interplay of GSCs and endothelial cells have been intensely studied. GSCs also located to hypoxic areas in close proximity to tumor necrosis, where TAMs/microglia have been described to support the stem like properties of GSCs (Codrici, Enciu et al. 2016).

Astrocytes, being part of the glioblastoma microenvironment, could potentially contribute to such GSC maintenance. Indeed, we demonstrate that astrocytes survive radiation and respond by a process called reactive gliosis, which is associated with a typical phenotypic shift towards cells with enlarged cell body and greater number of processions along with increased expression of intermediate filament proteins, particularly GFAP. Further we demonstrate that astrocytes sensitized glioma cells to irradiation. Irradiation of astrocytes has been demonstrated to induce secretions of soluble factors including IL-6, IL-8 and GM-CSF (Placone, Quinones-Hinojosa et al. 2016). Our data demonstrate that astrocytes that survive radiation change their expression profile. Indeed, human astrocytes exposed to 10 Gy radiation for example upregulated genes related to glioblastoma plasticity, cell cycle and oligodendrocyte differentiation.

There are several possible mechanisms by which astrocytes could contribute to the stem cell niche and maintain GSCs. In our study we addressed the contribution of soluble factors, secreted matrix components and direct cell-to cell signaling. Our data suggest that astrocytes primarily affect glioma stemness by changing the composition of the ECM. Similar effects could be seen under conditions of co-culture. Indeed, co-culture of irradiated astrocytes with glioma cells increased the ability of glioma cells to drug efflux though the ABCG2 transporter thus conferring increased stemness. Irradiated astrocytes further increased their activity

of lysyl oxidase (LOX), which is an enzyme that acts by crosslinking collagens or elastin. The increased activity of LOX is thus likely to affect ECM stiffness where more collagen crosslinking associates with a stiffer matrix. Both increased expression of LOX and increased matrix stiffness has been associated with tumor progression and increased metastasis (Madsen, Pedersen et al. 2015, Wang, Davis et al. 2017). As demonstrated by co-culture of glioma cells in astrocyte-conditioned media, soluble factors (including IL-6, IL-8 and GM-CSF) did not affect the side population or clonal survival of glioma cells in this particular setting.

Similar to irradiation hypoxia is a known inducer of reactive astrocytes (Floyd and Lyeth 2007). Interestingly, astrocytes exposed to hypoxia exerted somewhat different effects on glioma cells compared to astrocytes exposed to irradiation. Both hypoxia and irradiation induced the reactive phenotype in astrocytes. Glioma cells grown on astrocyte-derived matrix from irradiated astrocytes was, however, able to induce stemness in glioma cells to a greater degree than matrix from hypoxic astrocytes. By contrast, matrix derived from glioma cells promoted stemness when exposed to hypoxia to a higher extent when compared to normal and irradiated glioma-derived matrix. These data propose that the hypoxic cue produced by the glioma cells themselves, combined with the radiation-induced changes of astrocytes significantly promotes the presence of more stem-like tumors.

A still unanswered question is how one would address these radiation-induced changes in the clinic. Our data indicate that irradiate astrocytes may contribute to the stem-like phenotype of glioma cells. However, as radiation is associated with significant survival benefit, there is no rational to remove radiation therapy from the standard of care. It may however be beneficial to address the radiation-induced changes. For example, being able to provide a drug that reduces ECM components that promote stemness alongside radiation treatment might prevent development of radiation resistant cells.

# Conclusion and Future perspective

In this thesis I have studied HIF dependent signaling pathways in cancer and have been focusing on two types of cancer, ccRCC and glioma, where hypoxic signaling is of particular interest and importance. Indeed, ccRCC-development is associated with near obligatory loss of the *von Hippel Lindau tumor* (*VHL*) suppressor gene, leading to stabilization of the hypoxia inducible factors (even in tumor regions with high oxygen tensions) and results in subsequent pseudohypoxia. As for gliomas hypoxic signaling has been extensively described to maintain treatment resistance of glioma stem cells independently of oxygen availability.

In paper I we substantiated the notion that hypoxic signaling alone is unable to induce ccRCC tumorigenesis. We demonstrated that expression of Notch-ICD is elevated in ccRCC and further that Notch1 signaling significantly contributed to the ccRCC tumorigenesis by regulating lipid accumulation. In paper II we demonstrated that hypoxia induced expression of the dopamine transporter SLC6A3 in normal renal epithelium but not in other tissues. We further demonstrated that ccRCC tumors uniquely harbors a functional uptake of dopamine though the SLC6A3 transporter, which constitute a possible target in the clinic.

Tumor hypoxia is associated with a more aggressive phenotype along with therapeutic resistance thus rationalizing clinical targeting of the hypoxic phenotype. In paper III we demonstrate the CD44, expressed at the surface of glioma stem cells, may be targeted by proteolytic cleavages generating formation of CD44ICD that interacts with HIF-2α to modulate the stem- like phenotype of gliomas. We further demonstrated that pharmacological inhibition of CD44 cleavage reduced the stem-like phenotype of glioma cells and CD44 thus constitutes a possible target for therapeutic intervention in glioma. In paper IV we have studied radiation-induced changes in the glioma microenvironment, which is known to support and maintain glioma stem cells. Our studies focused on tumor-associated astrocytes which were demonstrated to enhance stem-like phenotypes of glioma cells, both trough direct cell-cell interactions and by changes in the ECM, thus underlining the potential of targeting the microenvironment in order to treat glioma stem cells.

