Exploring the benefits of inhibiting HIF and Notch to overcome resistance to cancer therapy

Citation for published version (APA):

Moreno Roig, E. (2018). *Exploring the benefits of inhibiting HIF and Notch to overcome resistance to cancer therapy*. [Doctoral Thesis, Maastricht University]. Maastricht University.
<https://doi.org/10.26481/dis.20181114em>

Document status and date:

Published: 01/01/2018

DOI:

[10.26481/dis.20181114em](https://doi.org/10.26481/dis.20181114em)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Exploring the benefits of inhibiting HIF and Notch to overcome resistance to cancer therapy

Eloy Moreno Roig

Copyright

PhD thesis, Maastricht University, The Netherlands

© Eloy Moreno Roig, Maastricht, 2018

All rights reserved. No part of this thesis may be reproduced or transmitted in any form or by any means without prior written permission from the author.

Cover design by Eloy Moreno Roig

Lay-out by Eloy Moreno Roig

Printed by Gilderprint, www.gilderprint.nl

ISBN 978-94-6323-405-4

Exploring the benefits of inhibiting HIF and Notch to overcome resistance to cancer therapy

DISSERTATION

to obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus, Prof. dr. Rianne M. Letschert
in accordance with the decision of the Board of Deans,
to be defended in public
on Wednesday November 14th 2018 at 13:45 hours

by

Eloy Moreno Roig

Promoter: Prof. dr. Marc Vooijs

Co-promoter: dr. Ludwig Dubois

Assessment Committee:

Prof dr. de Windt (chair, UM)

Prof dr. W. Annaert (KU Leuven)

Prof dr. P. van Diest (UMC Utrecht)

Prof dr. P. Peters (UM)

Prof dr. H. Schmidt (UM)

Table of contents

Chapter 1	9
Introduction	
Chapter 2	41
Prognostic role of Hypoxia-Inducible Factor-2 alpha tumor cell expression in cancer patients: a meta-analysis	
Chapter 3	75
HIF-1 α and HIF-2 α differently regulate the radiation sensitivity of NSCLC cells, partly by impairing their metabolic profile	
Chapter 4	113
NOTCH blockade in combination with radiotherapy increases the number of lung stem cells in differentiated cultures	
Chapter 5	139
γ -secretase complex composition differentially regulates Notch1 wild type and oncogenic pathways	
Chapter 6	163
General discussion	
Summary	183
Valorization addendum	189
Acknowledgements	195
Curriculum Vitae	203
List of publications	207

Abbreviations

SC: Stem cells

CSC: Cancer stem cells

TAM: Tumor-associated macrophages

HIF: Hypoxia-inducible factors

N-TAD: N-terminus domain

C-TAD: C-terminal domain

ODDD: Oxygen-dependent degradation domain

pVHL: Von Hippel–Lindau protein

PHDs: prolyl-4-hydroxylases

ARD-1: Arrest-defective-1

HREs: Hypoxia response elements

GLUT-1: Glucose transporter 1

VEGF: Vascular endothelial growth factor

CAIX: Carbonic anhydrase IX

MCT-1: Monocarboxylate transporter 1

MCT-4: Monocarboxylate transporter 4

ATP: Adenosine triphosphate

OXPHOS: Oxidative phosphorylation

NHE-1: Sodium-hydrogen exchanger 1

TCA: Tricarboxylic acid cycle

ROS: Reactive oxygen species

LD: Lipid droplets

DDR: DNA damage response

DII: Delta

S1: Site-1

S2: Site-2

S3: Site-3

NECD: Notch extracellular domain

TMIC: Transmembrane and intracellular fragment

ADAM10: A Disintegrin And Metalloprotease 10

GS: γ -secretase

GSI: γ -secretase inhibitors

APP: Amyloid Precursor Protein

A β 42: pathologic amyloid peptide A β

NCT: Nicastrin

PSEN: Presenilin

PEN-2: Presenilin-enhancer 2

APH1: Anterior Pharynx-Defective 1

NICD: Notch IntraCellular Domain

T-ALL: T cell acute lymphoblastic leukemia

NSCLC: Non-small cell lung cancer

HIF-1 α : Hypoxia-inducible factor-1 α

HIF-2 α : Hypoxia-inducible factor-2 α

CC-RCC: Renal cell carcinoma

HR: Hazard ratio

CI: Confidence interval

EPAS1: Endothelial PAS domain protein-1

OS: Overall survival

DFS: Disease-free survival

LC: Locoregional control

DSS: Disease-specific survival

MFS: Metastasis-free survival

PFS: Progression-free survival

LDH-A: Lactate dehydrogenase A

sgRNA: Small guide RNA

DFO: Desferrioxamine

ECAR: Extracellular acidification rate

OCR: Oxygen consumption rate

2-DG: 2-deoxyglucose

mTORC1: Rapamycin complex 1

DSB: Double-strand break

ALI: Air-liquid interface

PBEC: Primary bronchial epithelial cells

AD: Alzheimer's disease

CQ: Chloroquine

CHAPTER 1

Introduction

Eloy Moreno Roig

Introduction to cancer

Cancer is the second leading cause of death worldwide with approximately 14 million new cases and 8.2 million cancer deaths in 2012 [1]. A person's risk of developing cancer depends on natural factors such as age and genetics but also by life-style factors including smoking, alcohol consumption and diet. An increased exposure to carcinogens causes DNA damage in normal cells, which may lead to mutations and ultimately abnormal cellular growth. A bulk of cells that rapidly divide and have the ability to invade surrounding tissues and migrate to distant organs is often referred to as a cancer. The alteration of a normal cell to a neoplastic state is driven by a multistep process which involves the defect of several cellular mechanisms described in the hallmarks of cancer by Hanahan and Weinberg [2]. To note, it is estimated that metastasis causes around 90% of deaths from cancer [3]. Treatment of cancer is of great importance in order to increase survival and quality of life of cancer patients.

Introduction to resistance to cancer therapy

Most patients with cancer respond to initial therapy, but the responses are typically brief and many patients experience relapse. Drug resistance is the ability of cells to become tolerant to pharmaceutical treatments that would normally kill or limit their growth. This term started to be used around 1940 after the observation that bacteria have the ability to become resistant to certain antibiotics [4, 5]. Many of the mechanisms leading to drug resistance are conserved from bacteria to man and occur in other diseases, including cancer [6]. Chemotherapy and radiotherapy are two of the most common types of cancer treatment. Both of them are designed to effectively kill proliferating cells, such as cancer cells, while sparing slower proliferative cells. Chemotherapy delivers drugs to the entire body and selectively targets rapidly dividing cells by interacting with regulators and molecules involved in cell division such as the DNA [7]. Radiation therapy uses X-ray to locally kill cancer cells or cause genetic instability resulting in cell death [8]. These treatment options have led to an increase in the survival of cancer patients but also induce many side-effects which can seriously affect the quality of life of patients and even cause their death. However, many of these patients show drug resistance leading to progressive disease after initially responding to the treatment [9]. Resistance to therapy remains a major issue to achieve complete remission in cancer patients.

Cancer stem cells

Recent investigations have shown that cancer cells are not all the same and have defined a small sub-population of cells which possess characteristics associated with normal stem cells (SC). The discovery of cancer stem cells (CSC) started with the observation of D. Bonnet in 1997 as she noticed that leukemia cells organize as a hierarchy that originates from a primitive hematopoietic cell [10]. Future investigations confirmed her findings for other cancer types [11, 12]. CSCs are thereby defined as a small subpopulation of cells capable of self-renewal, dedifferentiation, tumorigenicity and intrinsically resistant to chemo-radiotherapy. The ability of CSC to survive and proliferate under extreme conditions ultimately results in tumor relapse by failure of conventional therapies [13]. CSCs display many features of normal SCs such as activity of the Notch, Wnt and Hedgehog pathways, which regulate the characteristics previously mentioned. When these pathways are inappropriately activated, signals are transduced to trigger tumorigenic properties in cancer cells [14]. SCs maintain their stemness in a specific microenvironment, the stem cell niche, which preserves their undifferentiated state or promote their differentiation. Several molecular pathways such as Delta-Notch, BMP and VEGF are associated with niche function and thereby play an important role in stem cell maintenance [15]. Tumor hypoxia is the situation where tumor cells have been deprived of oxygen and in consequence triggers resistance to anti-cancer therapies by modulating several cellular pathways [16]. Previous investigations have identified hypoxia as critical factor in promoting stem-like properties, partially by enhancing the expression of stem cell factors. Thereby, targeting the hypoxic CSC niche may be highly effective for controlling tumor growth, preventing metastasis and improve therapy effectiveness in cancer patients [17, 18]. Different cell types within the CSC niche produce factors to stimulate CSC properties and recruit immune cells, such as tumor-associated macrophages (TAM), which secrete additional factors to promote tumorigenesis, metastasis and resistance to therapy [19] (Figure 1).

FIGURE 1: Schematic representation of Cancer Stem Cell niche. CSC niche is a complex environment where many cellular components communicate using specific factors as mediators such as Notch, WNT and PTCH1. Many CSC niches show low oxygen concentrations as a results of inadequate oxygen supply, which contributes to the malignant phenotype of cancer.

Hypoxia in cancer

Hypoxia is defined as an inadequate supply of oxygen (O_2) which occurs very frequently within solid tumors, mainly as a result of abnormal blood vessel formation, defective blood perfusion and disturbed microcirculation [20]. In proliferating cancer cells, oxygen demand is exceeded by oxygen supply. Also, as the tumor grows, the distance between cells and the vasculature increases, preventing adequate oxygen diffusion and creating more hypoxic regions [16]. Hypoxia is associated with worse prognosis in cancer patients [21] and promotes treatment resistance to conventional therapy [22, 23]. The resistance to therapy is mostly caused by limited drug perfusion into the tumor in combination with a decrease in drug uptake [22]. Transient hypoxia triggers the expression of genes encoding P-glycoprotein and dihydrofolate reductase, which induce hampered drug uptake into cells leading to multidrug resistance [24, 25]. Decreased cellular uptake is also influenced by the low pH of the tumor

microenvironment. Uncharged molecules diffuse passively across the cell membrane. Due to a low extracellular pH, basic drugs that present an acid dissociation constant of 7.5-9.5 are protonated and display decreased cellular uptake [26]. Active transport of some drugs such as methotrexate is also hampered by acidic microenvironments [27]. Hypoxia and nutrient deprivation inhibits cell proliferation and induces cell cycle arrest, conferring resistance to therapy since anti-cancer drugs are more effective against fast proliferating cells [28]. Also, ionizing radiation induces reduced DNA damage when oxygen is not present [23].

HIF pathway

One of the key regulators of hypoxic signalling are the hypoxia-inducible factors (HIFs) proteins, which are responsible for the induction of genes that facilitate the survival of cells under oxygen-limiting conditions (Figure 2) [29, 30]. HIF are transcription factors which have an evolutionary conserved function to facilitate the response to decreases in availability of oxygen in the cellular environment [31]. The HIF dimer is composed of an alpha and beta subunit which are similar in structure. Both proteins contain an N-terminus domain (N-TAD) for DNA binding, a central region which facilitates heterodimerization and a C-terminal domain (C-TAD) which recruits coregulatory proteins essential for the transcriptional activity of the dimer [32, 33]. Both N-TAD and C-TAD are responsible for HIF transcriptional activity [34]. The HIF- α proteins consist of several members of which three, i.e. HIF-1 α , HIF-2 α and HIF-3 α , have been associated with cancer and are expressed in both the cytoplasm and nucleus. In parallel, the HIF-1 β protein is constitutively expressed and regulates the function of HIF- α by binding to them and forming a transcriptionally active dimer [35, 36]. Independently of the oxygen levels, HIF- α are constitutively transcribed upon the activation of a series of signalling events in cells [38]. Moreover, all HIF- α subunits contain an oxygen-dependent degradation domain (ODDD) in their structure which is important in mediating oxygen regulation stability [37]. When oxygen is present, HIF- α undergoes rapid ubiquitination and subsequent proteasomal degradation in a pathway involving the von Hippel-Lindau protein (pVHL), which is member of the E3 ubiquitin protein ligase family [39]. HIF- α contains two prolyl residues (P⁴⁰²/ P⁵⁶⁴) located in the ODD domain which were found to be good substrates for prolyl-4-hydroxylases (PHDs) or HIF-1 prolyl hydroxylases (HPH). These enzymes require oxygen for their hydroxylation action, besides other co-substrates such as iron and ascorbate [37]. Moreover, an extra residue in the ODDD (lysine,

K⁵³²) is acetylated by an acetyl transferase enzyme called arrest-defective-1 (ARD-1) [40]. Hydroxylated HIF- α subunits are recognized by pVHL and subsequently marked for proteolytic degradation [41]. Under hypoxic conditions, the activity of the oxygen dependent PHDs is inhibited and the ODD domain cannot be hydroxylated nor acetylated resulting in the stabilization of the HIF- α subunit and dimerization with HIF1- β . Once active, the dimer can travel into the nucleus and together with the co-factor P300 bind to the hypoxia response elements (HREs) into the regulatory elements of target genes [42]. Active HIF complex in the nucleus will trigger the expression of several hypoxia-related genes crucial for the adaptation to low-oxygen levels [43]. For instance, glucose uptake increases by inducing the expression of glucose transporter 1 (GLUT-1) and thus increases the intracellular levels of glycolytic substrate [44]. Angiogenesis is also induced by the expression of vascular endothelial growth factor (VEGF), which stimulates the generation of blood vessels increasing oxygen supply [45]. Also, both carbonic anhydrase IX (CAIX) and monocarboxylate transporter 4 (MCT-4) play a crucial role in regulating intracellular pH. High MCT-4 activity, in turn, removes the elevated concentration of lactate produced during glycolysis [46].

FIGURE 2: Schematic representation of the HIF-pathway. The HIF pathway is regulated by oxygen levels and triggers the expression of multiple hypoxia-related genes. In normoxic conditions, HIF-1 α binds to VHL at specific proline residues leading to proteosomal degradation. During hypoxia, HIF-1 α no longer binds to VHL and accumulates. This leads to the binding with HIF-1 β and together form a transcriptionally active dimer which triggers the activation of several hypoxia-response genes such as CAIX, MCT and GLUT-1.

Hypoxia and metabolic adaptation in cancer

In order to survive, hypoxic cells switch to a more glycolytic phenotype by upregulating several enzymes from the glycolytic pathway, resulting in the acidification of the extracellular environment (Figure 3) [47]. Due to the lack of oxygen, the production of adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS) is significantly decreased and thereby has to be compensated by actively upregulating the glycolytic pathway. This metabolic switch results in a decrease of ATP production per mol of glucose (glycolysis produces 2 mol ATP/mol glucose, whereas OXPHOS produces 36 mol ATP/mol glucose) [48]. The enhanced glycolytic rate increases the intracellular amounts of lactate and CO₂, contributing to an acidic pH. Hypoxic cells compensate the excess of intracellular acidification by upregulating the expression and activity of certain lactate transporters, such as the monocarboxylate transporter 4 (MCT4) [49]. Lactate is excreted from the cells resulting in extracellular acidification that contributes to malignant phenotype in cancer [50] [51]. Another key protein in acid/base regulation is CAIX, which catalyses the hydration of CO₂ to HCO₃⁻ + H⁺, leading to intracellular alkalization and extracellular acidification [52]. Glycolysis also stimulates the activity of the sodium-hydrogen exchanger 1 (NHE-1) which enhances extracellular acidification by exporting protons out from cells. The extracellular bicarbonate generated by CAIX is transported back into the cell by sodium bicarbonate transporters (NBC) and anion exchangers (AE). CAII is an intracellular protein that uses the intracellularly transported bicarbonate as a buffer forming a metabolon with CAIX, thus converting the bicarbonate and intracellular protons back to water and carbon dioxide [53] [54]. Glucose supply is critical for the maintenance of cancer cells not only to generate cellular ATP, but also supply biosynthetic building blocks such as ribose-PP and precursors of nucleotides and amino acids. Low glucose availability can be compensated by enhancing glucose transport inside the cell, but also increasing the conversion of glycogen into glucose in cancer cells which helps maintaining cell viability and proliferation [55]. In contrast with the enhanced glycolytic activity, several studies have demonstrated that the stabilization HIF proteins reduce both the tricarboxylic acid cycle (TCA) and OXPHOS, thereby protecting cancer cells from the generation of reactive oxygen species (ROS) that cause DNA damage [56]. Besides, HIF also negatively regulates mitochondrial biogenesis resulting in a further decrease of OXPHOS capacity and ROS in hypoxic cells [57]. Mitochondrial autophagy is one of the mechanisms proposed to explain the reduction of mitochondrial mass in cancer cells [58]. The stabilization of HIF proteins originated by a lack of oxygen in cells thereby

affect genes involving glucose uptake, glycolysis and other metabolic pathways necessary to re-establish the levels of ATP needed for cells to survive.

Recent studies demonstrated that both HIF-1 α and HIF-2 α proteins also play an important role in lipid metabolism [59, 60]. As with glucose metabolism, cancer cells show an altered regulation of many pathways involving lipid transport and metabolism. Lipids are molecules which are important in all organisms due to their structural and functional roles in cells. Lipids are not only the major components of cellular membranes but also an important source of energy. In cancer, due to the active proliferation rate and high need of energy, cells require more production of fatty acids necessary for the synthesis of cellular membranes and lipid storage [61]. The accumulation of intracellular lipids in cells leads to the generation of a cellular organelle, the lipid droplets (LD). LD are associated with various malignant phenotypes such as endoplasmic reticulum (ER) stress, ROS scavenging and drug resistance [62]. Under hypoxic conditions, the flux from Acetyl-CoA into fatty acid in the cytoplasm is increased by the synthesis of carbohydrate precursors generated via the glycolytic pathway. Furthermore, hypoxia has been shown to inhibit lipid catabolism thus increasing the accumulation of LD in cells [63]. Disorders in lipid regulation has been seen in many types of solid tumors, such as liver [59], pancreas [64] and colon [65] in which hypoxia is present. Previous studies showed that hypoxia-induced lipid accumulation relies on HIF-1 α and HIF-2 α in cancer cells [66, 67]. However it is still not clear what the real contribution of both proteins is in these phenotypes.

FIGURE 3: Schematic representation glucose and lipid metabolic pathways in relation with HIF activation. HIF-1 activates transcription of transporters and enzymes regulating glycolysis and other metabolic pathways. HIF-1-regulated transporters and enzymes are shown in boxes. CAIX, carbonic anhydrase IX; GLUT1, glucose transporter 1; NHE1, sodium-hydrogen exchanger 1; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle; HK, hexokinase; LDHA, lactate dehydrogenase A; MCT4, monocarboxylate transporter 4.

Hypoxia on radiation response

The first studies on radiation response were performed by Schwarz as far as back as the early 1990s, in which he demonstrated that radiotherapy was less effective in less perfused areas from the skin. Later studies by Müller showed that tissues in which perfusion was stimulated with diathermia (a commonly used muscle relaxer) were more responsive to radiotherapy. These studies were the first proof indicating that a sufficient blood supply is needed for an

adequate radiation response. Eventually this led researchers to confirm that hypoxia exists in human cancer as a result of lack of oxygen [68] and that oxygen deficiency is the main cause of radiotherapy failure [69].

Tumor hypoxic fraction has been negatively correlated with prognosis in cancer patients that undergo radiotherapy [70, 71]. Oxygen is an electron-affinic molecule that participates in the chemical reaction that stabilizes the damage in the DNA upon exposure with ionizing radiation [72]. Irradiation primarily induces water ionization leading to the formation of $\text{OH}\cdot$ radicals which reacts with the DNA resulting in DNA damage. Oxygen, in turn, stabilizes the damage produced in the DNA by changing its chemical composition. In the absence of oxygen, damage produced by the free radicals may be repaired by the intrinsic DNA damage response (DDR) [73]. Therefore, oxygenated cells during radiation show a more radiosensitive phenotype than cells that are poorly oxygenated at the time of radiation. Metabolic reprogramming caused by hypoxia contributes in the generation of metabolites and other molecules, such as lactate [74] and pyruvate [75], that protect the cells from ionizing radiation and promoting cell survival [76]. These molecules are capable of quenching ROS and other reactive DNA damage molecules generated by radiation, thus decreasing their harmful effects on the DNA. Certain antioxidant molecules generated during this metabolic switch reduce the consequences of radiation exposure, reducing DNA damage and thus increasing cell survival [77]. For instance, the substantial increase of lactate production during hypoxia allows the scavenging of ROS molecules generated after radiation exposure due to the antioxidant properties of lactate [78, 79] [74]. Another mechanism to explain this phenomenon is that HIF proteins protect endothelial cells from apoptosis induced by radiotherapy and promote their survival via induction of vascular endothelial growth factor (VEGF) [80]. However, due to an excessive VEGF signal, tumor vessels are leaky and poor functional which causes a further reduction of oxygen perfusion in tumor cells [81].

Previous research has also demonstrated that irradiation alone is capable of stabilizing the HIF proteins even when oxygen is present. For instance, HIF stability may occur upon irradiation through a direct interaction between the heat shock protein 90 (Hsp90) and HIF-1 α [82]. Also, the activation of different proteins from the DNA damage response pathway, such as ATR/ATM, leads to the phosphorylation of the HIF-1 α protein which prevents its degradation [83, 84]. Furthermore, it has been described that ROS itself is capable of inducing HIF-1 α stabilization by attenuating its degradation and enhancing its transcriptional activity [85].

HIF as a target for cancer treatment

Considering the multiple roles of the HIF pathway on several aspects of cancer, many years of research have focused on developing HIF inhibitors and some of them are currently under investigation in clinical trials. Many HIF inhibitors, mainly against HIF-1 α and HIF-2 α , are therefore being designed, which are hypothesized to lead to total or partial suppression of the HIF pathway and tumor-specific cell death [86]. These inhibitors are classified in different types depending on their mechanism of action: Interfere with mRNA transcription, protein translation, degradation and dimerization, DNA binding and transcriptional activity of HIF. Some of the agents may target more than one mechanism and also both HIF-1 α and HIF-2 α proteins at the same time. EZN-2698 is a commonly used anti-sense oligodeoxynucleotide molecule comprised of 16 nucleotide residues which binds to the mRNA of HIF-1 α with high affinity causing its downregulation and consequent reduction of HIF-1 α protein levels [87]. Although the mechanisms underlying HIF-1 α translation are still poorly understood, several agents have been shown to suppress the synthesis of HIF proteins via unknown or indirect mechanisms. For instance, the inhibition of topoisomerase I and II [88, 89], receptor tyrosine kinase [90], cyclin-dependent kinase [91] and microtubule disrupting agents [92] were shown to promote HIF-1 α inhibition through different mechanisms. Hsp90 is a cellular chaperone essential for the correct folding and localization of proteins that when inhibited promote HIF degradation in a VHL-independent manner [93]. Several other drugs are currently being investigated for its inhibitory effect on HIF; unfortunately no drugs targeting HIF have been approved for treating cancer patients due to safety and limited therapeutic efficacy. Nevertheless, there are currently many HIF targeting drugs under clinical evaluation for different kinds of cancer. Furthermore, the depletion of one HIF subunit could activate a compensatory mechanism by increasing the levels of the other one, leading to a more aggressive phenotype and drug resistance [94, 95]. Therefore, developing agents that simultaneously target both HIF-1 α and HIF-2 α proteins might increase the therapeutic efficacy of HIF inhibitors. The lack of patient selection also contributes to the failure in clinical trials since many of these studies test the efficacy of HIF inhibitors regardless of the expression levels of HIF in tumors.