# Populärvetenskaplig sammanfattning

Medan normala celler har en begränsad tillväxtpotential kan cancerceller genom mutationer i sitt DNA förvärva egenskaper som gör att de växer och delar sig okontrollerat. Cancerceller har även förmågan att invadera normal vävnad och därmed störa dess funktion.

Syre behövs generellt för att generera energi till kroppens vävnader. Även cancerceller behöver syre för att stödja deras snabba expansion. Cancerceller har förmågan att anpassa sig till låga syrenivåer (hypoxi) genom att sätta igång cellulära processer för att spara på energi och för att kunna rekrytera nya blodkärl för att syrebristen ska upphöra. Även normala celler har förmågan att under korta perioder anpassa sig till låga syrenivåer, medan en längre period syrebrist gör att normala celler genomgår programmerad celldöd. Anpassningen till låga syrenivåer styrs till stora delar av två proteiner; hypoxi inducerade faktorer (HIF) (HIF-1α och HIF-2α). Dessa proteiner finns i normala fall bara i celler som upplever syrebrist, men uttryck av dessa proteiner är vanligare i tumörer, dels för att tumörer oftare upplever syrebrist då de expanderar snabbare än det hinner bildas nya blodkärl, och dels för att vissa cancerceller har förvärvat förmågan att uttrycka HIF proteiner trots normala syrenivåer.

I den första delen av avhandlingen har vi studerat klarcellig njurcancer. I stort sett alla fall av klarcellig njurcancer har förlorat den normala funktionen av von Hippel Lindau proteinet (pVHL) vilket gör att tumörerna uttrycker HIF trots att de har normala syrenivåer. I delarbete I, visar vi att klarcellig njurcancer uttrycker högre nivåer av signalmolekylen Notch1. Vidare visar vi att Notchsignalering samverkar med pVHL förlust för att främja tidiga tecken på tumörutveckling exempelvis genom att öka cellernas lagring av fett, vilket gör att cellerna ser ”klara” ut. Klara celler är ett av de typiska tecken som skiljer klarcellig njurcancer från andra typer av njurcancer. I delarbete II, visar vi att klarcellig njurcancer uttrycker höga nivåer av dopamintransportören SLC6A3. Dessa höga nivåer av SLC6A3 är unika för just denna tumörform. I normal vävnad är SLC6A3 endast uttryckt i dopaminerga neuron i hjärnan där förlust av dessa har kopplats till Parkinsons sjukdom. I resten av kroppen utrycks endast väldigt låga nivåer SLC6A3 vilket gör att man skulle kunna rikta behandling mot denna dopamintransportör utan att skada övriga kroppen.

I den andra delen av avhandlingen har vi studerat gliom som är den vanligaste formen av hjärntumör bland vuxna. Den mest aggressiva formen, glioblastom multiform (GBM) har en genomsnittlig överlevnad på ca 15 månader. Typiskt för GBM är att en liten andel av tumörcellerna ha specifika egenskaper som gör att överlever behandling så som strålning och kemoterapi. Dessa celler kallas cancerstamceller och har förmågan att dela sig och bilda en ny tumör. Cancerstamceller tycks finnas på specifika ställen i gliomtumörer, dels alldeles i närheten av blodkärl (med god syretillförsel) och dels nära tumörregioner med svår syrebrist. Oavsett om cancerstamcellerna befinner sig i regioner med god eller väldigt dålig syretillförsel uttrycker de HIF-2α protein, medan endast cancerstamceller med dålig syretillförsel verkar uttrycka HIF-1α. I delarbete III, visar vi att dessa cancerstamceller (både i syrerik och syrefattig miljö) uttrycker en markör och signalmolekyl som kallas CD44. Vi visar också CD44 kan modulera det hypoxiska svaret genom att binda till HIF-2α proteinet och att det gör cancercellerna mer stamcellslika. Farmakologisk hämning av CD44 medförde att tumörcellerna blev mindre aggressiva, vilket öppnar upp för att man skulle kunna rikta behandling mot CD44 för att minska cancercellernas förmåga att anpassa sig till syrebrist.

I delarbete IV, visar vi slutligen att behandling av hjärntumörer genom strålning kan påverka miljön runt tumörcellerna och även påverka de normala cellerna som finns i närheten av tumörceller. En av de celltyper som normalt finns i hjärnan är astrocyter. Astrocyter är stjärnformade celler som bland annat har i uppgift att läka skador som uppstår i hjärnan. Vi har tittat specifikt på effekten av syrebrist och strålning och har sett att astrocyter (både efter strålning och vid syrebrist) kan påverka gliomcellerna så att de blir mer stamcellslika och därmed svarar sämre på behandling. Det behövs en ökad förståelse för de molekylära processerna som ligger bakom anledningen till att astrocyter kan göra gliomceller mer stamcellslika för att vi ska kunna erbjuda bättre framtida behandlingar.

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