In summary, the HIF pathway mediates the response to hypoxia and is required for cancer stem cell function [96]. Another potential candidate for CSC maintenance is Notch, which plays an important role in regulating functions and features of stem cells such as self-renewal and differentiation [97]. Additionally, these pathways interact through different mechanisms.

Firstly, HIF induces the expression the Notch ligand JAGGED2 [98] and Delta4 [99], leading to the stimulation of the Notch signalling pathway. Secondly, HIF requires the activation of Notch signalling to promote the undifferentiated cell state of some cell types such as myogenic and neuronal progenitors. This process is effective through a direct binding of HIF-1 α with the intracellular domain of NOTCH in the nucleus, leading to its stabilization and further activation of relevant target genes [100].

The Notch pathway

The Notch pathway is a highly conserved signalling system which operates in multicellular eukaryotic organisms and it is important for spatial patterning, morphogenesis and homeostasis. Notch signalling regulates activities that have key roles during embryonic development and also in tissue maintenance of adult tissues [101]. In mammals, the NOTCH pathway consists of 4 NOTCH receptors (NOTCH 1-4) which are located on the surface of the cellular membrane. The 4 receptors interact with five membrane-anchored ligands from the Delta (Dll1, 3 and 4) and Jagged (Jagged1 and 2) family which are expressed on neighboring cells [102]. Cells communicate with each other via direct physical contact and stimulate multiple cellular processes like differentiation, proliferation and apoptosis [101]. The Notch signalling pathways is often deregulated in many inherited developmental and adult onset syndromes [103]. For instance, NOTCH is often mutated in certain types of cancer leading to the over activation or repression of the pathway [104].

The NOTCH receptors encode single-pass transmembrane receptors that are located on the cell surface. Maturation and activation of the NOTCH receptors (Figure 4) rely on the activity of three distinct proteolytic processing steps which cleave NOTCH at distinct sites [105] [106]. NOTCH receptor maturation begins in the cytoplasm. NOTCH receptor precursors need to undergo cleavage at Site-1 (S1) in the trans-golgi network by a Furin-like protease. This first cleavage results in the formation of a heterodimeric Type 1 receptor with the Notch extracellular domain (NECD) bound non-covalently to a transmembrane and intracellular fragment (TMIC) at the cell surface [107-109]. Subsequently, the mature NOTCH receptor is transported to the cellular membrane which can bind to ligands expressed at the surface of neighbouring cells [109]. In the absence of stimulation by ligand, NOTCH receptors are kept in an *off* state called the auto-inhibitory state. This state keeps the NOTCH receptor in a closed conformation which protects the receptor from proteolytic cleavage at the site 2 (S2)

[110]. Upon ligand binding, the receptor changes into an unfolded state allowing the cleavage at S2 by A Disintegrin And Metalloprotease 10 (ADAM10) and releasing the ectodomain of the receptor (NECD) into the extracellular matrix [105]. Next, transendocytosis of NECD into Delta-expressing cells occurs and results in notch proteolytic activation independently of ADAM cleavage [111]. Immediately after the removal of the large NOTCH ectodomain by ADAM10, NOTCH1 receptor is cleaved by the γ -secretase complex at site 3 (S3) which is located at valine 1744 [112]. S3 cleavage can be prevented using γ -secretase inhibitors (GSI) which specifically target the catalytic activity of the γ -secretase complex [113]. The γ -secretase complex is responsible for the cleavage of a series of membrane proteins, such as the Amyloid Precursor Protein (APP). An aberrant cleavage results in the generation of a pathologic amyloid peptide A β (A β 42), which is the main constituent of the amyloid plaques in the brains of patients suffering from Alzheimer's disease. The polytopic GS enzyme is composed of four proteins Nicastrin (NCT), Presenilin (PSEN), Presenilin-enhancer 2 (PEN-2) and Anterior Pharynx-Defective 1 (APH1). Both PSEN and APH1 subunits present two different paralogs (PSEN1, PSEN2, APH1A, APH1B) that are encoded by different genes and are capable of forming an active complex [114]. Different subunit combination result in four possibilities of γ -secretase complexes, which may result in different affinities to their substrates and different biological functions [115]. γ -secretase subunit composition regulates the generation of different A β peptide profiles that will contribute to the accumulation of pathological A β plaques [116]. NCT and PEN-2 are the only common subunits amongst the different complex combinations. The PSEN subunit is essential for the proper assembly of the complex, being also the catalytic subunit of the complex. PSEN is an enzyme with aspartyl protease activity localized in the lipid bilayer that cleaves transmembrane domains of several proteins such as NOTCH and APP [114]. The PEN-2 subunit controls the presenilinase activity of PSEN. NCT participates in the assembly of the complex by stabilizing the PSEN and PEN-2 subunits. The APH1 works as a scaffold protein binding the PSEN with the NCT subunits. In particular, the PSEN subunit mediates the NOTCH cleavage at S3 leading to the release of the Notch IntraCellular Domain (NICD) which translocates to the nucleus. Previous findings showed that the γ -secretase complex cleavage at S3 can also occur in acidic environments such as the endosomes and lysosomes [117]. Furthermore, recent findings revealed that the PSEN2 isoform selectively cleaves endosomal localized substrates and generates an intracellular A β pool which can contribute to Alzheimer pathogenesis [118]. The location of the S3 cleavage determines the precision of cleavage and also the residue aimed by the γ -secretase complex, which in acidic conditions occurs at serine 1747 [117] [119-121]. In

the nucleus, the NICD binds to CSL and mediates the conversion from a transcriptional repression complex to its activation resulting in transcriptional activation of the target genes [122, 123].

NOTCH activation triggers the expression of several target genes involved in cell-cycle regulation like cyclin D3 [124], cellular differentiation like Hes1 [125], stem cell maintenance like Abcg2 [126] and metabolism like c-Myc [127]. The NOTCH pathway is thereby crucial in regulating numerous down-stream signalling networks that if deregulated may generate a pathologic phenotype such as cancer.

FIGURE 4: Schematic representation of Notch receptor proteolysis. The Notch receptor is located in Golgi where S1 processing occurs by Furin. This results in the generation of a heterodimeric receptor that is transported to the membrane as a mature form. When ligand is present, the receptor undergoes a conformational change activating the Adam enzyme to cleave the Notch receptor at S2, resulting in the shedding of the extracellular domain. S2

cleavage is followed by a cleavage at S3 site mediated by the γ -secretase complex releasing the intracellular domain of the receptor (NICD). S3 cleavage also occurs in acidic environments such as the one in intracellular vesicles (IV), contributing to the total amount of released NICD into the cytosol. The NICD will travel into the nucleus and activate several Notch-related genes such as Hes1 and Hey1.

The role of Notch in lung development

Notch signalling is necessary in many tissues during embryonic development but also plays a crucial role in adult tissue renewal and maintenance of several organs such as the intestine, lung, nervous system and skin [128]. Understanding the roles of Notch regulation in these organs is critical to comprehend the basic mechanisms of organ development and the possible implications of Notch in disease.

Notch plays an essential role in coordinating events during lung development including airway epithelial cell fate decision and alveogenesis. Notch promotes proximal cell fates in early lung development, impacting on the differentiation of specific cellular lineages at the different stages of human and mice development. For instance, Notch is a negative regulator of pulmonary neuroendocrine cell (PNEC) differentiation by activating Hes1 which in turn leads the differentiation of progenitors into other cell types [129]. Also, when constitutively activated Notch1-NICD is expressed in distal lung epithelium, alveolar development is completely abolished [130]. These processes are strictly regulated by the activation of the different types of Notch receptor and ligand isoforms, such as Notch1, Notch2, Notch3, Jagged1, Jagged2 and Delta1. Each of them is crucial for the induction of a specific cellular lineage during the different stages of lung development. Multiple cell types participate in giving and receiving signals that contribute in generating cellular responses related with survival, proliferation, differentiation and cell fate [131]. Notch determines the cellular fate of the cell populations covering the proximal airways including club (formerly Clara cells), goblet, ciliated and basal cells. The airway epithelium is maintained by progenitor cells from which ciliated, goblet and club cells differentiate. Pulmonary function relies on the slow renewal capacity of basal cells, which is required for ongoing maintenance of normal lung structure. In turn, lung tissue needs rapid regenerative responses upon airway cell injury e.g after radiation exposure. Abnormalities in the distribution and balance of the cell types mentioned above affect the repair of airway epithelial cells leading to (irreversible)

pathological processes such as pulmonary fibrosis, COPD and lung cancer. Notch signalling is necessary for cell progenitor self-renewal and also for differentiation into secretory mucous cells upon SO₂ mediated airway epithelial injury. The effects of Notch signalling inducing mucous cell differentiation is balanced by a reduction in the amount of ciliated cells [132, 133]. Other studies have shown that ROS is capable of activating Notch via Nrf2 signalling [134]. The link Notch-ROS signalling is important for progenitor self-renewal by regulating proliferative capacity, which is critical for maintaining a balance with the different cell types in upper airways of human and mouse lungs.

Since Notch is necessary for proper tissue maintenance and cell fate, Notch inhibition affects the ability of basal cells to differentiate into some cell types, hence inducing a pathological balance of cell populations in the lung [135]. The proximal cartilaginous respiratory tract receives radiation in the course of treatment for lung, oesophageal, breast cancer and Hodgkin lymphoma. Because Notch signalling has been shown to sensitize tumor cell to RT it would be important to know what the response of normal irradiated tissue to Notch inhibition is. To date there is not much information on how RT affects the regeneration of the upper airways and how Notch signalling is involved.

Notch in cancer

Aberrant Notch signalling is documented to participate in various types of cancers through activation or repression of the pathway. The first proof indicating that Notch plays a role in cancer was described in patients with T cell acute lymphoblastic leukemia (T-ALL) [136]. T-ALL is a very aggressive form of cancer of the white blood cells which mainly occurs during childhood. Gain of function mutations in NOTCH1 are frequently found in T-ALL (60% of T-ALL patients) [137, 138]. Mutations in the HD domain of NOTCH lead to a conformational change of the receptor, which allows an easier access to the S2 site to metalloproteases, thereby resulting in the activation and cleavage of NOTCH independently of ligand [137]. Other mutations cause the disruption of the PEST domain in NOTCH, leading to a decreased recycling and degradation of the active NICD fragment, prolonging an active NOTCH signalling [137]. Other types of tumors such as lung adenocarcinoma [139] and glioblastoma [140] also exhibit an oncogenic role of NOTCH. In contrast with the oncogenic activity of Notch seen in T-ALL, solid tumors usually show a deregulated Notch signalling, which is due to a loss of negative regulation or impaired expression of the Notch receptors and ligands.

Furthermore, the cooperation of Notch with other signalling pathways like RAS, EGFR, PI3K-AKT and JAK-STAT causes proliferative effects, malignant transformation and promotes stem-cell like properties such as self-renewal and differentiation [104].

A role for aberrant Notch signalling has been characterized for many of the oncogenic processes that take place in cancer [141]. The best-characterized oncogenic function of Notch in human cancers is its ability to turn on programs of gene expression regulating cellular energetics. In particular, Notch interacts with the pro-growth PI3K/AKT pathway in different cell types conferring oncogenic activity. Akt phosphorylates a large number of substrates regulating several oncogenic processes which include cell survival via inhibiting FOXO, activating NF- κ B and phosphorylating apoptotic regulators such as Bad [142]. Importantly, mutations in the suppressor gene PTEN confer resistance to Notch inhibition treatment in T-cell leukemia [143]. Also, Notch directly regulates *c-Myc* expression, another master regulator of pro-growth metabolism. Interaction between Notch and *c-Myc* composes a feed-forward-loop transcriptional regulatory motif regulating leukemic cell growth [127].

As explained above, Notch plays a crucial role in determining cell fate within multiple progenitor cell types. Perturbation of Notch signalling might lead to hyperplasia of a specific cell population, frequently at the expense of alternative fates [144]. Research from *Lafkles, et al.* established a model for Notch regulating transdifferentiation of secretory cells into ciliated cells upon Jagged inhibition. Additionally, due to its importance as a “stemness” factor, several studies have found that Notch has a similar role in maintaining cancer stem cell populations in different types of solid tumors such as brain [145], ovarian [146] and breast cancer [147]. Notch signalling in solid tumors is also associated with cancer metastasis [148] and therapy resistance [149, 150].

Notch as a target for cancer treatment

Notch inhibition is an interesting therapeutic strategy due to the observed deregulation in many types of haematological malignancies and impaired expression and activity in solid tumors. The NOTCH pathway can be inhibited at many levels by using different therapeutic options. Blockade of total NOTCH signalling pathway can be achieved by inhibiting the formation of NICD, which ultimately leads to the upregulation of downstream NOTCH target genes. Thus, a pharmacological approach using GSIs to prevent the S3 cleavage of NOTCH

will decrease total levels of NICD and consequently reduce the adverse effects due to NOTCH deregulation [151]. This therapeutic strategy could be used either as a single agent or in combination with targeted treatments, cytotoxic chemotherapy or radiotherapy for a subset of cancer patients [152-154]. For instance, inhibition of human-Dll4 suppresses tumor growth and synergizes with the antitumoral effect of the chemotherapeutic agent irinotecan in a mouse model [155]. Dual Notch2 and Notch3 inhibition by tarextumab, as a single agent or in combination with chemotherapeutic agents, exhibited tumor growth delay and decreased tumor-initiating cell frequency [156]. Other promising strategies are currently being tested to more efficiently inhibit Notch signalling diminishing its side-effects. Dll4-Notch signalling mediated resistance to VEGF-inhibition treatment by inducing dysfunctional blood vessels in tumors. The VEGF pathway interacts with DLL4-Notch signalling in regulating vasculature generation. The abnormal blood vessels generated by DLL4 signalling no longer depend on VEGF signalling leading to resistance against anti-VEGF treatment [157]. As a consequence, the combination of specific DLL4-Notch blockade with radiotherapy improves the anti-tumoral effects of single therapies by promoting non-functional tumor angiogenesis and extensive tumor necrosis [158]. Furthermore, monoclonal antibodies against DLL4 [159] and NCT [160] can be used as an approach to target Notch signalling, showing at the same time anti-tumoral effects. Other natural agents, such as 3, 3'-diindolylmethane (DIM), and molecular inhibitors have been reported to be alternative strategies for cancer treatment via repressing the Notch pathway [161, 162]. Targeting the Notch pathway could be a useful strategy to enhance the efficacy of current treatment options. For example, Notch inhibition combined with radiation therapy and the chemotherapeutic agent Temozolomide results in anti-glioma stem cell effect which provides a survival benefit in orthotopic models of glioblastoma [163]. Drug resistance is also a major cause for therapeutic failure in many types of cancers, leading to recurrence, cancer progression and ultimately mortality. Monotherapy anti-Notch treatment like for most treatments is not sufficient to induce tumor control or cure. Combination therapies that have potent interactions with systemic treatments are more likely to yield sustained anti-tumoral responses.

Although GSIs are efficient in blocking the NOTCH signalling pathway, animal studies have demonstrated severe toxicity associated with inhibition of NOTCH signalling in the intestinal epithelium [164]. Besides, prolonged Notch inhibitions can also lead to vascular abnormalities and endothelium cancer [165]. A potential way to reverse intestinal toxicity is to combine anti-NOTCH therapy with glucocorticoid treatment, thereby inhibiting the

development of goblet cell metaplasia [166]. Another way to avoid GSI induced toxicity is by targeting specific NOTCH receptors, thus reducing total NOTCH blocking [167]. Notch inhibition therapy in combination with other therapeutic agents in selected patients might result in a potentially improved cancer treatment by balancing anti-tumoral effect with dose-limiting normal tissue complication.

Thesis outline

The aim of this thesis was to investigate the role of Notch and HIF proteins as targets for anti-cancer treatment and the possible implementation in a personalized treatment approach. HIF expression is closely associated with the hypoxic areas of a tumor and is important for cell survival at low nutrient and oxygen conditions. Together with the hypoxic-specific expression, HIF might be a promising target for targeting CSC within the tumor. HIF inhibition can modulate treatment efficiency through interacting with surviving and DDR pathways, thereby it is very interesting to study the biological effects of inhibiting HIF in combination with radiotherapy. In the second part of the thesis, new insights were obtained on how Notch receptor is regulated by the γ -secretase complex and how its inhibition affects normal tissue homeostasis. In **Chapter 1**, a general introduction is given on the general roles of hypoxia and notch signalling in cancer, the mechanisms by which cells achieve resistance to therapy as well as their potential role as therapeutic targets. In **Chapter 2** the prognostic significance of HIF-2 α expression is first evaluated, since conflicting data exists in literature about its prognostic value. Therefore, a meta-analysis has been performed, which included selected literature based on specific criteria, to make a general conclusion about the prognostic value of HIF-2 α expression in cancer cells. In **Chapter 3**, we studied the roles of HIF-1 α and HIF-2 α in radiation sensitivity and metabolic dependence in non-small cell line cancer (NSCLC) cells. Therefore, we generated a genetically-engineered H1299 cell models deficient on HIF-1 α , HIF-2 α or both proteins to obtain information on the ability of HIF proteins in regulating different aspects of cancer disease and resistance to therapy. We observed that HIF-1 α and HIF-2 α show overlapping and unique roles as regulators of several aspects of tumor metabolism, such as OXPHOS, glycolysis or lipid metabolism. Besides, single inhibition of HIF-1 α expression increases clonogenic survival and reduces double-strand break formation in cells irradiated under hypoxic conditions. Double- HIF-1 α and HIF-2 α inhibition confers a radiosensitive phenotype in H1299 cells.

γ -secretase dependent S3 cleavage activates Notch signalling at the cellular membrane. This leads to the release of a transcriptional active-intracellular domain which travels to the nucleus and mediates the transcription of Notch target genes. The γ -secretase complex consists of four individual subunits that present different isoforms and affects the execution of the proteolytic cleavage. In **chapter 4** we focused on the ability of different isoform combination of the γ -secretase complex in cleaving wild type and mutant Notch receptors. Here, we observed that the different subunit composition of the γ -secretase complex generates characteristic Notch cleavage profiles, which may aid in the development of Notch specific γ -secretase inhibitors. To note, we showed that PSEN2:APH1B γ -secretase complex combination cleaves oncogenic mutant Notch1 receptors more efficiently than wildtype Notch receptors. This finding might lead to the development of specific inhibitors targeting pathological Notch, leaving physiological notch signal intact. Notch signalling is necessary in lung development but also plays a crucial role during tissue maintenance and repair e.g. after radiation exposure. Therefore, in **Chapter 5** we investigated the effects of inhibiting Notch in irradiated primary bronchial lung epithelial cells derived from patients. Finally, **Chapter 6** summarizes the previous findings from the earlier chapters and outlines the remaining challenges to be addressed in future research.

References

1. Ferlay, J., et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012*. Int J Cancer, 2015. **136**(5): p. E359-86.
2. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation*. Cell, 2011. **144**(5): p. 646-74.
3. Chaffer, C.L. and R.A. Weinberg, *A perspective on cancer cell metastasis*. Science, 2011. **331**(6024): p. 1559-64.
4. Jacobson, L.O., C.L. Spurr, and et al., *Nitrogen mustard therapy; studies on the effect of methyl-bis (beta-chloroethyl) amine hydrochloride on neoplastic diseases and allied disorders of the hemopoietic system*. J Am Med Assoc, 1946. **132**: p. 263-71.
5. Davies, J. and D. Davies, *Origins and evolution of antibiotic resistance*. Microbiol Mol Biol Rev, 2010. **74**(3): p. 417-33.
6. Housman, G., et al., *Drug resistance in cancer: an overview*. Cancers (Basel), 2014. **6**(3): p. 1769-92.
7. Chan, K.S., C.G. Koh, and H.Y. Li, *Mitosis-targeted anti-cancer therapies: where they stand*. Cell Death Dis, 2012. **3**: p. e411.
8. Baskar, R., et al., *Cancer and radiation therapy: current advances and future directions*. Int J Med Sci, 2012. **9**(3): p. 193-9.
9. Liu, F.S., *Mechanisms of chemotherapeutic drug resistance in cancer therapy--a quick review*. Taiwan J Obstet Gynecol, 2009. **48**(3): p. 239-44.
10. Bonnet, D. and J.E. Dick, *Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell*. Nat Med, 1997. **3**(7): p. 730-7.
11. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells*. Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3983-8.
12. Singh, S.K., et al., *Identification of human brain tumour initiating cells*. Nature, 2004. **432**(7015): p. 396-401.
13. Aponte, P.M. and A. Caicedo, *Stemness in Cancer: Stem Cells, Cancer Stem Cells, and Their Microenvironment*. Stem Cells Int, 2017. **2017**: p. 5619472.
14. Takebe, N., et al., *Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update*. Nat Rev Clin Oncol, 2015. **12**(8): p. 445-64.
15. Kise, K., Y. Kinugasa-Katayama, and N. Takakura, *Tumor microenvironment for cancer stem cells*. Adv Drug Deliv Rev, 2016. **99**(Pt B): p. 197-205.

16. Muz, B., et al., *The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy*. Hypoxia (Auckl), 2015. **3**: p. 83-92.
17. Li, Z. and J.N. Rich, *Hypoxia and hypoxia inducible factors in cancer stem cell maintenance*. Curr Top Microbiol Immunol, 2010. **345**: p. 21-30.
18. Yun, Z. and Q. Lin, *Hypoxia and regulation of cancer cell stemness*. Adv Exp Med Biol, 2014. **772**: p. 41-53.
19. Ye, J., et al., *The cancer stem cell niche: cross talk between cancer stem cells and their microenvironment*. Tumour Biol, 2014. **35**(5): p. 3945-51.
20. Hockel, M. and P. Vaupel, *Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects*. J Natl Cancer Inst, 2001. **93**(4): p. 266-76.
21. Nordsmark, M., et al., *Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study*. Radiother Oncol, 2005. **77**(1): p. 18-24.
22. Wojtkowiak, J.W., et al., *Drug resistance and cellular adaptation to tumor acidic pH microenvironment*. Mol Pharm, 2011. **8**(6): p. 2032-8.
23. Good, J.S. and K.J. Harrington, *The hallmarks of cancer and the radiation oncologist: updating the 5Rs of radiobiology*. Clin Oncol (R Coll Radiol), 2013. **25**(10): p. 569-77.
24. Rice, G.C., C. Hoy, and R.T. Schimke, *Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells*. Proc Natl Acad Sci U S A, 1986. **83**(16): p. 5978-82.
25. Comerford, K.M., et al., *Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene*. Cancer Res, 2002. **62**(12): p. 3387-94.
26. Tannock, I.F. and D. Rotin, *Acid pH in tumors and its potential for therapeutic exploitation*. Cancer Res, 1989. **49**(16): p. 4373-84.
27. Cowan, D.S. and I.F. Tannock, *Factors that influence the penetration of methotrexate through solid tissue*. Int J Cancer, 2001. **91**(1): p. 120-5.
28. Tredan, O., et al., *Drug resistance and the solid tumor microenvironment*. J Natl Cancer Inst, 2007. **99**(19): p. 1441-54.
29. Carmeliet, P., et al., *Role of HIF-1 α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis*. Nature, 1998. **394**(6692): p. 485-90.
30. Ziello, J.E., I.S. Jovin, and Y. Huang, *Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia*. Yale J Biol Med, 2007. **80**(2): p. 51-60.

31. Smith, T.G., P.A. Robbins, and P.J. Ratcliffe, *The human side of hypoxia-inducible factor*. Br J Haematol, 2008. **141**(3): p. 325-34.
32. Zhulin, I.B., B.L. Taylor, and R. Dixon, *PAS domain S-boxes in Archaea, Bacteria and sensors for oxygen and redox*. Trends Biochem Sci, 1997. **22**(9): p. 331-3.
33. Ponting, C.P. and L. Aravind, *PAS: a multifunctional domain family comes to light*. Curr Biol, 1997. **7**(11): p. R674-7.
34. Li, H., H.P. Ko, and J.P. Whitlock, *Induction of phosphoglycerate kinase 1 gene expression by hypoxia. Roles of Arnt and HIF1alpha*. J Biol Chem, 1996. **271**(35): p. 21262-7.
35. Wang, G.L., et al., *Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension*. Proc Natl Acad Sci U S A, 1995. **92**(12): p. 5510-4.
36. Jiang, B.H., et al., *Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1*. J Biol Chem, 1996. **271**(30): p. 17771-8.
37. Bruick, R.K. and S.L. McKnight, *A conserved family of prolyl-4-hydroxylases that modify HIF*. Science, 2001. **294**(5545): p. 1337-40.
38. Kallio, P.J., et al., *Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor*. Proc Natl Acad Sci U S A, 1997. **94**(11): p. 5667-72.
39. Iwai, K., et al., *Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex*. Proc Natl Acad Sci U S A, 1999. **96**(22): p. 12436-41.
40. Jeong, J.W., et al., *Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation*. Cell, 2002. **111**(5): p. 709-20.
41. Maxwell, P.H., et al., *The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis*. Nature, 1999. **399**(6733): p. 271-5.
42. Wenger, R.H., D.P. Stiehl, and G. Camenisch, *Integration of oxygen signaling at the consensus HRE*. Sci STKE, 2005. **2005**(306): p. re12.
43. Liu, W., et al., *Targeted genes and interacting proteins of hypoxia inducible factor-1*. Int J Biochem Mol Biol, 2012. **3**(2): p. 165-78.
44. Hayashi, M., et al., *Induction of glucose transporter 1 expression through hypoxia-inducible factor 1alpha under hypoxic conditions in trophoblast-derived cells*. J Endocrinol, 2004. **183**(1): p. 145-54.

45. Ramakrishnan, S., V. Anand, and S. Roy, *Vascular endothelial growth factor signaling in hypoxia and inflammation*. J Neuroimmune Pharmacol, 2014. **9**(2): p. 142-60.
46. Jamali, S., et al., *Hypoxia-induced carbonic anhydrase IX facilitates lactate flux in human breast cancer cells by non-catalytic function*. Sci Rep, 2015. **5**: p. 13605.
47. Tennant, D.A., et al., *Metabolic transformation in cancer*. Carcinogenesis, 2009. **30**(8): p. 1269-80.
48. Eales, K.L., K.E. Hollinshead, and D.A. Tennant, *Hypoxia and metabolic adaptation of cancer cells*. Oncogenesis, 2016. **5**: p. e190.
49. Damaghi, M., J.W. Wojtkowiak, and R.J. Gillies, *pH sensing and regulation in cancer*. Front Physiol, 2013. **4**: p. 370.
50. Gatenby, R.A. and E.T. Gawlinski, *Mathematical models of tumour invasion mediated by transformation-induced alteration of microenvironmental pH*. Novartis Found Symp, 2001. **240**: p. 85-96; discussion 96-9.
51. Ullah, M.S., A.J. Davies, and A.P. Halestrap, *The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism*. J Biol Chem, 2006. **281**(14): p. 9030-7.
52. Sedlakova, O., et al., *Carbonic anhydrase IX, a hypoxia-induced catalytic component of the pH regulating machinery in tumors*. Front Physiol, 2014. **4**: p. 400.
53. Swietach, P., et al., *Tumor-associated carbonic anhydrase 9 spatially coordinates intracellular pH in three-dimensional multicellular growths*. J Biol Chem, 2008. **283**(29): p. 20473-83.
54. Swietach, P., et al., *The role of carbonic anhydrase 9 in regulating extracellular and intracellular pH in three-dimensional tumor cell growths*. J Biol Chem, 2009. **284**(30): p. 20299-310.
55. Pescador, N., et al., *Hypoxia promotes glycogen accumulation through hypoxia inducible factor (HIF)-mediated induction of glycogen synthase 1*. PLoS One, 2010. **5**(3): p. e9644.
56. Goda, N. and M. Kanai, *Hypoxia-inducible factors and their roles in energy metabolism*. Int J Hematol, 2012. **95**(5): p. 457-63.
57. Zhang, H., et al., *HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity*. Cancer Cell, 2007. **11**(5): p. 407-20.

58. Zhang, H., et al., *Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia*. J Biol Chem, 2008. **283**(16): p. 10892-903.
59. Huang, et al., *HIF-1-mediated suppression of acyl-CoA dehydrogenases and fatty acid oxidation is critical for cancer progression*. Cell Rep, 2014. **8**(6): p. 1930-1942.
60. Valli, A., et al., *Hypoxia induces a lipogenic cancer cell phenotype via HIF1alpha-dependent and -independent pathways*. Oncotarget, 2015. **6**(4): p. 1920-41.
61. Huang, D., C. Li, and H. Zhang, *Hypoxia and cancer cell metabolism*. Acta Biochim Biophys Sin (Shanghai), 2014. **46**(3): p. 214-9.
62. Koizume, S. and Y. Miyagi, *Lipid Droplets: A Key Cellular Organelle Associated with Cancer Cell Survival under Normoxia and Hypoxia*. Int J Mol Sci, 2016. **17**(9).
63. Huss, J.M., F.H. Levy, and D.P. Kelly, *Hypoxia inhibits the peroxisome proliferator-activated receptor alpha/retinoid X receptor gene regulatory pathway in cardiac myocytes: a mechanism for O₂-dependent modulation of mitochondrial fatty acid oxidation*. J Biol Chem, 2001. **276**(29): p. 27605-12.
64. Swierczynski, J., A. Hebanowska, and T. Sledzinski, *Role of abnormal lipid metabolism in development, progression, diagnosis and therapy of pancreatic cancer*. World J Gastroenterol, 2014. **20**(9): p. 2279-303.
65. Accioly, M.T., et al., *Lipid bodies are reservoirs of cyclooxygenase-2 and sites of prostaglandin-E₂ synthesis in colon cancer cells*. Cancer Res, 2008. **68**(6): p. 1732-40.
66. Shen, G.M., et al., *Hypoxia-inducible factor-1 (HIF-1) promotes LDL and VLDL uptake through inducing VLDLR under hypoxia*. Biochem J, 2012. **441**(2): p. 675-83.
67. Hu, C.J., et al., *Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation*. Mol Cell Biol, 2003. **23**(24): p. 9361-74.
68. Thomlinson, R.H. and L.H. Gray, *The histological structure of some human lung cancers and the possible implications for radiotherapy*. Br J Cancer, 1955. **9**(4): p. 539-49.
69. Gray, L.H., et al., *The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy*. Br J Radiol, 1953. **26**(312): p. 638-48.
70. Aebbersold, D.M., et al., *Expression of hypoxia-inducible factor-1alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer*. Cancer Res, 2001. **61**(7): p. 2911-6.
71. Moon, E.J., et al., *The potential role of intrinsic hypoxia markers as prognostic variables in cancer*. Antioxid Redox Signal, 2007. **9**(8): p. 1237-94.

72. Quintiliani, M., *The oxygen effect in radiation inactivation of DNA and enzymes*. Int J Radiat Biol Relat Stud Phys Chem Med, 1986. **50**(4): p. 573-94.
73. Grimes, D.R. and M. Partridge, *A mechanistic investigation of the oxygen fixation hypothesis and oxygen enhancement ratio*. Biomed Phys Eng Express, 2015. **1**(4): p. 045209.
74. Grotius, J., et al., *Impact of exogenous lactate on survival and radioresponse of carcinoma cells in vitro*. Int J Radiat Biol, 2009. **85**(11): p. 989-1001.
75. Huang, C.Y., et al., *Resistance to hypoxia-induced necroptosis is conferred by glycolytic pyruvate scavenging of mitochondrial superoxide in colorectal cancer cells*. Cell Death Dis, 2013. **4**: p. e622.
76. Bristow, R.G. and R.P. Hill, *Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability*. Nat Rev Cancer, 2008. **8**(3): p. 180-92.
77. Okunieff, P., et al., *Antioxidants reduce consequences of radiation exposure*. Adv Exp Med Biol, 2008. **614**: p. 165-78.
78. Groussard, C., et al., *Free radical scavenging and antioxidant effects of lactate ion: an in vitro study*. J Appl Physiol (1985), 2000. **89**(1): p. 169-75.
79. Hirschhaeuser, F., U.G. Sattler, and W. Mueller-Klieser, *Lactate: a metabolic key player in cancer*. Cancer Res, 2011. **71**(22): p. 6921-5.
80. Meijer, T.W., et al., *Targeting hypoxia, HIF-1, and tumor glucose metabolism to improve radiotherapy efficacy*. Clin Cancer Res, 2012. **18**(20): p. 5585-94.
81. Li, J.L., et al., *Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo*. Cancer Res, 2007. **67**(23): p. 11244-53.
82. Kim, W.Y., et al., *Targeting heat shock protein 90 overrides the resistance of lung cancer cells by blocking radiation-induced stabilization of hypoxia-inducible factor-1alpha*. Cancer Res, 2009. **69**(4): p. 1624-32.
83. Cam, H., et al., *mTORC1 signaling under hypoxic conditions is controlled by ATM-dependent phosphorylation of HIF-1alpha*. Mol Cell, 2010. **40**(4): p. 509-20.
84. Fallone, F., et al., *ATR controls cellular adaptation to hypoxia through positive regulation of hypoxia-inducible factor 1 (HIF-1) expression*. Oncogene, 2013. **32**(37): p. 4387-96.
85. Jung, S.N., et al., *Reactive oxygen species stabilize hypoxia-inducible factor-1 alpha protein and stimulate transcriptional activity via AMP-activated protein kinase in DU145 human prostate cancer cells*. Carcinogenesis, 2008. **29**(4): p. 713-21.

86. Yu, T., B. Tang, and X. Sun, *Development of Inhibitors Targeting Hypoxia-Inducible Factor 1 and 2 for Cancer Therapy*. Yonsei Med J, 2017. **58**(3): p. 489-496.
87. Greenberger, L.M., et al., *A RNA antagonist of hypoxia-inducible factor-1alpha, EZN-2968, inhibits tumor cell growth*. Mol Cancer Ther, 2008. **7**(11): p. 3598-608.
88. Rapisarda, A., et al., *Topoisomerase I-mediated inhibition of hypoxia-inducible factor 1: mechanism and therapeutic implications*. Cancer Res, 2004. **64**(4): p. 1475-82.
89. Creighton-Gutteridge, M., et al., *Cell type-specific, topoisomerase II-dependent inhibition of hypoxia-inducible factor-1alpha protein accumulation by NSC 644221*. Clin Cancer Res, 2007. **13**(3): p. 1010-8.
90. Laughner, E., et al., *HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression*. Mol Cell Biol, 2001. **21**(12): p. 3995-4004.
91. Newcomb, E.W., et al., *Flavopiridol downregulates hypoxia-mediated hypoxia-inducible factor-1alpha expression in human glioma cells by a proteasome-independent pathway: implications for in vivo therapy*. Neuro Oncol, 2005. **7**(3): p. 225-35.
92. Mabeesh, N.J., et al., *2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF*. Cancer Cell, 2003. **3**(4): p. 363-75.
93. Isaacs, J.S., et al., *Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway*. J Biol Chem, 2002. **277**(33): p. 29936-44.
94. Zhao, D., et al., *Upregulation of HIF-2alpha induced by sorafenib contributes to the resistance by activating the TGF-alpha/EGFR pathway in hepatocellular carcinoma cells*. Cell Signal, 2014. **26**(5): p. 1030-9.
95. Koh, M.Y., et al., *The hypoxia-associated factor switches cells from HIF-1alpha- to HIF-2alpha-dependent signaling promoting stem cell characteristics, aggressive tumor growth and invasion*. Cancer Res, 2011. **71**(11): p. 4015-27.
96. Keith, B. and M.C. Simon, *Hypoxia-inducible factors, stem cells, and cancer*. Cell, 2007. **129**(3): p. 465-72.
97. Liu, J., et al., *Notch signaling in the regulation of stem cell self-renewal and differentiation*. Curr Top Dev Biol, 2010. **92**: p. 367-409.
98. Diaz, B., et al., *Notch increases the shedding of HB-EGF by ADAM12 to potentiate invadopodia formation in hypoxia*. J Cell Biol, 2013. **201**(2): p. 279-92.

99. Diez, H., et al., *Hypoxia-mediated activation of Dll4-Notch-Hey2 signaling in endothelial progenitor cells and adoption of arterial cell fate*. Exp Cell Res, 2007. **313**(1): p. 1-9.
100. Gustafsson, M.V., et al., *Hypoxia requires notch signaling to maintain the undifferentiated cell state*. Dev Cell, 2005. **9**(5): p. 617-28.
101. Artavanis-Tsakonas, S., M.D. Rand, and R.J. Lake, *Notch signaling: cell fate control and signal integration in development*. Science, 1999. **284**(5415): p. 770-6.
102. Kopan, R. and M.X. Ilagan, *The canonical Notch signaling pathway: unfolding the activation mechanism*. Cell, 2009. **137**(2): p. 216-33.
103. Gridley, T., *Notch signaling and inherited disease syndromes*. Hum Mol Genet, 2003. **12 Spec No 1**: p. R9-13.
104. Ranganathan, P., K.L. Weaver, and A.J. Capobianco, *Notch signalling in solid tumours: a little bit of everything but not all the time*. Nat Rev Cancer, 2011. **11**(5): p. 338-51.
105. van Tetering, G. and M. Vooijs, *Proteolytic cleavage of Notch: "HIT and RUN"*. Curr Mol Med, 2011. **11**(4): p. 255-69.
106. Groot, A.J. and M.A. Vooijs, *The role of Adams in Notch signaling*. Adv Exp Med Biol, 2012. **727**: p. 15-36.
107. Kopan, R., et al., *Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain*. Proc Natl Acad Sci U S A, 1996. **93**(4): p. 1683-8.
108. Logeat, F., et al., *The Notch1 receptor is cleaved constitutively by a furin-like convertase*. Proc Natl Acad Sci U S A, 1998. **95**(14): p. 8108-12.
109. Blaumueller, C.M., et al., *Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane*. Cell, 1997. **90**(2): p. 281-91.
110. Gordon, W.R., et al., *Structural basis for autoinhibition of Notch*. Nat Struct Mol Biol, 2007. **14**(4): p. 295-300.
111. Nichols, J.T., et al., *DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur*. J Cell Biol, 2007. **176**(4): p. 445-58.
112. De Stropper, B., et al., *A Presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain*. Nature, 1999. **398**(6727):518-22.
113. Curry, C.L., et al., *Gamma secretase inhibitor blocks Notch activation and induces apoptosis in Kaposi's sarcoma tumor cells*. Oncogene, 2005. **24**(42): p. 6333-44.

114. De Strooper, B., *Aph-1, Pen-2, and Nicastrin with Presenilin generate an active gamma-Secretase complex*. Neuron, 2003. **38**(1): p. 9-12.
115. Serneels, L., et al., *Differential contribution of the three Aph1 genes to gamma-secretase activity in vivo*. Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1719-24.
116. Acx, H., et al., *Signature amyloid beta profiles are produced by different gamma-secretase complexes*. J Biol Chem, 2014. **289**(7): p. 4346-55.
117. Pasternak, S.H., et al., *Presenilin-1, nicastrin, amyloid precursor protein, and gamma-secretase activity are co-localized in the lysosomal membrane*. J Biol Chem, 2003. **278**(29): p. 26687-94.
118. Sannerud, R., et al., *Restricted Location of PSEN2/gamma-Secretase Determines Substrate Specificity and Generates an Intracellular Abeta Pool*. Cell, 2016. **166**(1): p. 193-208.
119. Kaether, C., C. Haass, and H. Steiner, *Assembly, trafficking and function of gamma-secretase*. Neurodegener Dis, 2006. **3**(4-5): p. 275-83.
120. Fukumori, A., et al., *Presenilin-dependent gamma-secretase on plasma membrane and endosomes is functionally distinct*. Biochemistry, 2006. **45**(15): p. 4907-14.
121. Tagami, S., et al., *Regulation of Notch signaling by dynamic changes in the precision of S3 cleavage of Notch-1*. Mol Cell Biol, 2008. **28**(1): p. 165-76.
122. Kovall, R.A., et al., *The Canonical Notch Signaling Pathway: Structural and Biochemical Insights into Shape, Sugar, and Force*. Dev Cell, 2017. **41**(3): p. 228-241.
123. Petcherski, A.G. and J. Kimble, *Mastermind is a putative activator for Notch*. Curr Biol, 2000. **10**(13): p. R471-3.
124. Lewis, H.D., et al., *Apoptosis in T cell acute lymphoblastic leukemia cells after cell cycle arrest induced by pharmacological inhibition of notch signaling*. Chem Biol, 2007. **14**(2): p. 209-19.
125. Ross, D.A., P.K. Rao, and T. Kadesch, *Dual roles for the Notch target gene Hes-1 in the differentiation of 3T3-L1 preadipocytes*. Mol Cell Biol, 2004. **24**(8): p. 3505-13.
126. Bhattacharya, S., et al., *Maintenance of retinal stem cells by Abcg2 is regulated by notch signaling*. J Cell Sci, 2007. **120**(Pt 15): p. 2652-62.
127. Palomero, T., et al., *NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth*. Proc Natl Acad Sci U S A, 2006. **103**(48): p. 18261-6.

128. Sato, C., G. Zhao, and M.X. Ilagan, *An overview of notch signaling in adult tissue renewal and maintenance*. Curr Alzheimer Res, 2012. **9**(2): p. 227-40.
129. Ito, T., et al., *Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium*. Development, 2000. **127**(18): p. 3913-21.
130. Guseh, J.S., et al., *Notch signaling promotes airway mucous metaplasia and inhibits alveolar development*. Development, 2009. **136**(10): p. 1751-9.
131. Xu, K., N. Moghal, and S.E. Egan, *Notch signaling in lung development and disease*. Adv Exp Med Biol, 2012. **727**: p. 89-98.
132. Rock, J.R., et al., *Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition*. Proc Natl Acad Sci U S A, 2011. **108**(52): p. E1475-83.
133. Xing, Y., et al., *NOTCH1 is required for regeneration of Clara cells during repair of airway injury*. Stem Cells, 2012. **30**(5): p. 946-55.
134. Paul, M.K., et al., *Dynamic changes in intracellular ROS levels regulate airway basal stem cell homeostasis through Nrf2-dependent Notch signaling*. Cell Stem Cell, 2014. **15**(2): p. 199-214.
135. Whitsett, J.A. and V.V. Kalinichenko, *Notch and basal cells take center stage during airway epithelial regeneration*. Cell Stem Cell, 2011. **8**(6): p. 597-8.
136. Ellisen, L.W., et al., *TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms*. Cell, 1991. **66**(4): p. 649-61.
137. Weng, A.P., et al., *Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia*. Science, 2004. **306**(5694): p. 269-71.
138. Mansour, M.R., et al., *High incidence of Notch-1 mutations in adult patients with T-cell acute lymphoblastic leukemia*. Leukemia, 2006. **20**(3): p. 537-9.
139. Licciulli, S., et al., *Notch1 is required for Kras-induced lung adenocarcinoma and controls tumor cell survival via p53*. Cancer Res, 2013. **73**(19): p. 5974-84.
140. Chu, Q., et al., *Prolonged inhibition of glioblastoma xenograft initiation and clonogenic growth following in vivo Notch blockade*. Clin Cancer Res, 2013. **19**(12): p. 3224-33.
141. Aster, J.C., W.S. Pear, and S.C. Blacklow, *The Varied Roles of Notch in Cancer*. Annu Rev Pathol, 2017. **12**: p. 245-275.

142. Manning, B.D. and L.C. Cantley, *AKT/PKB signaling: navigating downstream*. Cell, 2007. **129**(7): p. 1261-74.
143. Palomero, T., et al., *Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia*. Nat Med, 2007. **13**(10): p. 1203-10.
144. Lafkas, D., et al., *Therapeutic antibodies reveal Notch control of transdifferentiation in the adult lung*. Nature, 2015. **528**(7580): p. 127-31.
145. Fan, X., et al., *NOTCH pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts*. Stem Cells, 2010. **28**(1): p. 5-16.
146. Jiang, L.Y., et al., *gamma-Secretase Inhibitor, DAPT Inhibits Self-renewal and Stemness Maintenance of Ovarian Cancer Stem-like Cells In Vitro*. Chin J Cancer Res, 2011. **23**(2): p. 140-6.
147. Dontu, G., et al., *Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells*. Breast Cancer Res, 2004. **6**(6): p. R605-15.
148. Sahlgren, C., et al., *Notch signaling mediates hypoxia-induced tumor cell migration and invasion*. Proc Natl Acad Sci U S A, 2008. **105**(17): p. 6392-7.
149. Santagata, S., et al., *JAGGED1 expression is associated with prostate cancer metastasis and recurrence*. Cancer Res, 2004. **64**(19): p. 6854-7.
150. Miyamoto, Y., et al., *Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis*. Cancer Cell, 2003. **3**(6): p. 565-76.
151. Olsauskas-Kuprys, R., A. Zlobin, and C. Osipo, *Gamma secretase inhibitors of Notch signaling*. Onco Targets Ther, 2013. **6**: p. 943-55.
152. Meng, R.D., et al., *gamma-Secretase inhibitors abrogate oxaliplatin-induced activation of the Notch-1 signaling pathway in colon cancer cells resulting in enhanced chemosensitivity*. Cancer Res, 2009. **69**(2): p. 573-82.
153. Akiyoshi, T., et al., *Gamma-secretase inhibitors enhance taxane-induced mitotic arrest and apoptosis in colon cancer cells*. Gastroenterology, 2008. **134**(1): p. 131-44.
154. Osipo, C., et al., *ErbB-2 inhibition activates Notch-1 and sensitizes breast cancer cells to a gamma-secretase inhibitor*. Oncogene, 2008. **27**(37): p. 5019-32.
155. Hoey, T., et al., *DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency*. Cell Stem Cell, 2009. **5**(2): p. 168-77.
156. Yen, W.C., et al., *Targeting Notch signaling with a Notch2/Notch3 antagonist (tarextumab) inhibits tumor growth and decreases tumor-initiating cell frequency*. Clin Cancer Res, 2015. **21**(9): p. 2084-95.

157. Li, J.L., et al., *DLL4-Notch signaling mediates tumor resistance to anti-VEGF therapy in vivo*. Cancer Res, 2011. **71**(18): p. 6073-83.
158. Liu, S.K., et al., *Delta-like ligand 4-notch blockade and tumor radiation response*. J Natl Cancer Inst, 2011. **103**(23): p. 1778-98.
159. Jenkins, D.W., et al., *MEDI0639: a novel therapeutic antibody targeting Dll4 modulates endothelial cell function and angiogenesis in vivo*. Mol Cancer Ther, 2012. **11**(8): p. 1650-60.
160. Filipovic, A., et al., *Anti-nicastrin monoclonal antibodies elicit pleiotropic anti-tumour pharmacological effects in invasive breast cancer cells*. Breast Cancer Res Treat, 2014. **148**(2): p. 455-62.
161. Ahmad, A., W.A. Sakr, and K.M. Rahman, *Novel targets for detection of cancer and their modulation by chemopreventive natural compounds*. Front Biosci (Elite Ed), 2012. **4**: p. 410-25.
162. Dong, X., et al., *Aspartate beta-Hydroxylase expression promotes a malignant pancreatic cellular phenotype*. Oncotarget, 2015. **6**(2): p. 1231-48.
163. Yahyanejad, S., et al., *NOTCH blockade combined with radiation therapy and temozolomide prolongs survival of orthotopic glioblastoma*. Oncotarget, 2016. **7**(27): p. 41251-41264.
164. Milano, J., et al., *Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation*. Toxicol Sci, 2004. **82**(1): p. 341-58.
165. Yan, M., et al., *Chronic DLL4 blockade induces vascular neoplasms*. Nature, 2010. **463**(7282): p. E6-7.
166. Samon, J.B., et al., *Preclinical analysis of the gamma-secretase inhibitor PF-03084014 in combination with glucocorticoids in T-cell acute lymphoblastic leukemia*. Mol Cancer Ther, 2012. **11**(7): p. 1565-75.
167. Wu, Y., et al., *Therapeutic antibody targeting of individual Notch receptors*. Nature, 2010. **464**(7291): p. 1052-7.

CHAPTER 2

Prognostic Role of Hypoxia-Inducible Factor-2 Alpha Tumor Cell Expression in Cancer Patients: a Meta-analysis

Eloy Moreno Roig¹, Ala Yaromina¹, Ruud Houben², Arjan J. Groot¹, Ludwig Dubois¹, and
Marc Vooijs^{1*}

¹Department of Radiotherapy (MAASTRO)/GROW – School for Developmental Biology & Oncology, Maastricht University, Maastricht, Netherlands

²Department of Radiation Oncology, MAASTRO Clinic, Maastricht, Netherlands

* Corresponding author: Marc Vooijs, Department of Radiotherapy (MAASTRO)/GROW – School for Developmental Biology & Oncology, Maastricht University, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands; E-mail: marc.vooijs@maastrichtuniversity.nl

Keywords: cancer, hypoxia-inducible factor-2, meta-analysis, prognosis, EPAS1

Running title: HIF2 expression in cancer.

Published in: Frontiers Oncology 2018 Jun 11;8:224

Abstract

Hypoxia-inducible factor-2 α (HIF-2 α) plays an important role in tumor progression and metastasis. A number of studies have evaluated the correlation between hypoxia-inducible factor-2 α (HIF-2 α) overexpression and clinical outcome in cancer patients but yielded inconsistent results. To comprehensively and quantitatively summarize the evidence on the capability of HIF-2 α to predict the prognosis of cancer patients with solid tumors, a meta-analysis was carried out. Renal cell carcinoma (CC-RCC) was separately analyzed due to an alternative mechanism of regulation. Systematic literature searches were performed in PubMed and Embase databases for relevant original articles until Feb 2018. Forty nine studies with 6,052 patients were included in the present study. The pooled hazard ratios (HRs) with corresponding confidence intervals (CIs) were calculated to assess the prognostic value of HIF-2 α protein expression in tumor cells. The meta-analysis revealed strong significant negative associations between HIF-2 α expression and five endpoints: overall survival (HR = 1.69, 95% confidence interval (95%CI) 1.39–2.06), disease-free survival (HR = 1.87, 95%CI 1.2–2.92), disease-specific survival (HR = 1.57, 95%CI 1.06–2.34), metastasis-free survival (HR = 2.67, 95%CI 1.32–5.38) and progression-free survival (HR = 2.18, 95%CI 1.25–3.78). Subgroup analyses revealed similar associations in the majority of tumor sites. Overall, this data demonstrate a negative prognostic role of HIF-2 α in patients suffering from different types of solid tumors.

Introduction

Hypoxia is a common feature of most of solid tumors resulting from an imbalance between oxygen supply and consumption by tumor cells. Hypoxic tumor areas are characterized by a disrupted vasculature causing inefficient oxygen and nutrient supply to neighboring cells [1]. Hypoxia is one of the key factors in inducing the development of resistant cells with an aggressive phenotype [2], which leads to poor prognosis in patients and decreases the efficacy of chemoradiotherapy [3, 4]. Accurate measurement of tumor hypoxia in patients together with the design of novel anti-hypoxia treatments has largely been a major goal in cancer research [5-8].

Hypoxia triggers important cellular stress responses allowing tumor cells to survive under extreme conditions, including the stabilization of the hypoxia inducible factor (HIF) proteins [9, 10]. Under normoxic conditions, prolyl-hydroxylation promotes HIF- α degradation via the von Hippel-Lindau (VHL) ubiquitin/proteasome pathway. Under hypoxia, this regulation is suppressed, leading to the stabilization of three independent HIF- α subunits (HIF-1 α , HIF-2 α and HIF-3 α) that dimerize with the constitutively expressed HIF-1 β and activate the transcription of genes via HREs (Hypoxia Responsive Elements) in their promoter region. The HIF-2 α protein, also named endothelial PAS domain protein-1 (EPAS1), is equally oxygen-regulated as its counterpart HIF-1 α , and both present over 50% similarity in their amino acid sequence identity [11]. In physiologic conditions, HIF-1 α has a broad activity in several tissues which contain hypoxic regions while HIF-2 α is more restricted to specific cell types from e.g. kidney, lung and heart [12]. HIF proteins distinctly contribute to the up-regulation of genes involved in proliferation, glucose metabolism and angiogenesis and genes involved in invasion and metastasis in different types of cancer [13]. HIF isoforms also differ in their ability to promote treatment resistance in cancer by playing highly divergent or even opposite roles, leading to distinct clinicopathologic features and prognosis [14]. Specific activity of HIF-2 α differently contributes to total HIF target gene expression among many types of cancers, which may influence the characteristics of these tumors and the outcome of patients [15]. To date, much effort has been made to better understand the roles of HIF-2 α in cancer and the consequences for patients suffering from high-HIF-2 α expressing tumors. So far, there is clear evidence suggesting that HIF-2 α is a crucial protein for the development and progression of many types of cancer. Indeed, HIF-2 α seems to be crucial in regulating multiple aspects in cancer, including cell proliferation, apoptosis, epithelial to mesenchymal transition, cell metabolism, angiogenesis and resistance to therapy [16, 17]. Hypoxia-induced

HIF-2 α expression and its subsequent chain of events make this protein a relevant marker of tumor hypoxia and a promising target for anti-cancer therapies with novel inhibitors.

Several clinical studies describe the prognostic value of HIF-1 α in cancer, including the elaboration of many meta-analyses for several kinds of cancer and different HIF-1 α polymorphisms [18-20]. For some of the clinical studies included in these meta-analyses, prognostic data on HIF-2 α can be found which often differed from HIF-1 α data [21]. In addition, other studies specifically looked at the role of HIF-2 α independently of HIF-1 α expression. Nonetheless, many discrepancies are seen among the investigations that were performed during the last years, in which HIF-2 α has been reported to be either a positive or negative prognostic factor in cancer. The purpose of this meta-analysis was to provide an updated comprehensive analysis regarding the prognostic value of HIF-2 α expression in solid tumors.

Methods

Literature Search

The research question of this meta-analysis was defined as follows: “what is the prognostic value of tumoral HIF-2 α expression in patients with solid tumors?” Meta-analysis included patients with solid tumors of different types (Table 1) independent of stage and/or grade or treatment modality. Various treatment outcomes (see below) were compared between patients with high and low tumoral HIF-2 α expression. PubMed and Embase were used to identify studies that investigated the prognostic significance of HIF-2 α in solid tumors to be included in the present meta-analysis. The search for literature was performed including papers published until 1st of February 2018. Three main key words were identified to address the research question of this meta-analysis, i.e., *prognosis*, *cancer* and *HIF2*. Several combinations of the selected keywords (in any of the formulations or truncations) were tested as free text searches to identify potential articles to be included in this meta-analysis (Supplementary file 1). A total of 636 papers were identified from both databases (Figure 1).

Screening of Papers

Three independent reviewers assessed the eligibility of the studies. The first round of screening was based on the title and abstract (EMR and AG). 99 review articles without original data, conference records, commentaries, meta-analyses, editorials, or book chapters were excluded, as were 10 over-lapping or non-English papers. The total number of papers for further screening was thereby reduced to 527 (Figure 1). The second round consisted of a detailed evaluation of the full-text (EMR and AY).

In order to be included in the meta-analysis, a study had to fulfil predetermined inclusion/exclusion criteria: 1) only solid primary tumors of various types were included, 2) all endpoints were included with the minimal median follow-up of 1 year, 3) all treatments were included, 4) none of the patient populations were excluded by distinctive diagnosis, tumor grade or stage and 5) pre-treatment protein expression by immunohistochemistry was the only technique for HIF-2 α detection included. Discrepancies between the included papers by both reviewers were discussed and consensus was reached on all. A total of 49 papers were included in the meta-analysis (Figure 1) [21-69].

Data extraction

Reported parameters were extracted from each paper, i.e., the number and origin of patients, treatment modalities, tumor organ, tumor stage, tumor type, group dichotomization, antibody supplier, expression pattern, cellular localization, positivity in macrophages and outcome variables (see below). The univariate hazard ratio (HR) and 95% confidence interval (95% CI) were directly obtained from the information available in the text. If not reported, the method from Tierney et al. was used to calculate HR and thereby assess prognostic value of HIF-2 α expression [70]. Multivariate HR was only considered when the univariate HR could not be estimated. Authors were contacted to obtain additional data when not all the information was reported for estimating HR.

Kidney cancer was excluded from the main analysis because the loss of the von Hippel-Lindau (VHL) gene, a common mutation in CC-RCC, results in the stabilization of HIFs independent of oxygen, i.e. an alternative mechanism of HIF-2 α activation in cancer absent in the majority of other solid tumors [74]. Therefore prognostic value of HIF-2 α in this tumor type has been analyzed separately.

Quality assessment

The methodological quality of the included papers was evaluated with an adjusted version of the Newcastle–Ottawa scale (NOS) to better suit the study design of the included papers (Supplementary file 2) [71]. The NOS was proposed in the 2011 version of the Cochrane Collaboration handbook being an easy method to evaluate the methodological quality of cohort studies (available at http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp).

Statistical Analysis and Sensitivity Analysis

Distribution and frequencies of the extracted data parameters were analyzed using SPSS (version 22). Meta-analysis was performed using R statistical software with the Metafor Library (version 2.0-0) [72]. Fixed-effect modeling was performed when no statistical significant heterogeneity between studies was observed. When the heterogeneity between studies was statistically significant ($p < 0.05$), random-effects modeling was applied based on the DerSimonian and Laird method [73]. The inverse variance of each study was used to assign an independent weight value. Six different endpoints were considered for the analysis: overall survival (OS), disease-free survival (DFS), locoregional control (LC), disease-specific survival (DSS), metastasis-free survival (MFS), and progression-free survival (PFS). OS is

based on death at any cause. In DFS the event is relapse rather than death and is used to analyze the results of the treatment for the localized disease. LC evaluates the mobility and control of the primary tumor site. When assessing DSS, we evaluate the amount patients which died from a specific disease in a defined period of time. In MSF is the event of metastasis rather than death for patients diagnosed with cancer. Finally, the event of PFS refers to the progression of the disease meaning that it gets worse or progresses, or the patient dies from any cause.

Sensitivity analysis was performed by analyzing subgroups of studies based on organ site. We assessed the possibility of publication bias and heterogeneity among studies by generating and visually analyzing funnel plots. Asymmetric funnel plots and studies outside the pyramid suggest heterogeneity between them.

Results

This meta-analysis includes a total number of 6,052 patients across 49 independent studies [21-69]. The median follow-up time ranged between 27 and 391 months and mostly included only a small number of patients (median 90, range 21 – 695). Selected papers were published between 2001 and 2017 of which 56% were published after 2010. Depending on the study, patients followed different treatment schedules. In most of the selected studies patients were treated with surgery alone (56%), in combination with either chemotherapy (8%) or standard radiotherapy (10%), or a combination of all three modalities (14%). Other treatment alternatives such as hormonal therapy and tyrosine-kinase inhibitors were used in 8% of the studies. Overall, the majority of the patients (61%) were treated with surgery alone. Most studies report on head and neck and kidney cancer patients (both 18%) followed by colon, liver, pancreas and lung cancer patients (all of them 8%). By contrast, cancers of the bladder, cartilage, cervix, endometrium, ovary and salivary glands were only described once.

Immunohistochemical staining of HIF-2 α was most commonly performed using the EP190b Ab (38%) from Novus Biologicals. Other studies used anti-HIF2 α antibodies obtained from other suppliers. Cytoplasmic expression of HIF-2 α was described in 14% of the studies and 18% were positive in the nucleus. A combination of both positive cytoplasmic and nuclear staining was reported in 46% of the studies. HIF-2 α expression was quantified using different methods and patient stratification into groups with low and high tumoral HIF-2 α expression was performed using different thresholds. Taken together, 45% of the total tumors were classified as expressing high levels of HIF-2 α . Also, 26% of these studies stated positive staining in macrophages.

Overall, patients suffering from tumors with high HIF-2 α expression had a worse treatment outcome (Figure 2). This association was significant for OS ($p<0.0001$), DFS ($p=0.0057$), DSS ($p=0.0249$), MFS ($p=0.0061$) and PFS ($p=0.0058$). No association was found between HIF-2 α expression and locoregional control ($p=0.1281$). Sub-group analyses based on tumor type and treatment option were not performed due to the low number of studies. Studies on renal cell cancer were eliminated from the overall HR estimation as HIF-2 α plays a different role in this cancer type [74]. Funnel plots demonstrated systematic heterogeneity for almost all the endpoints. This can be due to publication bias, variation across the reports or small number of studies (Supplementary Figure 1).

Overall Survival

A total of 38 from the selected 49 studies investigated the association between HIF-2 α and OS. All the necessary information to estimate the HR could not be obtained from 2 papers and were therefore not included in the analysis (Supplementary Table 1) [21, 23, 24, 26-31, 33-35, 37-39, 41-43, 45, 46, 48, 49, 51, 52, 54-57, 59, 60, 62-69]. Based on these studies, high HIF-2 α expression was statistically significantly associated with a decreased OS (HR = 1.69, 95%CI 1.39–2.06, $p < 0.0001$, Figure 3). Sub-group analysis based on the different organ sites indicated a similar negative association between tumoral HIF-2 α expression and OS: head and neck (HR = 1.55, 95%CI 1.24–1.92, $p < 0.0001$), lung (HR = 2.15, 95%CI 1.65–2.81, $p < 0.0001$), stomach (HR = 1.71, 95%CI 1.25–2.32, $p = 0.0007$) and pancreas (HR = 2.11, 95%CI 1.38–3.24, $p = 0.0006$). In contrast, no association between tumoral HIF-2 α expression and OS was observed for breast (HR = 1.18, 95%CI 0.95–1.47, $p = 0.1225$), colon (HR = 1.46, 95%CI 0.7–3.07, $p = 0.3121$), liver (HR = 1.06, 95%CI 0.43–2.61, $p = 0.89$) and kidney (HR = 0.61, 95%CI 0.27–1.35, $p = 0.2239$) cancer (Table 1).

Disease-Free Survival

Effect of pre-treatment expression of HIF-2 α on DFS could be evaluated in 6 studies [36, 37, 54, 66, 67, 69]. Overall, high HIF-2 α expression was significantly associated with a decreased DFS (HR = 1.87, 95%CI 1.2–2.92, $p = 0.0057$, Figure 4). Sub-group analysis indicated that elevated HIF-2 α levels were marginally significantly associated with DFS in head and neck cancer (HR = 1.41, 95%CI 0.99–2, $p = 0.0577$) (Table 1).

Disease-Specific Survival

A total of 12 studies evaluated the association of HIF-2 α expression with DSS, of which 2 studies provided incomplete data to estimate the HR (Supplementary Table 1) and 3 were excluded for being CC-RCC. In the remaining 7 studies, patients suffering from tumors with high HIF-2 α had significantly shorter DSS (HR = 1.57, 95%CI 1.06–2.34, $p = 0.0249$, Figure 5) [24, 25, 32, 34, 35, 47, 49, 50, 53, 58, 61, 66]. Sub-group analysis by organ site revealed not association between high HIF-2 α expression and worse DSS in tumors of the head and neck (HR = 1.45, 95%CI 0.8–2.64, $p = 0.2219$) and kidney (HR = 1.21, 95%CI 0.57–2.6, $p = 0.6138$) cancer (Table 1).

Locoregional-Control

Ten studies were included to analyze the association of HIF-2 α expression with risk of locoregional control. High tumoral HIF-2 α expression was not associated with a higher risk of locoregional recurrences compared to patients with low expression of HIF-2 α in tumors (HR = 1.31, 95%CI 0.92–1.86, p = 0.1281, Figure 6) [35, 40, 44-46, 52, 56-59]. However, subgroup analysis for the association between high HIF-2 α expression in tumors and worse LC was significant in head and neck tumors (HR = 1.94, 95%CI 1.43–2.63, p <0.0001). No significant association was observed in kidney tumors (HR = 0.54, 95%CI 0.11–2.57, p =0.4409) (Table 1).

Metastasis-Free Survival

Based on the available data reported in 2 studies, we analyzed the relationship of HIF-2 α expression with MFS [53, 57]. We found that the pooled HR for MFS was 2.67 (95%CI 1.32–5.38, p = 0.0061), indicating that HIF-2 α expression is a negative prognostic factor for MFS in patients with prostate and salivary gland cancer (Figure 7). In kidney cancer, one paper reported an inverse correlation between HIF-2 α positivity and metastasis-free survival (HR = 0.08, 95%CI 0.03–0.27, p <0.001 [23]).

Progression-Free Survival

Progression-free survival was reported in 5 out the 49 included studies, of which 1 study provided incomplete data (Supplementary Table 1) and 2 described patients with renal cell cancer [29, 39, 48, 51, 53]. Similar to the other endpoints, PFS was significantly shorter (HR = 2.18, 95%CI 1.25–3.78, p = 0.0058) in patients with tumors expressing high levels of HIF-2 α (Figure 8). In kidney cancers, this data showed no association between HIF-2 α expression and PFS (HR = 0.75, 95%CI 0.32–1.72, p =0.498) (Table 1).

High-Quality Papers

This meta-analysis used an adjusted version of the NOS to evaluate the quality of a study. The scores of this quality assessment ranged between 1 and 7 stars, i.e. the maximum, awarded per study. Approximately 78% of these studies were considered as high-quality studies, i.e., with a number of stars greater or equal to 5.

Discussion

There is growing evidence that overexpression of HIF-2 α in cancer can contribute to differences in treatment outcome between patients. Although HIF-2 α oncogenic activity has been proven, there is significant variability in its value as a biomarker for patient's prognosis. In view of its role in regulating oncogenic processes triggered by hypoxia, the evaluation of its prognostic value in cancer is of great clinical importance, which may lead to a more accurate patient prognosis and the generation of targeted therapies in the future. This meta-analysis is the first complete overview to summarize all reported clinical studies investigating the impact of HIF-2 α expression on treatment outcome in solid tumors.

Here we show that high HIF-2 α levels in cancer are correlated with worse prognosis for OS, DFS, DSS, MFS and PFS. No association between HIF-2 α expression and locoregional control was found. This data suggests that HIF-2 α might not be involved in treatment resistance directly but is indicative of more malignant phenotype with greater metastatic potential. Sub-group analyses were performed to explore the source of heterogeneity based on different organ sites. We found that this variable did not alter the prognostic value of HIF-2 α for most of the endpoints assessed.

We excluded CC-RCCs from the main analysis due to a different regulatory mechanism of HIF-2 α , which might affect the final outcome. A common mutation in CC-RCC is the loss of the von Hippel-Lindau (VHL) gene, which results in the stabilization of HIFs upon normoxia. This oncogenic process is specific for CC-RCC with an abundance of 80% in patients, resulting in an alternative mechanism of HIF-2 α activation in cancer [74]. However, the negative prognostic value of HIF-2 α does not change when CC-RCC is included in the overall meta-analysis (data not shown). In CC-RCC, we found that HIF-2 α is a positive prognostic biomarker for metastasis-free survival only (Table 1). Subcellular localization might also affect the prognostic significance of HIF-2 α in CC-RCC as previously noted [75]. Their data show that high cytoplasmic expression of HIF-2 α was significantly associated with poor DSS which is consistent with our findings. In contrast, high nuclear expression of HIF-2 α is associated with better DSS in patients (Supplementary Table 2). Therefore, it might be that subcellular localization of HIF-2 α is crucial in determining the prognostic value in CC-RCC patients, which requires further investigation. Another study demonstrated that HIF-2 α can be used as a predictive biomarker related to drug selection for CC-RCC patients treated with

Sunitinib and Sorafenib [51]. Therefore, assessment of sub-group categories is necessary to better evaluate the prognostic and predictive value of HIF-2 α in CC-RCC.

Previous studies have shown that increased infiltration of tumor-associated macrophages (TAMs) in cancer patients is associated with worse overall survival [76]. In comparison with normal macrophages, TAMs express high levels of HIF-2 α , which seems to be an indicator of poor prognosis in cancer patients [77]. Using *in vivo* models of acute inflammation, it has been shown that HIF-2 α expression in macrophages is essential for inflammatory responses by regulating proinflammatory cytokine expression [78]. Together, these studies show that HIF-2 α tightly regulates macrophage functions, which in turn may impact the patient prognosis. We excluded studies in which only macrophage data was reported, since the main goal of this meta-analysis is to determine the prognostic role of HIF-2 α expression in tumor cells. To note, 26% of the studies included in this meta-analysis stated HIF-2 α reactivity in macrophages together with tumor cells.

The papers included in the current meta-analysis were all published between 2001 and 2018, which is likely attributed to the fact that HIF-2 α was first identified by independent groups a few years earlier [79, 80]. First studies showed the importance of HIF-2 α on the transcription of hypoxia-regulated genes such as VEGF in endothelial cells, fibroblasts and epithelial cells. In addition, researchers described the novel role of HIF-2 α in comparison with the already studied HIF-1 α , its counteractive protein, in hypoxia and tissue homeostasis [80-83]. The discovery of this new hypoxia-activated transcription factor encouraged research to further evaluate the role of HIF-2 α *in vivo* and during development in mice. Their data show that HIF-2 α displays a specific pattern of developmental expression at different embryonic stages [84]. HIF-2 α was thereby defined as a novel bHLH-PAS protein involved in the response to hypoxia showing overlapping but also independent roles with HIF-1 α . Basic HLH (helix-loop-helix)-PER-ARNT-SIM (bHLH-PAS) proteins are a family of transcription factors, which respond to environmental signals such as low oxygen levels. This family of proteins is involved in dimerization, DNA binding and signal transduction [85]. Future studies demonstrated that HIF-2 α was expressed in a much larger number of cell types [86]. Apart from its regulatory role in normal tissue homeostasis, HIF-2 α was seen to be commonly upregulated in a broad range of cancers and to contribute to multiple aspects of tumorigenesis such as altered metabolism, angiogenesis, epithelial-mesenchymal transition (EMT) and metastasis. This is supported by data showing that HIF-2 α is involved in

promoting resistance of tumor cells to several treatment modalities and increased patient mortality [87]. The important role of HIF-2 α in cancer prognosis is also supported by the results of the current meta-analysis, which shows that patients with high HIF-2 α expression have shorter overall survival. HIF-1 α protein, in turn, has been shown to induce more aggressive phenotype in tumors cells by regulating similar cellular mechanisms [88]. Therefore, assessment of oxygen-sensing proteins in tumors prior and/or during therapy may represent a powerful prognostic and predictive biomarker as well as important targets for new anti-cancer treatments, which warrants further investigations.

Importantly, there is also the risk of encountering publication bias since positive results are more likely to be published than negative ones. This meta-analysis identified a total of 49 studies of which 4 could not be included in final analysis because the HR could not be estimated due to incomplete reporting. Three out of four studies stated non-significant association between HIF-2 α and outcome (Supplementary Table 1). Including these 4 papers in the analysis might therefore decrease the magnitude of the prognostic value of HIF-2 α expression reported here. Nevertheless, since the prognostic value of HIF-2 α expression is highly statistically significant and the number of excluded studies is very low, we believe that the possible effect of publication bias on this association is negligible.

There are also other limitations in this meta-analysis. The approach of extrapolating the HRs could potentially introduce the source of bias. First, when it was not possible to extract HR directly from the article, survival curves were used to extract data to estimate HR following the method of Tierney, et al. [70]. Second, significant heterogeneity was found for most of the endpoints tested, which might confirm the high variability among studies. Reduced variability could be achieved by better stratifying tumors into high and low expressing. Third, the use of different antibodies with varying dilutions to detect HIF-2 α , different staining protocols, different scoring methods, sub-cellular localization and cut-off values may contribute to heterogeneity. Finally, due to the lack of papers referring to each specific endpoint, treatment modality and/or organ site, it is difficult to set a robust outcome and achieve significant data for different sub-groups. Therefore, more high-quality, large-sample, prospectively designed studies are needed to strengthen the prognostic and predictive relevance of HIF-2 α in solid cancers.

The results presented here clearly indicate that HIF-2 α expression is associated with worse prognosis in a global patient population and in some tumor sites. Altogether, the results of

this meta-analysis support the development of a clinical test to determine patient prognosis and/or predict treatment outcome based on HIF-2 α expression, although standardized protocols remains to be developed and validated. While potent inhibitors targeting HIF-2 α are being translated into clinical trials for renal cancer [89] such predictive tests would be crucial in advancing anti-HIF2 inhibitors not only in renal cancer but also in other solid cancers.

Acknowledgements

This work was supported by a H2020 grant from the European Research Council (ERC-CoG, 617060) and the Kankeronderzoekfonds Limburg from the Health Foundation Limburg.

Author contributions

Study was conceived and designed by EM, AY and MV. Screening of papers and data extraction was performed by EMR, AY and AJ. Statistical analyses were performed by RH. Writing of the first draft of the manuscript was performed by EM. AY, RH, AJ, LD and MV contributed to the writing of the manuscript.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Vaupel, P. and A. Mayer, *Hypoxia in cancer: significance and impact on clinical outcome*. Cancer Metastasis Rev, 2007. **26**(2): p. 225-39.
2. Gort, E.H., A.J. Groot, E. van der Wall, P.J. van Diest, and M.A. Vooijs, *Hypoxic regulation of metastasis via hypoxia-inducible factors*. Curr Mol Med, 2008. **8**(1): p. 60-7.
3. Rockwell, S., I.T. Dobrucki, E.Y. Kim, S.T. Marrison, and V.T. Vu, *Hypoxia and radiation therapy: past history, ongoing research, and future promise*. Curr Mol Med, 2009. **9**(4): p. 442-58.
4. Walsh, J.C., A. Lebedev, E. Aten, K. Madsen, L. Marciano, and H.C. Kolb, *The clinical importance of assessing tumor hypoxia: relationship of tumor hypoxia to prognosis and therapeutic opportunities*. Antioxid Redox Signal, 2014. **21**(10): p. 1516-54.
5. Dubois, L.J., R. Niemans, S.J. van Kuijk, K.M. Panth, N.K. Parvathaneni, S.G. Peeters, C.M. Zegers, N.H. Rekers, M.W. van Gisbergen, R. Biemans, N.G. Lieuwes, L. Spiegelberg, A. Yaromina, J.Y. Winum, M. Vooijs, and P. Lambin, *New ways to image and target tumour hypoxia and its molecular responses*. Radiother Oncol, 2015. **116**(3): p. 352-7.
6. Hammond, E.M., M.C. Asselin, D. Forster, J.P. O'Connor, J.M. Senra, and K.J. Williams, *The meaning, measurement and modification of hypoxia in the laboratory and the clinic*. Clin Oncol (R Coll Radiol), 2014. **26**(5): p. 277-88.
7. Pettersen, E.O., P. Ebbesen, R.G. Gieling, K.J. Williams, L. Dubois, P. Lambin, C. Ward, J. Meehan, I.H. Kunkler, S.P. Langdon, A.H. Ree, K. Flatmark, H. Lyng, M.J. Calzada, L.D. Peso, M.O. Landazuri, A. Gorlach, H. Flamm, J. Kieninger, G. Urban, A. Weltin, D.C. Singleton, S. Haider, F.M. Buffa, A.L. Harris, A. Scozzafava, C.T. Supuran, I. Moser, G. Jobst, M. Busk, K. Toustrup, J. Overgaard, J. Alsner, J. Pouyssegur, J. Chiche, N. Mazure, I. Marchiq, S. Parks, A. Ahmed, M. Ashcroft, S. Pastorekova, Y. Cao, K.M. Rouschop, B.G. Wouters, M. Koritzinsky, H. Mujcic, and D. Cojocari, *Targeting tumour hypoxia to prevent cancer metastasis. From biology, biosensing and technology to drug development: the METOXIA consortium*. J Enzyme Inhib Med Chem, 2015. **30**(5): p. 689-721.
8. Yaromina, A., M. Granzier, R. Biemans, N. Lieuwes, W. van Elmpt, G. Shakirin, L. Dubois, and P. Lambin, *A novel concept for tumour targeting with radiation: Inverse dose-painting or targeting the "Low Drug Uptake Volume"*. Radiother Oncol, 2017. **124**(3): p. 513-520.
9. Eales, K.L., K.E. Hollinshead, and D.A. Tennant, *Hypoxia and metabolic adaptation of cancer cells*. Oncogenesis, 2016. **5**: p. e190.
10. Wouters, B.G. and M. Koritzinsky, *Hypoxia signalling through mTOR and the unfolded protein response in cancer*. Nat Rev Cancer, 2008. **8**(11): p. 851-64.
11. Ke, Q. and M. Costa, *Hypoxia-inducible factor-1 (HIF-1)*. Mol Pharmacol, 2006. **70**(5): p. 1469-80.
12. Akeno, N., M.F. Czyzyk-Krzeska, T.S. Gross, and T.L. Clemens, *Hypoxia induces vascular endothelial growth factor gene transcription in human osteoblast-like cells through the hypoxia-inducible factor-2alpha*. Endocrinology, 2001. **142**(2): p. 959-62.

13. Carroll, V.A. and M. Ashcroft, *Role of hypoxia-inducible factor (HIF)-1 α versus HIF-2 α in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway*. Cancer Res, 2006. **66**(12): p. 6264-70.
14. Shah, T., B. Krishnamachary, F. Wildes, Y. Mironchik, S.M. Kakkad, D. Jacob, D. Artemov, and Z.M. Bhujwalla, *HIF isoforms have divergent effects on invasion, metastasis, metabolism and formation of lipid droplets*. Oncotarget, 2015. **6**(29): p. 28104-19.
15. Imamura, T., H. Kikuchi, M.T. Herraiz, D.Y. Park, Y. Mizukami, M. Mino-Kenduson, M.P. Lynch, B.R. Rueda, Y. Benita, R.J. Xavier, and D.C. Chung, *HIF-1 α and HIF-2 α have divergent roles in colon cancer*. Int J Cancer, 2009. **124**(4): p. 763-71.
16. Wigerup, C., S. Pahlman, and D. Bexell, *Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer*. Pharmacol Ther, 2016. **164**: p. 152-69.
17. Bertout, J.A., A.J. Majmundar, and J.D. Gordan, *HIF2 α inhibition promotes p53 pathway activity, tumor cell death, and radiation responses*. in *Proceedings of the* 2009.
18. Li, Y., C. Li, H. Shi, L. Lou, and P. Liu, *The association between the rs11549465 polymorphism in the hif-1 α gene and cancer risk: a meta-analysis*. Int J Clin Exp Med, 2015. **8**(2): p. 1561-74.
19. Ren, H.Y., Y.H. Zhang, H.Y. Li, T. Xie, L.L. Sun, T. Zhu, S.D. Wang, and Z.M. Ye, *Prognostic role of hypoxia-inducible factor-1 α expression in osteosarcoma: a meta-analysis*. Onco Targets Ther, 2016. **9**: p. 1477-87.
20. Zheng, S.S., X.H. Chen, X. Yin, and B.H. Zhang, *Prognostic significance of HIF-1 α expression in hepatocellular carcinoma: a meta-analysis*. PLoS One, 2013. **8**(6): p. e65753.
21. Wu, X.H., C. Qian, and K. Yuan, *Correlations of hypoxia-inducible factor-1 α /hypoxia-inducible factor -2 α expression with angiogenesis factors expression and prognosis in non-small cell lung cancer*. Chinese Medical Journal, 2011.
22. Abel, E.J., T.M. Bauman, M. Weiker, F. Shi, T.M. Downs, D.F. Jarrard, and W. Huang, *Analysis and validation of tissue biomarkers for renal cell carcinoma using automated high-throughput evaluation of protein expression*. Hum Pathol, 2014. **45**(5): p. 1092-9.
23. Szendrői, A., A.M. Szász, M. Kardos, A.M. Tőkés, R. Idan, M. Szücs, J. Kulka, P. Nyirády, M. Szendrői, Z. Szállási, B. Györffy, and J. Tímár, *Opposite prognostic roles of HIF1 α and HIF2 α expressions in bone metastatic clear cell renal cell cancer*. Oncotarget, 2016.
24. Baba, Y., K. Nosho, K. Shima, N. Irahara, A.T. Chan, J.A. Meyerhardt, D.C. Chung, E.L. Giovannucci, C.S. Fuchs, and S. Ogino, *HIF1A overexpression is associated with poor prognosis in a cohort of 731 colorectal cancers*. Am J Pathol, 2010. **176**(5): p. 2292-301.
25. Biswas, S., P.J. Charlesworth, G.D. Turner, R. Leek, P.T. Thamboo, L. Campo, H. Turley, P. Dildy, A. Protheroe, D. Cranston, K.C. Gatter, F. Pezzella, and A.L. Harris, *CD31 angiogenesis and combined expression of HIF-1 α and HIF-2 α are prognostic in primary clear-cell renal cell carcinoma (CC-RCC), but HIF1 α*

transcriptional products are not: implications for antiangiogenic trials and HIF α biomarker studies in primary CC-RCC. Carcinogenesis, 2012. **33**(9): p. 1717-25.

26. Chen, C., Q. Ma, X. Ma, Z. Liu, and X. Liu, *Association of elevated HIF-2 α levels with low Beclin 1 expression and poor prognosis in patients with chondrosarcoma.* Ann Surg Oncol, 2011. **18**(8): p. 2364-72.

27. Ebru, T., O.P. Fulya, A. Hakan, Y.C. Vuslat, S. Necdet, C. Nuray, and O. Filiz, *Analysis of various potential prognostic markers and survival data in clear cell renal cell carcinoma.* Int Braz J Urol, 2017. **43**(3): p. 440-454.

28. Gao, Z.J., Y. Wang, W.D. Yuan, J.Q. Yuan, and K. Yuan, *HIF-2 α not HIF-1 α overexpression confers poor prognosis in non-small cell lung cancer.* Tumour Biol, 2017. **39**(6): p. 1010428317709637.

29. Garcia-Donas, J., L.J. Leandro-Garcia, A. Gonzalez Del Alba, M. Morente, I. Alemany, E. Esteban, J.A. Arranz, M.A. Climent, E. Gallardo, D.E. Castellano, J. Bellmunt, B. Mellado, J. Puente, F. Moreno, A. Font, S. Hernando, M. Robledo, and C. Rodriguez-Antona, *Prospective study assessing hypoxia-related proteins as markers for the outcome of treatment with sunitinib in advanced clear-cell renal cell carcinoma.* Ann Oncol, 2013. **24**(9): p. 2409-14.

30. Bangoura, G., Z.S. Liu, Q. Qian, C.Q. Jiang, G.F. Yang, and S. Jing, *Prognostic significance of HIF-2 α EPAS1 expression in hepatocellular carcinoma.* World Journal of Gastroenterology, 2007.

31. Giatromanolaki, A., E. Sivridis, N.E. Bechrakis, G. Willerding, G. St Charitoudis, M.H. Foerster, K.C. Gatter, A.L. Harris, and M.I. Koukourakis, *Phosphorylated pVEGFR2/KDR receptor expression in uveal melanomas: relation with HIF2 α and survival.* Clin Exp Metastasis, 2012. **29**(1): p. 11-7.

32. Giatromanolaki, A., E. Sivridis, C. Kouskoukis, K.C. Gatter, A.L. Harris, and M.I. Koukourakis, *Hypoxia-inducible factors 1 α and 2 α are related to vascular endothelial growth factor expression and a poorer prognosis in nodular malignant melanomas of the skin.* Melanoma Res, 2003. **13**(5): p. 493-501.

33. Giatromanolaki, A., M.I. Koukourakis, E. Sivridis, H. Turley, K. Talks, F. Pezzella, K.C. Gatter, and A.L. Harris, *Relation of hypoxia inducible factor 1 α and 2 α in operable non-small cell*

lung cancer to angiogenicmolecular profile of tumours and survival. British Journal of Cancer, 2001.

34. Griffiths, E.A., S.A. Pritchard, S.M. McGrath, H.R. Valentine, P.M. Price, I.M. Welch, and C.M. West, *Hypoxia-associated markers in gastric carcinogenesis and HIF-2 α in gastric and gastro-oesophageal cancer prognosis.* Br J Cancer, 2008. **98**(5): p. 965-73.

35. Helczynska, K., A.M. Larsson, L. Holmquist-Mengelbier, E. Bridges, E. Fredlund, S. Borgquist, G. Landberg, S. Pahlman, and K. Jirstrom, *Hypoxia-inducible factor-2 α correlates to distant recurrence and poor outcome in invasive breast cancer.* Cancer Res, 2008. **68**(22): p. 9212-20.

36. Higashi, K., T. Yamagishi, Y. Ueda, Y. Ishigaki, M. Shimasaki, Y. Nakamura, M. Oguchi, T. Takegami, M. Sagawa, and H. Tonami, *Correlation of HIF-1 α /HIF-*

- 2alpha expression with FDG uptake in lung adenocarcinoma.* Ann Nucl Med, 2016. **30**(10): p. 708-715.
37. Yoshimura, H., D.K. Dhar, H. Kohno, H. Kubota, T. Fujii, S. Ueda, S. Kinugasa, M. Tachibana, and N. N., *Prognostic impact of hypoxia-inducible factors 1alpha and 2alpha in colorectal cancer patient..pdf*>. Clinical Cancer Research, 2004.
38. Holmquist-Mengelbier, L., E. Fredlund, T. Lofstedt, R. Noguera, S. Navarro, H. Nilsson, A. Pietras, J. Vallon-Christersson, A. Borg, K. Gradin, L. Poellinger, and S. Pahlman, *Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype.* Cancer Cell, 2006. **10**(5): p. 413-23.
39. Hui, E.P., A.T. Chan, F. Pezzella, H. Turley, K.F. To, T.C. Poon, B. Zee, F. Mo, P.M. Teo, D.P. Huang, K.C. Gatter, P.J. Johnson, and A.L. Harris, *Coexpression of hypoxia-inducible factors 1alpha and 2alpha, carbonic anhydrase IX, and vascular endothelial growth factor in nasopharyngeal carcinoma and relationship to survival.* Clin Cancer Res, 2002. **8**(8): p. 2595-604.
40. Ioachim, E., M. Michael, M. Salmas, M.M. Michael, N.E. Stavropoulos, and V. Malamou-Mitsi, *Hypoxia-inducible factors HIF-1alpha and HIF-2alpha expression in bladder cancer and their associations with other angiogenesis-related proteins.* Urol Int, 2006. **77**(3): p. 255-63.
41. Fuady, J.H., K. Gutsche, S. Santambrogio, Z. Varga, D. Hoogewijs, and R. Wenger, *Estrogen-dependent downregulation of hypoxia-inducible factor (HIF)-2` in invasive breast cancer cells.* Oncotarget, 2016.
42. Yang, J., D.M. Zhu, X.G. Zhou, N. Yin, Y. Zhang, Z.X. Zhang, D.C. Li, and J. Zhou, *HIF-2 α promotes the formation of vasculogenic mimicry in pancreatic cancer by regulating the binding of Twist1 to the VE-cadherin promoter.* Oncotarget, 2017.
43. Jubb, A.M., H. Turley, H.C. Moeller, G. Steers, C. Han, J.L. Li, R. Leek, E.Y. Tan, B. Singh, N.J. Mortensen, I. Noguera-Troise, F. Pezzella, K.C. Gatter, G. Thurston, S.B. Fox, and A.L. Harris, *Expression of delta-like ligand 4 (Dll4) and markers of hypoxia in colon cancer.* Br J Cancer, 2009. **101**(10): p. 1749-57.
44. Kim, M.K., T.J. Kim, C.O. Sung, C.H. Choi, J.W. Lee, D.S. Bae, and B.G. Kim, *Clinical significance of HIF-2alpha immunostaining area in radioresistant cervical cancer.* J Gynecol Oncol, 2011. **22**(1): p. 44-8.
45. Koukourakis, M.I., S.M. Bentzen, A. Giatromanolaki, G.D. Wilson, F.M. Daley, M.I. Saunders, S. Dische, E. Sivridis, and A.L. Harris, *Endogenous markers of two separate hypoxia response pathways (hypoxia inducible factor 2 alpha and carbonic anhydrase 9) are associated with radiotherapy failure in head and neck cancer patients recruited in the CHART randomized trial.* J Clin Oncol, 2006. **24**(5): p. 727-35.
46. Koukourakis, M.I., A. Giatromanolaki, V. Danielidis, and E. Sivridis, *Hypoxia inducible factor (Hif1alpha and HIF2alpha) and carbonic anhydrase 9 (CA9) expression and response of head-neck cancer to hypofractionated and accelerated radiotherapy.* Int J Radiat Biol, 2008. **84**(1): p. 47-52.
47. Kroeger, N., D.B. Seligson, S. Signoretti, H. Yu, C.E. Magyar, J. Huang, A.S. Belldegrun, and A.J. Pantuck, *Poor prognosis and advanced clinicopathological features*

- of clear cell renal cell carcinoma (ccRCC) are associated with cytoplasmic subcellular localisation of Hypoxia inducible factor-2alpha.* Eur J Cancer, 2014. **50**(8): p. 1531-40.
48. Li, W., C. Chen, X. Zhao, H. Ye, Y. Zhao, Z. Fu, W. Pan, S. Zheng, L. Wei, T. Nong, Z. Li, and R. Chen, *HIF-2alpha regulates non-canonical glutamine metabolism via activation of PI3K/mTORC2 pathway in human pancreatic ductal adenocarcinoma.* J Cell Mol Med, 2017. **21**(11): p. 2896-2908.
49. Liang, X., M. Zheng, J. Jiang, G. Zhu, J. Yang, and Y. Tang, *Hypoxia-inducible factor-1 alpha, in association with TWIST2 and SNIP1, is a critical prognostic factor in patients with tongue squamous cell carcinoma.* Oral Oncol, 2011. **47**(2): p. 92-7.
50. Liu, Y., X. Tan, W. Liu, X. Chen, X. Hou, D. Shen, Y. Ding, J. Yin, L. Wang, H. Zhang, Y. Yu, J. Hou, T.C. Thompson, and G. Cao, *Follistatin-like protein 1 plays a tumor suppressor role in clear-cell renal cell carcinoma.* Chin J Cancer, 2018. **37**(1): p. 2.
51. Ma, X., L. Wang, H. Li, Y. Zhang, Y. Gao, G. Guo, K. Liu, Q. Meng, C. Zhao, D. Wang, Z. Song, and X. Zhang, *Predictive Immunohistochemical Markers Related to Drug Selection for Patients Treated with Sunitinib or Sorafenib for Metastatic Renal Cell Cancer.* Sci Rep, 2016. **6**: p. 30886.
52. Koukourakis, M.I., A. Giatromanolaki, E. Sivridis, C. Simopoulos, H. Turley, K. Talks, K.C. Gatter, and A.L. Harris, *Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer.* Clinical Investigation, 2002.
53. Nanni, S., V. Benvenuti, A. Grasselli, C. Priolo, A. Aiello, S. Mattiussi, C. Colussi, V. Lirangi, B. Illi, M. D'Eletto, A.M. Cianciulli, M. Gallucci, P. De Carli, S. Sentinelli, M. Mottotese, P. Carlini, L. Strigari, S. Finn, E. Mueller, G. Arcangeli, C. Gaetano, M.C. Capogrossi, R.P. Donnorso, S. Bacchetti, A. Sacchi, A. Pontecorvi, M. Loda, and A. Farsetti, *Endothelial NOS, estrogen receptor beta, and HIFs cooperate in the activation of a prognostic transcriptional pattern in aggressive human prostate cancer.* J Clin Invest, 2009. **119**(5): p. 1093-108.
54. Beasley, N.J.P., R. Leek, M. Alam, H. Turley, G.J. Cox, K. Gatter, P. Millard, S. Fuggle, and A.L. Harris, *Hypoxia-inducible Factors HIF1 and HIF2 in Head and Neck Cancer.* Cancer Research, 2002.
55. Osada, R., A. Horiuchi, N. Kikuchi, J. Yoshida, A. Hayashi, M. Ota, Y. Katsuyama, G. Melillo, and I. Konishi, *Expression of hypoxia-inducible factor 1alpha, hypoxia-inducible factor 2alpha, and von Hippel-Lindau protein in epithelial ovarian neoplasms and allelic loss of von Hippel-Lindau gene: nuclear expression of hypoxia-inducible factor 1alpha is an independent prognostic factor in ovarian carcinoma.* Hum Pathol, 2007. **38**(9): p. 1310-20.
56. Rasheed, S., A.L. Harris, P.P. Tekkis, H. Turley, A. Silver, P.J. McDonald, I.C. Talbot, R. Glynne-Jones, J.M. Northover, and T. Guenther, *Hypoxia-inducible factor-1alpha and -2alpha are expressed in most rectal cancers but only hypoxia-inducible factor-1alpha is associated with prognosis.* Br J Cancer, 2009. **100**(10): p. 1666-73.
57. Roh, J.L., K.J. Cho, G.Y. Kwon, S.H. Choi, S.Y. Nam, and S.Y. Kim, *Prognostic values of pathologic findings and hypoxia markers in 21 patients with salivary duct carcinoma.* J Surg Oncol, 2008. **97**(7): p. 596-600.

58. Roh, J.L., K.J. Cho, G.Y. Kwon, C.H. Ryu, H.W. Chang, S.H. Choi, S.Y. Nam, and S.Y. Kim, *The prognostic value of hypoxia markers in T2-staged oral tongue cancer*. Oral Oncol, 2009. **45**(1): p. 63-8.
59. Yang, S.L., L.P. Liu, L. Niu, Y.P. Sun, X.R. Yang, J. Fan, J.W. Ren, G.G. Chen, and P.B. Lai, *Downregulation and pro-apoptotic effect of hypoxia-inducible factor 2 alpha in hepatocellular carcinoma*. Oncotarget, 2016.
60. Sivridis, E., A. Giatromanolaki, K.C. Gatter, A.L. Harris, M.I. Koukourakis, Tumor, and G. Angiogenesis Research, *Association of hypoxia-inducible factors 1alpha and 2alpha with activated angiogenic pathways and prognosis in patients with endometrial carcinoma*. Cancer, 2002. **95**(5): p. 1055-63.
61. Smeland, E., T.K. Kilvaer, S. Sorbye, A. Valkov, S. Andersen, R.M. Bremnes, L.T. Busund, and T. Donnem, *Prognostic impacts of hypoxic markers in soft tissue sarcoma*. Sarcoma, 2012. **2012**: p. 541650.
62. Sun, H.X., Y. Xu, X.R. Yang, W.M. Wang, H. Bai, R.Y. Shi, S.K. Nayar, R.P. Devbhandari, Y.Z. He, Q.F. Zhu, Y.F. Sun, B. Hu, M. Khan, R.A. Anders, and J. Fan, *Hypoxia inducible factor 2 alpha inhibits hepatocellular carcinoma growth through the transcription factor dimerization partner 3/ E2F transcription factor 1-dependent apoptotic pathway*. Hepatology, 2013. **57**(3): p. 1088-97.
63. Tong, W.W., G.H. Tong, X.X. Chen, H.C. Zheng, and Y.Z. Wang, *HIF2alpha is associated with poor prognosis and affects the expression levels of survivin and cyclin D1 in gastric carcinoma*. Int J Oncol, 2015. **46**(1): p. 233-42.
64. Wang, H.X., C. Qin, F.Y. Han, X.H. Wang, and N. Li, *HIF-2alpha as a prognostic marker for breast cancer progression and patient survival*. Genet Mol Res, 2014. **13**(2): p. 2817-26.
65. Wang, M., M.Y. Chen, X.J. Guo, and J.X. Jiang, *Expression and significance of HIF-1alpha and HIF-2alpha in pancreatic cancer*. J Huazhong Univ Sci Technolog Med Sci, 2015. **35**(6): p. 874-9.
66. Winter, S.C., K.A. Shah, C. Han, L. Campo, H. Turley, R. Leek, R.J. Corbridge, G.J. Cox, and A.L. Harris, *The relation between hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression with anemia and outcome in surgically treated head and neck cancer*. Cancer, 2006. **107**(4): p. 757-66.
67. Yang, S.L., L.P. Liu, J.X. Jiang, Z.F. Xiong, Q.J. He, and C. Wu, *The correlation of expression levels of HIF-1alpha and HIF-2alpha in hepatocellular carcinoma with capsular invasion, portal vein tumor thrombi and patients' clinical outcome*. Jpn J Clin Oncol, 2014. **44**(2): p. 159-67.
68. Zhang, Q., Y. Lou, J. Zhang, Q. Fu, T. Wei, X. Sun, Q. Chen, J. Yang, X. Bai, and T. Liang, *Hypoxia-inducible factor-2alpha promotes tumor progression and has crosstalk with Wnt/beta-catenin signaling in pancreatic cancer*. Mol Cancer, 2017. **16**(1): p. 119.
69. Zhu, G.Q., Y.L. Tang, L. Li, M. Zheng, J. Jiang, X.Y. Li, S.X. Chen, and X.H. Liang, *Hypoxia inducible factor 1alpha and hypoxia inducible factor 2alpha play distinct and functionally overlapping roles in oral squamous cell carcinoma*. Clin Cancer Res, 2010. **16**(19): p. 4732-41.
70. Tierney, J.F., L.A. Stewart, D. Ghersi, S. Burdett, and M.R. Sydes, *Practical methods for incorporating summary time-to-event data into meta-analysis*. Trials, 2007. **8**: p. 16.

71. van Kuijk, S.J., A. Yaromina, R. Houben, R. Niemans, P. Lambin, and L.J. Dubois, *Prognostic Significance of Carbonic Anhydrase IX Expression in Cancer Patients: A Meta-Analysis*. Front Oncol, 2016. **6**: p. 69.
72. Viechtbauer, W., *Conducting meta-analyses in R with the metafor package*. Journal of Statistical Software, 2010. **36**(3): p. 1-48.
73. DerSimonian, R. and N. Laird, *Meta-analysis in clinical trials*. Control Clin Trials, 1986. **7**(3): p. 177-88.
74. Razorenova, O.V., E.C. Finger, R. Colavitti, S.B. Chernikova, A.D. Boiko, C.K. Chan, A. Krieg, B. Bedogni, E. LaGory, I.L. Weissman, M. Broome-Powell, and A.J. Giaccia, *VHL loss in renal cell carcinoma leads to up-regulation of CUB domain-containing protein 1 to stimulate PKC $\{\delta\}$ -driven migration*. Proc Natl Acad Sci U S A, 2011. **108**(5): p. 1931-6.
75. Fan, Y., H. Li, X. Ma, Y. Gao, L. Chen, X. Li, X. Bao, Q. Du, Y. Zhang, and X. Zhang, *Prognostic Significance of Hypoxia-Inducible Factor Expression in Renal Cell Carcinoma: A PRISMA-compliant Systematic Review and Meta-Analysis*. Medicine (Baltimore), 2015. **94**(38): p. e1646.
76. Leek, R.D., C.E. Lewis, R. Whitehouse, M. Greenall, J. Clarke, and A.L. Harris, *Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma*. Cancer Res, 1996. **56**(20): p. 4625-9.
77. Leek, R.D., K.L. Talks, F. Pezzella, H. Turley, L. Campo, N.S. Brown, R. Bicknell, M. Taylor, K.C. Gatter, and A.L. Harris, *Relation of hypoxia-inducible factor-2 α (HIF-2 α) expression in tumor-infiltrative macrophages to tumor angiogenesis and the oxidative thymidine phosphorylase pathway in Human breast cancer*. Cancer Res, 2002. **62**(5): p. 1326-9.
78. Imtiyaz, H.Z., E.P. Williams, M.M. Hickey, S.A. Patel, A.C. Durham, L.J. Yuan, R. Hammond, P.A. Gimotty, B. Keith, and M.C. Simon, *Hypoxia-inducible factor 2 α regulates macrophage function in mouse models of acute and tumor inflammation*. J Clin Invest, 2010. **120**(8): p. 2699-714.
79. Ema, M., S. Taya, N. Yokotani, K. Sogawa, Y. Matsuda, and Y. Fujii-Kuriyama, *A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1 α regulates the VEGF expression and is potentially involved in lung and vascular development*. Proc Natl Acad Sci U S A, 1997. **94**(9): p. 4273-8.
80. Tian, H., S.L. McKnight, and D.W. Russell, *Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells*. Genes Dev, 1997. **11**(1): p. 72-82.
81. Flamme, I., T. Frohlich, M. von Reutern, A. Kappel, A. Damert, and W. Risau, *HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 α and developmentally expressed in blood vessels*. Mech Dev, 1997. **63**(1): p. 51-60.
82. Ladoux, A. and C. Frelin, *Cardiac expressions of HIF-1 α and HLF/EPAS, two basic loop helix/PAS domain transcription factors involved in adaptative responses to hypoxic stresses*. Biochem Biophys Res Commun, 1997. **240**(3): p. 552-6.
83. Wiesener, M.S., H. Turley, W.E. Allen, C. Willam, K.U. Eckardt, K.L. Talks, S.M. Wood, K.C. Gatter, A.L. Harris, C.W. Pugh, P.J. Ratcliffe, and P.H. Maxwell, *Induction*

of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. Blood, 1998. **92**(7): p. 2260-8.

84. Jain, S., E. Maltepe, M.M. Lu, C. Simon, and C.A. Bradfield, *Expression of ARNT, ARNT2, HIF1 alpha, HIF2 alpha and Ah receptor mRNAs in the developing mouse*. Mech Dev, 1998. **73**(1): p. 117-23.

85. Bersten, D.C., A.E. Sullivan, D.J. Peet, and M.L. Whitelaw, *bHLH-PAS proteins in cancer*. Nat Rev Cancer, 2013. **13**(12): p. 827-41.

86. Wiesener, M.S., J.S. Jurgensen, C. Rosenberger, C.K. Scholze, J.H. Horstrup, C. Warnecke, S. Mandriota, I. Bechmann, U.A. Frei, C.W. Pugh, P.J. Ratcliffe, S. Bachmann, P.H. Maxwell, and K.U. Eckardt, *Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs*. FASEB J, 2003. **17**(2): p. 271-3.

87. Keith, B., R.S. Johnson, and M.C. Simon, *HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression*. Nat Rev Cancer, 2011. **12**(1): p. 9-22.

88. Liao, D., C. Corle, T.N. Seagroves, and R.S. Johnson, *Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression*. Cancer Res, 2007. **67**(2): p. 563-72.

89. Chen, W., H. Hill, A. Christie, M.S. Kim, E. Holloman, A. Pavia-Jimenez, F. Homayoun, Y. Ma, N. Patel, P. Yell, G. Hao, Q. Yousuf, A. Joyce, I. Pedrosa, H. Geiger, H. Zhang, J. Chang, K.H. Gardner, R.K. Bruick, C. Reeves, T.H. Hwang, K. Courtney, E. Frenkel, X. Sun, N. Zojwalla, T. Wong, J.P. Rizzi, E.M. Wallace, J.A. Josey, Y. Xie, X.J. Xie, P. Kapur, R.M. McKay, and J. Brugarolas, *Targeting renal cell carcinoma with a HIF-2 antagonist*. Nature, 2016. **539**(1476-4687 (Electronic)): p. 112-117.

Figures and Tables

FIGURE 1: Flowchart of selecting articles describing the association between tumoral HIF-2 α expression and prognosis.

FIGURE 2: Summary of the overall hazard ratios (HRs) for different endpoints. Symbols represent the HR and horizontal bars the 95%CI [21-69].

FIGURE 3: Forest plot of Hazard Ratios (HR) with 95% CI (horizontal bars) for the association of HIF-2 α expression and overall survival (OS). Symbol size represents the assigned weight of the study [21, 24, 26, 28, 30, 31, 33-35, 37, 38, 41-43, 45, 46, 48, 49, 52, 54-57, 59, 60, 62-64, 66-69].

FIGURE 4: Forest plot of Hazard Ratios (HR) with 95% CI (horizontal bars) for the association between HIF-2α expression and disease-free survival (DFS). Symbol size represents the assigned weight of the study [36, 37, 54, 66, 67, 69].

FIGURE 5: Forest plot of Hazard Ratios (HR) with 95% CI (horizontal bars) for the association between HIF-2α expression and disease-specific survival (DSS). Symbol size represents the assigned weight of the study [24, 32, 34, 35, 58, 61, 66].

FIGURE 6: Forest plot of Hazard Ratios (HR) with 95% CI (horizontal bars) for the association between HIF-2 α expression and locoregional control (LC). Symbol size represents the assigned weight of the study [35, 40, 44-46, 52, 56-59].

FIGURE 7: Forest plot of Hazard Ratios (HR) with 95% CI (horizontal bars) for the association between HIF-2 α expression and metastasis-free survival (MFS). Symbol size represents the assigned weight of the study [53, 57].

FIGURE 8: Forest plot of Hazard Ratios (HR) with 95% CI (horizontal bars) for the association between HIF-2 α expression and progression-free survival (PFS). Symbol size represents the assigned weight of the study [48, 53].

Organ site	OS	DFS	DSS	LC	MFS	PFS
Bladder				0.51 (0.33 – 0.77)*		
Brain	3.78 (1.64 – 8.75)*					
Breast	1.18 (0.95 – 1.47)		2.3 (1.3 – 4.1)*	1.6 (1 – 2.4)*		
Cartilage	4.13 (1.47 – 11.58)*					
Cervix				1.53 (1.14 – 2.03)*		
Colorectal	1.46 (0.7 – 3.04)	1.86 (0.89 – 3.92)*	0.8 (0.61 – 1.05)*	0.54 (0.25 – 1.17)*		
Endometrium	5.72 (1.51 – 21.6)*					
Head and Neck	1.55 (1.24 – 1.92)	1.41 (0.99 – 2)	1.45 (0.8 – 2.64)	1.94 (1.43 – 2.63)		
Kidney	0.61 (0.27 – 1.35)		1.21 (0.57 – 2.6)	0.54 (0.11 – 2.57)	0.08 (0.03 – 0.27)*	0.75 (0.32 – 1.73)
Liver	1.06 (0.43 – 2.61)	1.52 (0.82 – 2.84)*		1.22 (0.87 – 1.83)*		
Lung	2.15 (1.65 – 2.81)	8.47 (3.26 – 22.06)*				
Ovarium	2.72 (1.27 – 5.83)*					
Pancreas	2.11 (1.38 – 3.24)					1.67 (1.26 – 2.21)*
Prostate					2.44 (1.07 – 5.57)*	2.94 (1.99 – 4.36)*
Salivary Glands	4.52 (1.06 – 19.3)*			3.64 (0.72 – 18.42)*	3.36 (0.89 – 12.66)*	
Skin	3.21 (1.15 – 8.97)*		3.81 (1.66 – 8.78)*			
Soft tissues			1.53 (1.03 – 2.28)*			
Stomach	1.7 (1.25 – 2.32)		1.6 (1 – 2.4)*			

TABLE 1: Summary of results of sub-group meta-analyses of different organ sites. The HR with 95%CI is shown. Highlighted numbers indicate statistically significant association between HIF-2 α expression and prognosis ($p < 0.05$). *When HR was available from only one paper, the values were adopted from that single paper [21-69].

SUPPLEMENTARY FIGURE 1: Funnel plots of papers reporting on OS (A), DFS (B), DSS (C), LC (D), MFS (E), and PFS (F). The x-axis represents the HR for each paper with the corresponding standard error on the y-axis. Heterogeneity is significant for OS (A), DFS (B), LC (C), DSS (D), and PFS (F) [21-69].

Study	Endpoint	Organ	Patients	Comments
Nanni 2009	DSS	Prostate	88	“While no correlation was found between HIF-2 α expression and DSS”.
Liang 2011	DSS	Head and Neck	89	“However, HIF- 2 α had no significant association with either overall survival (P = 0.195, Fig. 1b) or disease-free survival (P = 0.356).” Only OS data shown.
Wang 2015	OS	Pancreas	90	Only P value reported (0.0078).
Hui 2002	OS	Head and Neck	89	“No significant association between HIF-2, CA IX, or VEGF expression with respect to OS or PFS was found”.
Hui 2002	PFS	Head and Neck	89	“No significant association between HIF-2, CA IX, or VEGF expression with respect to OS or PFS was found”.

SUPPLEMENTARY TABLE 1: Summary of papers that reported inadequate or insufficient data to estimate the HR. A brief description of the available data is given in the comments [39, 49, 53, 65].

Study	Subcellular localization	HR(95%CI)
Biswas 2012	Nuclear	0.77 (0.44 – 1.33)
Kroeger 2014	Nuclear	0.61 (0.39 – 0.97)
Kroeger 2014	Cytoplasm	2.33 (1.6 – 3.39)
Liu 2018	Cytoplasm	2.27 (0.84 – 6.17)

SUPPLEMENTARY TABLE 2: Summary of papers that reported association between HIF-2 α expression and disease-specific survival (DSS) depending on subcellular localization in clear cell renal cell carcinoma patients. Highlighted numbers indicate statistically significant associations [25, 47, 50].

Research question: What is the prognostic value of tumoral HIF2 α expression in patients with solid tumors?

Keywords identified as search terms:

Prognosis, Cancer, and HIF2

Search algorithm Pubmed:

Prognosis:

(Prognos*) OR (prognostic value) OR (clinicopathological) OR (surviv*) OR (hazard) OR (disease-free) OR (“disease free”) OR (progression-free) OR (“progression free”) OR (Kaplan-meier) OR (“Kaplan meier”) OR (predict*) OR (outcome) OR (efficacy) OR (effective*)

Cancer:

(tumor) OR (tumors*) OR (tumor’s) OR (tumoral*) OR (“tumor associated”) OR (tumor-associated) OR (“tumor related”) OR (tumor-related) OR (tumorigen*) OR (tumorous*) OR (tumour*) OR (cancer) OR (cancers) OR (cancer’s) OR (cancerogen*) OR (cancera*) OR (cancer-associate*) OR (cancerigen*) OR (cancerno*) OR (cancero*) OR (cancerp*) OR (“cancer related”) OR (cancer-related) OR (*carcinoma) OR (*sarcoma) OR (neoplas*) OR (malignanc*) OR (melanoma)

HIF2:

(HIF2) OR (HIF-2) OR (EPAS1) OR (Endothelial PAS domain-containing protein 1) OR (Endothelial PAS domain protein 1) OR (HIF-2 α) OR (HIF2 α) OR (hypoxia-inducible factor 2 α) OR (hypoxia-inducible factor-2 α)

Prognosis AND Cancer AND HIF2:

((Prognos*) OR (prognostic value) OR (clinicopathological) OR (surviv*) OR (hazard) OR (disease-free) OR (“disease free”) OR (progression-free) OR (“progression free”) OR (Kaplan-meier) OR (“Kaplan meier”) OR (predict*) OR (outcome) OR (efficacy) OR (effective*)) AND ((tumor) OR (tumors*) OR (tumor’s) OR (tumoral*) OR (“tumor associated”) OR (tumor-associated) OR (“tumor related”) OR (tumor-related) OR (tumorigen*) OR (tumorous*) OR (tumour*) OR (cancer) OR (cancers) OR (cancer’s) OR

(cancerogen*) OR (cancera*) OR (cancer-associate*) OR (cancerigen*) OR (cancerno*) OR (cancero*) OR (cancerp*) OR (“cancer related”) OR (cancer-related) OR (*carcinoma) OR (*sarcoma) OR (neoplas*) OR (malignanc*) OR (melanoma)) AND ((HIF2) OR (HIF-2) OR (EPAS1) OR (Endothelial PAS domain-containing protein 1) OR (Endothelial PAS domain protein 1) OR (HIF-2 α) OR (HIF2 α) OR (hypoxia-inducible factor 2 α) OR (hypoxia-inducible factor-2 α))

Hits: 626

Search algorithm Embase:

Prognosis:

(Prognos\$) OR (prognostic value) OR (clinicopathological) OR (surviv\$) OR (hazard) OR (disease-free) OR (“disease free”) OR (progression-free) OR (“progression free”) OR (Kaplan-meier) OR (“Kaplan meier”) OR (predict\$) OR (outcome) OR (efficacy) OR (effective\$)

Cancer:

(tumor) OR (tumors\$) OR (tumor’s) OR (tumoral\$) OR (“tumor associated”) OR (tumor-associated) OR (“tumor related”) OR (tumor-related) OR (tumorigen\$) OR (tumorous\$) OR (tumour\$) OR (cancer) OR (cancers) OR (cancer’s) OR (cancerogen\$) OR (cancera\$) OR (cancer-associate\$) OR (cancerigen\$) OR (cancerno\$) OR (cancero\$) OR (cancerp\$) OR (“cancer related”) OR (cancer-related) OR (\$carcinoma) OR (\$sarcoma) OR (neoplas\$) OR (malignanc\$) OR (melanoma)

HIF2:

(HIF2) OR (HIF-2) OR (EPAS1) OR (Endothelial PAS domain-containing protein 1) OR (Endothelial PAS domain protein 1) OR (HIF-2 α) OR (HIF2 α) OR (hypoxia-inducible factor 2 α) OR (hypoxia-inducible factor-2 α)

Prognosis AND Cancer AND HIF2:

((Prognos\$) OR (prognostic value) OR (clinicopathological) OR (surviv\$) OR (hazard) OR (disease-free) OR (“disease free”) OR (progression-free) OR (“progression free”) OR

(Kaplan-meier) OR (“Kaplan meier”) OR (predict\$) OR (outcome) OR (efficacy) OR (effective\$)) AND ((tumor) OR (tumors\$) OR (tumor’s) OR (tumoral\$) OR (“tumor associated”) OR (tumor-associated) OR (“tumor related”) OR (tumor-related) OR (tumorigen\$) OR (tumorous\$) OR (tumour\$) OR (cancer) OR (cancers) OR (cancer’s) OR (cancerogen\$) OR (cancera\$) OR (cancer-associate\$) OR (cancerigen\$) OR (cancerno\$) OR (cancero\$) OR (cancerp\$) OR (“cancer related”) OR (cancer-related) OR (\$carcinoma) OR (\$sarcoma) OR (neoplas\$) OR (malignanc\$) OR (melanoma)) AND ((HIF2) OR (HIF-2) OR (EPAS1) OR (Endothelial PAS domain-containing protein 1) OR (Endothelial PAS domain protein 1) OR (HIF-2 α) OR (HIF2 α) OR (hypoxia-inducible factor 2 α) OR (hypoxia-inducible factor-2 α))

Hits: 10

Literature search was performed in Pubmed and Embase on the 1th of February 2018.

SUPPLEMENTARY FILE 1. Literature search strategy to identify papers for the meta-analysis that describe an association between tumoral HIF2 α expression and prognosis.

CHAPTER 3

HIF-1 α and HIF-2 α differently regulate the radiation sensitivity of NSCLC cells, partly by impairing their metabolic profile

Eloy Moreno Roig, Arjan J. Groot*, Ala Yaromina*, Tessa C. Hendrickx, Lydie M.O. Barbeau, Lorena Giuranno, Glenn Dams, Jonathan Ient, Veronica Olivo Pimentel, Marike van Gisbergen, Ludwig Dubois and Marc Vooijs

Department of Radiotherapy (MAASTRO), GROW – School for Oncology and Developmental Biology, Maastricht University, Maastricht, Netherlands

Corresponding author: Marc Vooijs, Department of Radiotherapy (MAASTRO), GROW – School for Oncology and Developmental Biology, Maastricht University, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands; E-mail: marc.vooijs@maastrichtuniversity.nl

Submitted

Abstract

Nutrient and oxygen deprivation (hypoxia) are a widespread feature of rapidly proliferating tumors, caused by aberrant tumor vasculature and associated with a poor outcome and response to treatment. The hypoxia inducible transcription factor (HIF- α) is one of the main oxygen sensors which regulate the adaptation to intratumoral hypoxia. In mammals there are three highly conserved HIF- α orthologs (HIF1-3) of which HIF-1 α and HIF-2 α are most frequently implicated in tumorigenesis. To directly assess the unique and overlapping functions of HIF-1 α and HIF-2 α , we use CRISPR gene-editing to generate isogenic H1299 non-small cell lung carcinoma (NSCLC) cells lacking HIF-1 α (H1KO), HIF-2 α (H2KO) or both (dHKO). We found that in H1KO cells, HIF-2 α was strongly induced by hypoxia compared to wild type H1299 but the reverse was not seen in H2KO cells. Cells lacking HIF-1 α were more radiation resistant than H2KO and wild type cells upon hypoxia (0.2% O₂) and this was associated with a reduced recruitment of γ -H2AX foci directly after irradiation and not due to differences in proliferation. Conversely, dHKO cells were most radiation sensitive and had increased γ -H2AX recruitment and cell cycle delay. There were no differences in the activation of the DNA damage response proteins pATM and CHK2. When grown under hypoxia, H1KO cells uniquely had a strong increase in lactate production and decrease in extracellular pH. Moreover, HIF-1 α and HIF-2 α deficiency impacted in the glycolytic capacity under hypoxia. Finally, the absence of HIF-1 α reduced the number of lipid droplets (LD) in cells. Taken together, using genetically identical HIF- α isoform-deficient cells we identified a strong effect of HIF-1 α but not HIF-2 α on the radiation response, which was associated with a reduced extracellular pH and reduced glycolysis. We propose that HIF-2 α upregulation, in HIF-1 α null cells is in part responsible for these effects.

Introduction

Regions of oxygen deprivation (hypoxia) are a common feature of many solid cancers and are caused by an aberrant tumor vasculature. Hypoxic tumor cells activate stress response pathways in order to adapt to these low oxygen levels [1]. Within the tumor, some cells react to hypoxic stress with adaptive responses that, by changing their gene expression, confer an aggressive phenotype and resistance to therapy [2, 3]. Importantly, hypoxic tumors are more resistant to chemotherapy because they are poorly perfused, proliferate slower and also upregulate drug efflux pumps. Hypoxic tumor cells are also more radiation resistant. Irradiation primarily induces water ionization leading to the formation of OH $\cdot$ radicals, which impact and damage the DNA. In oxygenated cells, the OH $\cdot$ radical-induced DNA damage is irreversibly converted to a carboxyl group, thus stabilizing the DNA damage, whereas in hypoxic cells, damage produced by the free radicals is scavenged and consequently repaired [4]. Decreased treatment sensitivity of hypoxic tumor cells leads to chemo-radiotherapy treatment failure in cancer patients [5]. Despite the limited success of hypoxia modification therapy strategies in several clinical trials, hypoxia is still a promising tumor-selective therapeutic target. Accurate methods to evaluate the extent of hypoxic regions and their biological behavior in tumors however are essential to discriminate which patients will benefit from hypoxic modification [6, 7].

Mammalian cells encode for three hypoxia inducible factors (HIF)- α orthologs (HIF1-3) which all have been demonstrated to be oxygen-regulated. HIFs are bHLH-PAS domain heterodimers composed of a constitutively expressed β -subunit and an O $_2$ -regulated α -subunit, which is hydroxylated at two proline residues under aerobic conditions by O $_2$ -dependent prolyl hydroxylase domain proteins (PHDs) [8]. Factor inhibiting HIF-1 α (FIH) is an asparagine hydroxylase protein that suppresses HIF activity under normoxic conditions [9]. Other ways to induce HIF activity independently of the oxygen levels include the oncogene activation or tumor suppressor gene deletion for example the PI3K/AKT and PTEN signalling pathways [10]. When oxygen is present, prolyl-hydroxylated HIF- α undergoes rapid ubiquitination and subsequent proteasomal degradation involving the von Hippel–Lindau protein (pVHL), which is a member of the E3 ubiquitin protein ligase family. Under hypoxic conditions, the O $_2$ -dependent PHD/FIH are inhibited, HIF- α accumulates, forms a heterodimer with HIF- β and induces transcriptional activation of canonical HIF target genes such as carbonic anhydrase IX (CAIX), glucose transporter 1 (GLUT1) and vascular endothelial growth factor (VEGF) [11]. Additionally, HIF proteins interact with important

cell cycle and cell fate determination pathways such as Myc or Notch, to control malignant tumor cell behaviour [12]. HIF-1 α and HIF-2 α proteins bind to the same common hypoxia response elements (HRE) [13], but their transcriptional activities are determined by multiple factors including the presence of repressors, differential sensitivity to PHD/FIH hydroxylation and differential recruitment of co-activators [14]. In particular, differences in tissue-specific and temporal induction patterns of each isoform suggest that HIF-1 α and HIF-2 α may have distinct transcriptional targets that regulate specific aspects of hypoxic signalling and adaptation in different cell types [15] [16, 17]. This specific gene expression profile in hypoxic cells by either HIF-1 α and/or HIF-2 α can contribute differently to a malignant phenotype in cancer cells. For instance, genes with a role in Oct4-regulated cell pluripotency and therefore often deregulated in cancer are principally induced by HIF-2 α [18]. HIF stabilization is frequently observed in cancers associated with malignant phenotype and is an important prognostic factor for poor outcome in most solid cancers [19] [20, 21].

Preclinical studies have demonstrated the potential to pharmacologically block both HIF-1 α and/or HIF-2 α causing tumor growth delay and improved local control [22-24]. Pharmacological inhibition of HIF-1 α increases the therapeutic efficacy of radiation treatment both *in vitro* and *in vivo* [25, 26]. Following radiotherapy, cancer cells increase HIF-1 α accumulation in the nucleus, which leads to the secretion of survival factors that enhance endothelial cell radioresistance [27]. In particular, cells lacking HIF-1 α are markedly more sensitive to altered redox activity and glucose deprivation, suggesting a protective role upon stress conditions in tumors [28]. The metabolic switch towards increased glycolysis in hypoxic environments also affects the ability of these cells to survive therapy. For instance, the enhanced glycolytic rate increases the intracellular amounts of lactate and CO₂, which are transported out of the cell contributing to an extracellular acidic pH [29] and an increased therapy resistance [30, 31]. In addition, lactate can also be produced by elevated glutaminolysis activity, whereby glutamine is used as a metabolic source to generate pyruvate in the cytosol, which is ultimately reduced by lactate dehydrogenase A (LDH-A), producing lactate and NAD⁺ [32]. It has been demonstrated that HIF-1 α and HIF-2 α bind to the promoter region of LDH-A leading to its expression in human pancreatic cancer cells [33]. The substantial increase of lactate production during hypoxia allows the scavenging of ROS molecules generated after radiation exposure due to the antioxidant properties of lactate [34] [35, 36]. This metabolic reprogramming also leads to an increased accumulation of LD in the cytoplasm to cope with the higher energy demand of cancer cells [37]. LD accumulation in

cancer is associated with higher tumor aggressiveness by promoting epithelial-mesenchymal transition [38].

Previous studies using different tumor models have implicated HIF-1 α in radiation resistance via cell autonomous and non-cell autonomous mechanisms. As described above, activation of HIF-1 α in tumor cells alters metabolic response following radiation through mediating changes in lactate production and mitochondrial function [39]. Moreover, activation of HIF-1 α in tumor cells increases the secretion of surviving factors such as VEGF to promote the proliferation of tumor endothelial cells [27]. In turn, HIF-2 α expression has been seen to be crucial to promote aggressive phenotype and tumor growth of clear cell renal cell carcinoma (ccRCC) [40, 41] and neuroblastoma tumors [16]. Importantly, radiation enhances HIF-1 α stabilization and consequently its binding to the CXCR4 promoter, which induces an invasive and metastatic phenotype in NSCLC cells [42]. HIF proteins are important regulators of the tumorigenic capacity and therapy sensitivity of cancer cells; however, the regulatory mechanism of HIF-1 α and HIF-2 α mediating the response of NSCLC to radiation therapy needs further investigation. Previous studies have shown that the expression of HIF-1 α and HIF-2 α differently impact on patient prognosis and other clinicopathological characteristics in several cancers such as human NSCLC [43, 44]. Moreover, HIF-2 α is highly expressed in cancer stem cells, which has been associated with a radioresistant phenotype in lung cancer. Consequently, HIF-2 α negatively correlates with prognosis in patients treated with radiation [45].

Taken together, mounting evidence implicates both HIF-1 α and HIF-2 α proteins in a highly context dependent but orchestrated manner in tumor cell survival, hypoxia tolerance and treatment response. Here we used CRISPR/Cas9 gene-editing to generate loss-of-function deletions in HIF-1 α , HIF-2 α or both HIF-1 α and HIF-2 α in H1299 NSCLC cells. Using these cells, we assessed the contribution of HIF-1 α and HIF-2 α factors as important mediators of hypoxia response, radiation response and metabolic adaptation.

Materials and methods

Cell culture and hypoxia exposure

Human non-small cell lung carcinoma (NSCLC) H1299 (ATCC CRL-5803) cells were grown in RPMI 1640 (Westburg) supplemented with 10% FBS (Sigma-Aldrich), penicillin (10 U/ml) and streptomycin (10ug/ml). Normoxic cells were incubated in a humidified atmosphere with 5% CO₂/21% O₂ at 37 °C. Cells were exposed to hypoxic conditions in a hypoxic chamber (MACS VA500 microaerophilic workstation, Don Whitley Scientific, UK) to stabilize HIF proteins. The composition of the atmosphere in the incubator consisted of 5% CO₂, 0.2% O₂ and residual N₂. The identity of H1299 cells was verified by STR genotyping (Identicell Denmark).

CRISPR/Cas9-mediated knockout of human HIF1 α and HIF2 α genes

In order to generate HIF-1 α and HIF-2 α knockouts, H1299 cells were transfected with CRISPR/Cas9 using pSpCas9(BB)-2A-GFP (PX458) or pSpCas9(BB)-2A-Puro (PX459), a gift from Feng Zhang (Addgene plasmid #48139) [46]. Two small guide RNA (sgRNA) targeting the HIF-1 α and HIF-2 α locus were designed using the CRISPR Design Tool (<http://crispr.mit.edu>). Transfections were performed with Lipofectamine 2000 (Thermo fisher) and Puromycin resistant cells were detected after 3 days of puromycin selection (1 μ g/ml) followed by a recovery period of two weeks. Surviving cells were single cell seeded and expanded for further analysis. Genomic DNA was extracted from the potential candidates and the targeted regions of the HIF-1 α and HIF-2 α genes were PCR amplified. The product was cloned into TOPO-TA vector to isolate every single allele from the whole cell population. DNA fragments were sequenced and analysed for further selection of total HIF-1 α and HIF-2 α knockout clones. Multiple independent H1299 cell lines carrying mutations in HIF-1 α (H1KO) and HIF-2 α (H2KO) genes were established. Additionally, the H1KO-B5 clone was used as a cellular template to target the HIF-2 α proteins and thus create a double knockout cell line (dHKO) (Supplementary Figure 1).

Western blot

To validate HIF expression in the genetically modified cell lines, immunoblotting was performed after 24h of hypoxia exposure (0.2% O₂). Cells were washed with PBS quickly after either hypoxic or normoxic incubation and lysed in ice-cold RIPA buffer. Samples were kept on ice for 30 minutes and frozen in liquid N₂ for 1 minute. Protein lysates were collected

after thawing and spinning down the samples to discard cell debris. The protein concentrations were measured by using the Bradford protein assay (Bio-Rad). The proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (6 to 10% polyacrylamide), transferred onto nitrocellulose membranes and blocked for 1 hour in 5% skim milk in PBS or TBS, 0.2% Tween-20. Membranes were probed overnight at 4°C with primary antibodies and bound antibodies were visualized using HRP-linked secondary antibodies (Cell signalling) and ECL Luminescence (Pierce Biotechnology). Primary antibodies used included the anti-HIF1 α antibody (BD-biosciences), anti-HIF2 α antibody (Novus Biologicals), HIF1 β antibody (BD-biosciences), pATM pS 1981 (abcam), phospho-Chk2 (Thr68) (Bioke), Chk2 (Bioke) and anti-Lamin A/C (Sigma-Aldrich) as a loading control. Secondary anti-mouse and anti-rabbit antibodies (1:2500) were from Cell Signaling.

RNA isolation and quantitative PCR analysis

mRNA was extracted using the NucleoSpin RNA II kit (Bioke) and cDNA conversion was performed using the iScript cDNA Synthesis kit (BioRad) according to the manufacturers' instructions. Quantitative PCR was performed on the CFX96 (BioRad). The expression of the genes of interest was detected with SYBR Green I (Eurogentec). Values for each gene were normalized to HPRT expression levels. Primer sequences are listed in the S1 table.

Proliferation and clonogenic survival assay

Proliferation was monitored during 2 days by counting cells in an automatic cell counter. For clonogenic survival, cells were counted and seeded on day 0. Cells were placed under 0.2% O₂ the day after for 24h. Irradiation was performed using a 225kV Philips X-ray tube on day 2. Subsequently, cells were trypsinized and plated in triplicate for clonogenic survival. Cells were allowed to form colonies during 12 days, fixed and stained with a 0.4% methylene blue (Sigma-Aldrich) in 70% ethanol solution. Colonies were defined as >50 cells.

Cell viability – Hypoxia tolerance

Crystal violet staining was performed to assess cell viability. Cells were incubated in 6-well plates at 1.0×10^4 cells per well and cell viability was measured at 5 days after hypoxic incubation. Culture medium was removed and cells were fixed with 4% paraformaldehyde at room temperature for 10 min and then stained with 0.05% crystal violet for 30 min. The cells were then washed with tap water, after which the water was removed and the cells were dried out on filter paper. After the plates were photographed, blue dye was dissolved in 500 μ l of

methanol and emission spectra were measured at an excitation wavelength of 540 nm using a Multimode Microplate Reader.

γ -H2AX immunocytochemistry

Cells were fixed with ice-cold 100% methanol. Subsequently cells were permeabilized with 0.2% Triton-X solution (in PBS) and normal goat serum was used as blocking agent. Cells were stained with a primary anti-phospho(Ser139)-H2AX antibody (1:500, Millipore) followed by anti-rabbit Alexa Fluor 488 (1:500, Invitrogen) as secondary antibody. Hoechst 33342 (20ug/ml, Sigma-Aldrich) was used as nuclear counter stain.

Image analysis

For evaluation of γ -H2AX staining, fluorescent images were captured at 64x objective using a Leica TCS SPR confocal microscope. Images were taken in 2 μ m planes along the Z-axis and foci analysis was performed in maximal intensity projection Z-stacks using ImageJ software (v. 1.50, National Institutes of Health) in a semi-automated way. Nine images have been acquired per treatment condition resulting in 100 ± 20 total number of cells analyzed. First, DAPI positive nuclei were selected using a manual segmentation procedure based on signal intensity and background staining and nucleus area was recorded. Only complete intact nuclei per image field were evaluated. Second, raw integrated density (RID) of γ -H2AX per nucleus, i.e. the sum of the gray values of the pixels, was calculated. To correct for variability in nucleus size, RID was normalized to the nucleus area for each cell. Data are presented as mean $\pm$ SD of normalized RID per cell line and treatment group. To validate the method for γ -H2AX quantification, γ -H2AX foci were manually counted in randomly selected images in a manner blinded to the cell line and treatment group. Comparison of γ -H2AX foci with normalized RID shows significant correlation ($r=0.9608$, $p=0.000146$) indicating that normalized RID reflects the number of γ -H2AX foci.

Cell cycle analysis

For cell cycle analysis, cells were incubated either under normoxic or hypoxic conditions for 24 hours, exposed to radiation and placed under normoxia for 4 hours. Cells were washed with PBS, treated with trypsin and fixed in ice-cold 70% ethanol for at least 24h. Before analysis, cells were washed with PBS and stained with propidium iodide (PI) for 30 minutes at room temperature. Analysis was performed using a FACS CANTO II. Data obtained from the cell cycle distributions were analysed using a FlowJo_10.

pH and extracellular L-lactic acid measurements

Changes in extracellular pH were monitored using a pH meter (Beckman Coulter pH 350). Cells were seeded at different cell numbers and incubated for 24 hours under 0.2% O₂. Levels of extracellular L-Lactic acid were measured using the L-Lactic acid kit (Biosentec) according to manufacturer's guidelines. Both pH and L-Lactic acid levels were corrected for cell counts.

Metabolic profiling

Cells were seeded at an optimized cell density of 3x10⁴ cells/well. Metabolic profiles were generated by replacing the growth medium for assay media 1 hour before using the Seahorse XF96 extracellular Flux analyzer (Seahorse Bioscience) according to manufacturer's guidelines [47]. Cells were pre-incubated (24h) with 100 nM desferrioxamine (DFO), an iron chelator that stabilizes HIF, as well as during the Seahorse measurements. The glycolysis stress test was performed by measuring the extracellular acidification rate (ECAR) after sequential addition of 10 mM glucose, optimized oligomycin concentration 1.0 μ M (all cell lines) and 0.1 M 2-deoxyglucose (2-DG) (Sigma-Aldrich). Calculations of the glucose metabolism and glycolytic reserve were done according to the Seahorse Bioscience guidelines. Baseline OCR or ECAR was determined prior to the first compound injection using a mixing period of 5 minutes and a measurement period of 3 minutes followed by 3 loops of mixing and measuring for 3 minutes each. Every injection was followed by the same measurement protocol of a mixing period of 5 minutes and a measurement period of 3 minutes followed by 3 loops of mixing and measuring for 3 minutes.

Nile staining

To visualize LD after usual immunofluorescence microscopy procedure cells were stained with Nile Red (Sigma; 0.1 μ g in PBS) for 15 minutes, washed with PBS and mounted on slides [48].

Statistics

All assays were performed at least three times, and results are expressed as means $\pm$ standard deviations. Analyses were performed with GraphPad Prism 5. Unpaired two-tailed Student's *t* tests or Mann-Whitney U test were performed to determine

significance. Sum-square test for curve comparison was performed to determine significance of clonogenic data. p values ≤ 0.05 were considered significant.

Results

To examine the radiobiological and metabolic properties of HIF-1 α and HIF-2 α , we generated HIF-1 α and HIF-2 α loss-of-function mutants in H1299 cells using the type II CRISPR/Cas9 gene editing system. Single allele sequencing confirmed that H1KO, H2KO and dHKO cells carried mutations that led to premature termination of the HIF- α open reading frame. Each knockout harboured two or three different mutated alleles leading to one or several STOP codons (Supplementary Figure 2). We verified that H1299 clones did not have the Cas9 plasmid integrated (data not shown). Western blotting confirmed the absence of HIF proteins (Figure 1A). Interestingly, we observed a prominent increase in HIF-2 α stabilization following hypoxia incubation in H1KO cells, however without elevated HIF-2 α mRNA expression levels (Supplementary Figure 3). On the contrary, HIF-2 α -deficiency did not influence the hypoxic induction of HIF-1 α protein expression. In addition, the overall expression levels of HIF-1 β were decreased in all the knockout models in comparison with wild type cells (Figure 1A). Next we determined the mRNA expression levels of the canonical hypoxia-induced genes CAIX, GLUT1, CITED2 and TWIST1. We observed that the induction of these genes was severely compromised in the absence of HIF-1 α and/or HIF-2 α proteins under hypoxia (Figure 1B). Furthermore, only small differences were seen in the proliferative capacity of single HIF mutants in comparison with wild type cells, both under normoxic and low oxygen conditions. However, dHKO cells showed a significant ($p=0.0124$) proliferative delay under normoxic conditions (Figure 1C). Additionally, only dHKO cells demonstrated a small but significant ($p=0.0494$) reduction in survival under hypoxic conditions, whereas single HIF- α disrupted cells did not differ in their response to hypoxia (Figure 1D).

Next, we assessed the impact of HIF-1 α and HIF-2 α deletion on clonogenic survival following irradiation. Under normoxic conditions, no differences in survival were observed between wild type and H1KO or H2KO cells, while overall survival was significantly ($p=0.0006$) reduced for dHKO cells (Figure 2A). Additionally, clonogenic survival assays revealed that under hypoxic conditions the overall surviving fraction of H1KO cells was significantly ($p<0.0001$) higher than wild type cells (Figure 2B). Similar results were observed for an independently derived H1KO clone (Supplementary Figure 4). We also observed a more radiosensitive phenotype in dHKO cells irradiated under hypoxic conditions although to a lesser extent. Next, we investigated whether differences in the DNA damage response (DDR) or DNA repair were responsible for these differences in radiation sensitivity.

No DNA damage was observed in the absence of irradiation under normoxic or hypoxic conditions using γ -H2AX staining. Upon irradiation of cells under normoxia, γ H2AX recruitment increased in all genotypes, but significantly more in dHKO cells ($p=0.0007$) (Figure 3A). Upon hypoxia we observed a strong and significant decrease (85%, ($p=0.002$)) of γ H2AX labelling in irradiated H1KO cells compared to wild type and H2KO cells (Figure 3B). γ H2AX recruitment was highest in dHKO cells. These differences disappeared 24h after irradiation independently of the oxygen concentration. The γ H2AX labelling in irradiated cells under hypoxia and normoxia is consistent with the long-term survival outcome of dHKO and H1KO cells.

To identify the immediate effects of HIF-1 α and HIF-2 α on the expression and activation of important DDR proteins, we examined the expression of pATM and its downstream target p(Thr68)-CHK2 after irradiation (Figure 4). Levels of pATM were equally induced upon radiation independent of the oxygenation status and HIF genotype. Exposure to radiation enhanced pCHK2 levels both upon normoxia and hypoxia for all cells, except for dHKO cells where under normoxia a slight decrease was observed. Strikingly, levels of total and phosphorylated CHK2 protein were upregulated in single and double-HIFKO cells in comparison with wild type cells. Similar results were observed in an independent series of experiments (Supplementary Figure 5).

Flow cytometry was used to determine the effect on cell cycle distribution in irradiated cells lacking HIF-1 α , HIF-2 α or both. dHKO cells showed a slight delay in cell proliferation (Figure 1C), which was supported by a significant ($p=0.0305$) reduction in the G1 population (Figure 5A). As expected, irradiation induced a G2/M cell cycle arrest in all cells regardless of the presence of HIF. In line with the survival data, this effect was more prominent ($p=0.0074$) in dHKO (45%) in comparison with wild type (36%) cells. The increase in percentage of cells in G2/M was accompanied by a significant ($p=0.0180$) decrease in cells in S phase (Figure 5B). In addition, wild type and HIF-deficient cells incubated under hypoxic conditions for 24 hours showed an overall decrease in S phase and accumulation in the G2/M phase. Under hypoxia, a decrease ($p=0.0441$) in G1 phase was observed for the dHKO cells in comparison with wild type cells, which is consistent with an enhanced ($p=0.0297$) accumulation in G2/M phase (Figure 5C). However, when irradiation was performed under hypoxia, no additional effects on cell cycle distribution were observed (Figure 5D).

To determine whether changes in extracellular acidification are involved in the radiation response of HIF-deficient cells under hypoxia, we performed *in vitro* extracellular pH measurements and assessed lactic acid release into the medium both under normoxic and hypoxic conditions. No differences in extracellular pH and lactic acid concentration were observed under normoxic conditions (Figure 6A, C). After hypoxic incubation, the pH dropped for all cellular models, but extracellular pH was significantly ($p=0.0017$) more reduced in H1KO cells (Figure 6B), with a concomitant increase ($p=0.0008$) of lactic acid in medium (Figure 6D). In order to investigate whether lactate transporters are differently regulated in the genetic models, we measured the mRNA expression levels of the monocarboxylate transporter 1 (MCT-1) and 4 (MCT-4). While the expression levels of MCT-4 were regulated by hypoxia in H1299 cells, MCT-1 levels were unaffected in H1299 cells (Figure 6E). The hypoxia-induced expression of MCT4 expression was perturbed in all HIF-deficient cells and strongly reached significance ($p=0.0009$) in H1KO cells (Figure 6F).

To investigate the impact of HIF-1 α and HIF-2 α deletion on cellular respiratory metabolism, we measured extracellular acidification rate (ECAR) and LD accumulation. Basal ECAR was measured under control conditions and in the presence of desferrioxamine (DFO), an iron chelator that induces pseudo-hypoxia by blocking PHDs and stabilizing HIF proteins. Our results demonstrated that HIF-deficient cells did not show significant differences in ECAR compared to wild type cells under control conditions (Figure 7A). On the other hand, when cells were treated with DFO, dHKO cells showed a significant ($p=0.0286$) reduction in basal ECAR compared to wild type cells (Figure 7B). No differences in mitochondrial respiration were found when assessing basal oxygen consumption rate (OCR) levels (Supplementary Figure 6). Further analyses on the glycolytic profile of the genetic models was performed by measuring ECAR during a glycolytic stress test. As expected, the absence of HIF proteins did not result in changes in ECAR regulation under normoxic conditions (Figure 7C). In contrast, H1KO and H2KO cells induced a gain in glycolytic reserve accompanied by reduced glycolytic activity ($p=0.0024$ and $p=0.00199$ respectively) when stimulated with DFO. These results are consistent with the known role of HIF in anaerobic glycolysis. ECAR regulation of dHKO cells in comparison with wild type cells during a glycolytic stress test was not significant (Figure 7D). Furthermore, baseline levels of intracellular LD were significantly decreased in H1KO and H2KO cells ($p=0.0087$ and $p=0.0043$ respectively). Compared to wild type cells, lipid levels after hypoxia (0.2% O₂) were significantly lower in both H1KO ($p=0.0325$) and dHKO ($p=0.0127$) cells. Under anoxic (0% O₂) conditions, significantly

($p=0.0001$) fewer LD were observed in dHKO cells compared to control cells. Under these conditions, H1KO and H2KO cells did not show differences compared to wild type cells (Figure 8B). Moreover, we tested the capacity of H1299 cells to trigger LD deposition after radiation treatment under normoxia. Interestingly, we observed that only HIF-1 α expressing cells were capable of increasing the amount of intracellular lipids upon radiation (Figure 8C).

Discussion

Hypoxic tumor cells upregulate the HIF pathway which causes radiation resistance in many types of cancer including non-small cell lung carcinoma (NSCLC) [49, 50]. Furthermore, HIF stabilization promotes tumor progression and aggressiveness by altering blood vessel architecture and shifting the metabolic profile of tumor cells. Clinically, high levels of HIF-1 α and HIF-2 α positively correlate with tumor progression and poor patient outcome in NSCLC [43, 44]. In the present study, we investigated the role of HIF-1 α and HIF-2 α on the radiobiological and metabolic properties of the NSCLC cell line H1299. Therefore, we generated a novel *in vitro* genetically engineered model of NSCLC with intact or deleted HIF-1 α /HIF-2 α proteins. Deletion of HIF-1 α caused a compensatory increase in the levels of HIF-2 α when oxygen was limited. In contrast, the same effect has not been seen when knocking out HIF-2 α suggesting that HIF-1 α is sufficient to trigger the signalling pathways that regulate response to hypoxia. It was previously reported that cancer cells expressing both HIF-1 α and HIF-2 α proteins might mutually compensate by upregulating HIF-1 α under HIF-2 α knockdown conditions [51], favouring a malignant phenotype [52]. Conversely, other studies observed that HIF-1 α knockdown cells increased HIF-2 α protein levels compared with the non-silencing control [24]. In line with our data, the compensatory regulation of HIF proteins can be explained by an enhanced translation activity rather than increased mRNA production [53].

Previous studies have focused on unravelling the differential roles of HIF-1 α and HIF-2 α in hypoxic gene regulation. Both proteins seem to display distinct, non-overlapping biological functions and independently regulate specific target genes in a cell type-specific manner [16] [54]. For instance, hypoxic induction of HIF-1 α target genes is repressed in HIF-1 α -deficient endothelial cells, suggesting that its loss cannot be compensated by HIF-2 α or other hypoxia-inducible proteins [55]. Other hypoxia-regulated genes such as OCT-4, a transcription factor essential for maintaining stem cell pluripotency, is strictly regulated by HIF-2 α and not by HIF-1 α [56]. Here we show that the absence of either HIF-1 α or HIF-2 α is sufficient to block canonical HIF target gene expression, suggesting that both proteins are strictly necessary for the activation of the HIF pathway. Furthermore, we observed that endogenous HIF-1 β levels are also reduced in the knockout cells in comparison with HIF-expressing cells. HIF-1 β is necessary for the assembly and functionality of an active HIF complex [11], meaning that its downregulation could contribute to the reduction of hypoxia-related gene activation.

Our results raise some interesting questions about how HIF proteins regulate the radiobiological effects in NSCLC cells. First, it was shown that HIF-1 α -deficient cells pre-incubated and irradiated under hypoxic conditions exhibit a more radioresistant phenotype than wild type cells. This was a surprising finding, especially in light of data from other studies showing that HIF-1 α controls hundreds of genes involved in radioprotection in tumors [57]. It is widely accepted that HIF-1 α increases the radioresistance of a large variety of tumor types by inducing cell cycle arrest [58], inhibiting apoptosis [59], promoting angiogenesis [60] and enhancing glycolytic metabolism [61]. To note, only a few studies attempted to understand the biological mechanisms behind HIF-2 α regulating radiation response in cancer. A negative prognostic role of HIF-2 α has been seen in patients with different types of cancer [43] and is also associated with a poor response to radiotherapy [62, 63]. Pietras, et al. reported that the stem cell marker CD44 promoted cancer stem cell phenotypes and radiation resistance via the direct regulatory role of HIF-2 α in a murine glioblastoma model [64]. Moreover, HIF-2 α inhibition enhanced radiation sensitivity in a cellular model of lung cancer by promoting apoptotic activity via the p53 pathway [65]. We thereby hypothesise that the substantial accumulation of HIF-2 α seen in hypoxic HIF-1 α -deficient cells may be responsible for the radioresistant phenotype. Conversely, there were no differences in clonogenic survival between wild type and HIF-2 α -deficient cells treated with radiation, suggesting that HIF-1 α is able to compensate the lack of HIF-2 α without altering their radiobiological properties. In order to validate our hypothesis, we used HIF-1 α /2 α -deficient cells to demonstrate that the radioprotective effect seen in H1KO cells is dependent on HIF-2 α upregulation. As expected, deleting both HIF proteins rescued the radioprotective phenotype seen in HIF-1 α -deficient cells. Moreover, dHKO cells displayed a more radiosensitive phenotype in comparison with wild type cells, indicating an important role of both proteins when working together in determining the radiation response of tumor cells. Such an effect was also observed when dHKO cells were irradiated under normal oxygen conditions, suggesting a role for HIF proteins in determining radiation response also in normoxic tumor cells. As a result, we suggest that dual HIF-1 α /HIF-2 α inhibitors are potentially clinically relevant enhancers of tumor radiosensitivity. This approach has to be evaluated in order to assess the consequences of systematically inhibiting HIF-1 α and HIF-2 α in normal cells such as macrophages and endothelial cells.

It is shown here that in HIF-deficient cells, there is a substantial increase of the basal CHK2 protein levels without changes in activation of its upstream regulator ATM were observed.

Previously studies described the role of the DDR in relation with specific cellular stress modulators such as hypoxia and HIF. It appears that ATM is active in hypoxia and increases HIF-1 α stability through phosphorylation at serine 696, which in turn leads to a reduction in mammalian target of rapamycin complex 1 (mTORC1) signalling [66]. Given the high degree of crosstalk between ATM and hypoxia it is perhaps not surprising that CHK2 can be modulated by HIF expression. Further research is needed to better understand the link between these proteins. Interestingly, the activation of the DDR in relation with the status of HIF did not correlate with our cell survival outcome and double-strand break (DSB) formation results. ATM is considered the major regulator of H2AX protein phosphorylation in response to DSB formation, which consequently leads to cell cycle arrest and cell death [67]. Our data demonstrates that ATM regulation is not responsible for the differences seen in radiation sensitivity of the knock out cells versus wild type cells, suggesting an alternative mode of HIF regulating radiation response in cells. Importantly, it has been demonstrated that p53-deficient cells, such as H1299, rely on a different cell-cycle checkpoint pathway involving p38MAPK/MK2 for cell-cycle arrest and survival after DNA damage [68]. Furthermore, the influence of combined HIF-1 α and HIF-2 α subunits on radiation response was further validated by analysing the cell cycle distribution. The findings reported here indicate that irradiated dHKO cells undergo a higher G2/M phase arrest than control cells. To note, this effect was only significant when irradiation was performed under normoxic conditions. Other studies have observed an increased cell cycle arrest in irradiated cells under hypoxia when deleting HIF-1 α [58] and HIF-2 α [65], whereas our data show that the inhibition of both proteins is necessary for an increased cell cycle abrogation. Moreover, in line with the hypoxia tolerance experiments, H1299 cells abrogate G2/M cell cycle arrest when the levels of oxygen are low, which was more pronounced in dHKO cells. This data suggests that the combination of both HIF proteins contribute to an appropriated adaptation of cells to hypoxia.

To date, multiple lines of evidence support that certain metabolites, such as lactate, confer cells with a malignant behaviour and a pro-metastatic phenotype in cancer patients, resulting in a potential prognostic biomarker in cancer [69]. Tarnawski et al. have shown that lactate levels detected in glioma tumors are a prognostic parameter for predicting recurrence and overall survival after postoperative radiotherapy [70]. Lactate regulates different biological activities within the cell, which ultimately lead to an increased angiogenesis capacity, antioxidative and immunosuppressive effects, contributing to tumor evasion and resistance to

therapy [35, 71]. Moreover, decreasing the levels of lactate in tumors enhances response to high-dose single-fraction radiotherapy in solid tumors [72]. The high levels of lactic acid and consequently low pH seen in the medium of H1KO cells are a potential explanation for the radioresistant phenotype seen in these cells. The specific mechanisms explaining the increased lactate secretion in H1KO cells need further investigation. However, we hypothesise that the compensatory upregulation of HIF-2 α proteins in H1KO cell promotes an increased lactate production and consequently confers a radioresistant phenotype. Future experiments should examine the individual contribution of HIF-2 α in mediating these effects and may be a viable target for therapeutic interventions. Despite this, the lactate transporter MCT-4 was strongly downregulated in HIF-1 α -deficient cells, suggesting that elevated extracellular lactic acid levels are not caused by increased expression of MCT-4. Nonetheless, other studies previously reported anti-proliferative effects when blocking lactate influx by MCT-1 and MCT-4, truncating the capacity to take up lactate as an energetic fuel [73]. Therefore, reduced levels of the lactate transporter MCT-4 might also influence the capacity of H1299 cells to recycle extracellular lactate back into cells and thus cause its accumulation. The same phenotype was seen when investigating other hypoxia-related genes involving pH and metabolite balance such as CAIX and GLUT1. Here, we also show that combined HIF-1/2 α deficiency caused a substantial decrease in the baseline ECAR levels, also named as non-glycolytic acidification, in DFO-treated cells, possibly contributing to the enhanced radiation sensitivity observed in dHKO cells. However, the real glycolytic activity measured in DFO-treated cells was diminished by HIF-1 α deficiency in comparison with the other genetic models. Previous reports have investigated the effects of lactic acidosis in relation with the capacity of cells to metabolize glucose. These studies demonstrated that cells with increased lactic acidosis and higher proton density became less glycolytic by impairing several enzymes of the glycolytic pathway [74]. The above results suggest that HIF-1 α -deficient cells have a defect in the glycolytic pathway possibly caused by elevated lactate levels, which in turn leads to a more radioresistant phenotype. However, the specific mechanism regarding the role of HIF-2 α in regulating the levels of lactate needs further investigation.

HIF-1 α and HIF-2 α may have an important role in regulating lipid metabolism in cancer [75]. Indeed, it was noted that the net number of LD was reduced in HIF-deficient cells under basal levels, suggesting again a role of the HIF proteins under normoxic conditions. In line with our data, Liu et al showed that HIF-1 α and HIF-2 α are involved in hypoxia-induced lipid

accumulation in hepatocyte cells through reducing PGC-1 α mediated fatty acid β -oxidation [76]. Besides, here we show that the capacity of HIF proteins to increase lipid storage differs depending on the levels of oxygen restriction, suggesting a differential response of HIF under various stressing conditions. These experiments also demonstrate that there is a response dependent on HIF-1 α in the ability of radiation to increase intracellular lipid storage, probably as a mechanism to protect cells against ROS molecules generated upon radiation treatment [77]. Unfortunately, there is currently no data available on the relation between radiation and cellular LD accumulation.

Overall, our work may have relevant implications how HIF-1 α inhibitors are currently used in the clinic. Our data suggest that HIF-1 α inhibition in combination with radiation might be antagonistically effective if used to target hypoxic cells within the tumor. As an alternative, we propose that HIF-1 α blockade should be used concurrently with the inhibition of HIF-2 α in order to radiosensitize tumor cells, partly by reducing their basal-glycolytic activity. Importantly, the specific inhibition of HIF-1 α accounts for critical changes in the metabolic profile of H1299 cells, including an increase in extracellular lactate which might result in a defect in the using of glucose as an energetic fuel.

Acknowledgements

This work was supported by a grant from the European Research Council (ERC-CoG, 617060).

References

1. Brown, J.M. and W.R. Wilson, *Exploiting tumour hypoxia in cancer treatment*. Nat Rev Cancer, 2004. **4**(6): p. 437-47.
2. Axelson, H., et al., *Hypoxia-induced dedifferentiation of tumor cells--a mechanism behind heterogeneity and aggressiveness of solid tumors*. Semin Cell Dev Biol, 2005. **16**(4-5): p. 554-63.
3. Wilson, W.R. and M.P. Hay, *Targeting hypoxia in cancer therapy*. Nat Rev Cancer, 2011. **11**(6): p. 393-410.
4. Grimes, D.R. and M. Partridge, *A mechanistic investigation of the oxygen fixation hypothesis and oxygen enhancement ratio*. Biomed Phys Eng Express, 2015. **1**(4): p. 045209.
5. Rofstad, E.K., et al., *Hypoxia-induced treatment failure in advanced squamous cell carcinoma of the uterine cervix is primarily due to hypoxia-induced radiation resistance rather than hypoxia-induced metastasis*. Br J Cancer, 2000. **83**(3): p. 354-9.
6. Bentzen, S.M. and V. Gregoire, *Molecular imaging-based dose painting: a novel paradigm for radiation therapy prescription*. Semin Radiat Oncol, 2011. **21**(2): p. 101-10.
7. Horsman, M.R. and J. Overgaard, *The impact of hypoxia and its modification of the outcome of radiotherapy*. J Radiat Res, 2016. **57 Suppl 1**: p. i90-i98.
8. Bruick, R.K. and S.L. McKnight, *A conserved family of prolyl-4-hydroxylases that modify HIF*. Science, 2001. **294**(5545): p. 1337-40.
9. Mahon, P.C., K. Hirota, and G.L. Semenza, *FIH-1: a novel protein that interacts with HIF-1 α and VHL to mediate repression of HIF-1 transcriptional activity*. Genes Dev, 2001. **15**(20): p. 2675-86.
10. Semenza, G.L., *Targeting HIF-1 for cancer therapy*. Nat Rev Cancer, 2003. **3**(10): p. 721-32.
11. Lee, J.W., et al., *Hypoxia-inducible factor (HIF-1) α : its protein stability and biological functions*. Exp Mol Med, 2004. **36**(1): p. 1-12.
12. Gustafsson, M.V., et al., *Hypoxia requires notch signaling to maintain the undifferentiated cell state*. Dev Cell, 2005. **9**(5): p. 617-28.
13. Wenger, R.H., D.P. Stiehl, and G. Camenisch, *Integration of oxygen signaling at the consensus HRE*. Sci STKE, 2005. **2005**(306): p. re12.
14. Hu, C.J., et al., *Differential regulation of the transcriptional activities of hypoxia-inducible factor 1 α (HIF-1 α) and HIF-2 α in stem cells*. Mol Cell Biol, 2006. **26**(9): p. 3514-26.
15. Rosenberger, C., et al., *Expression of hypoxia-inducible factor-1 α and -2 α in hypoxic and ischemic rat kidneys*. J Am Soc Nephrol, 2002. **13**(7): p. 1721-32.
16. Holmquist-Mengelbier, L., et al., *Recruitment of HIF-1 α and HIF-2 α to common target genes is differentially regulated in neuroblastoma: HIF-2 α promotes an aggressive phenotype*. Cancer Cell, 2006. **10**(5): p. 413-23.
17. Rankin, E.B., et al., *Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo*. J Clin Invest, 2007. **117**(4): p. 1068-77.

18. Schodel, J., et al., *High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq*. Blood, 2011. **117**(23): p. e207-17.
19. Gort, E.H., et al., *The TWIST1 oncogene is a direct target of hypoxia-inducible factor-2 α* . Oncogene, 2008. **27**(11): p. 1501-10.
20. Vaupel, P. and A. Mayer, *Hypoxia in cancer: significance and impact on clinical outcome*. Cancer Metastasis Rev, 2007. **26**(2): p. 225-39.
21. Keith, B., R.S. Johnson, and M.C. Simon, *HIF1 α and HIF2 α : sibling rivalry in hypoxic tumour growth and progression*. Nat Rev Cancer, 2011. **12**(1): p. 9-22.
22. Ryan, H.E., J. Lo, and R.S. Johnson, *HIF-1 α is required for solid tumor formation and embryonic vascularization*. EMBO J, 1998. **17**(11): p. 3005-15.
23. Burkitt, K., et al., *Targeting both HIF-1 and HIF-2 in human colon cancer cells improves tumor response to sunitinib treatment*. Mol Cancer Ther, 2009. **8**(5): p. 1148-56.
24. Carroll, V.A. and M. Ashcroft, *Role of hypoxia-inducible factor (HIF)-1 α versus HIF-2 α in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway*. Cancer Res, 2006. **66**(12): p. 6264-70.
25. Williams, K.J., et al., *Enhanced response to radiotherapy in tumours deficient in the function of hypoxia-inducible factor-1*. Radiother Oncol, 2005. **75**(1): p. 89-98.
26. Luo, Z., et al., *Silencing of HIF-1 α enhances the radiation sensitivity of human glioma growth in vitro and in vivo*. Neuropharmacology, 2015. **89**: p. 168-74.
27. Moeller, B.J., et al., *Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules*. Cancer Cell, 2004. **5**(5): p. 429-41.
28. Williams, K.J., et al., *A protective role for HIF-1 in response to redox manipulation and glucose deprivation: implications for tumorigenesis*. Oncogene, 2002. **21**(2): p. 282-90.
29. Damaghi, M., J.W. Wojtkowiak, and R.J. Gillies, *pH sensing and regulation in cancer*. Front Physiol, 2013. **4**: p. 370.
30. van Kuijk, S.J., et al., *The Sulfamate Small Molecule CAIX Inhibitor S4 Modulates Doxorubicin Efficacy*. PLoS One, 2016. **11**(8): p. e0161040.
31. Quennet, V., et al., *Tumor lactate content predicts for response to fractionated irradiation of human squamous cell carcinomas in nude mice*. Radiother Oncol, 2006. **81**(2): p. 130-5.
32. DeBerardinis, R.J., et al., *Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis*. Proc Natl Acad Sci U S A, 2007. **104**(49): p. 19345-50.
33. Cui, X.G., et al., *HIF1/2 α mediates hypoxia-induced LDHA expression in human pancreatic cancer cells*. Oncotarget, 2017. **8**(15): p. 24840-24852.
34. Groussard, C., et al., *Free radical scavenging and antioxidant effects of lactate ion: an in vitro study*. J Appl Physiol (1985), 2000. **89**(1): p. 169-75.
35. Hirschhaeuser, F., U.G. Sattler, and W. Mueller-Klieser, *Lactate: a metabolic key player in cancer*. Cancer Res, 2011. **71**(22): p. 6921-5.

36. Grotius, J., et al., *Impact of exogenous lactate on survival and radioresponse of carcinoma cells in vitro*. Int J Radiat Biol, 2009. **85**(11): p. 989-1001.
37. Tirinato, L., et al., *An Overview of Lipid Droplets in Cancer and Cancer Stem Cells*. Stem Cells Int, 2017. **2017**: p. 1656053.
38. Nieva, C., et al., *The lipid phenotype of breast cancer cells characterized by Raman microspectroscopy: towards a stratification of malignancy*. PLoS One, 2012. **7**(10): p. e46456.
39. Zhang, M., et al., *HIF-1 Alpha Regulates the Response of Primary Sarcomas to Radiation Therapy through a Cell Autonomous Mechanism*. Radiat Res, 2015. **183**(6): p. 594-609.
40. Kondo, K., et al., *Inhibition of HIF2alpha is sufficient to suppress pVHL-defective tumor growth*. PLoS Biol, 2003. **1**(3): p. E83.
41. Raval, R.R., et al., *Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma*. Mol Cell Biol, 2005. **25**(13): p. 5675-86.
42. Gu, Q., et al., *Hypoxia-inducible factor 1alpha (HIF-1alpha) and reactive oxygen species (ROS) mediates radiation-induced invasiveness through the SDF-1alpha/CXCR4 pathway in non-small cell lung carcinoma cells*. Oncotarget, 2015. **6**(13): p. 10893-907.
43. Moreno Roig, E., et al., *Prognostic Role of Hypoxia-Inducible Factor-2 α Tumor Cell Expression in Cancer Patients: A Meta-Analysis*. Frontiers in Oncology, 2018. **8**(224).
44. Wu, X.H., C. Qian, and K. Yuan, *Correlations of hypoxia-inducible factor-1alpha/hypoxia-inducible factor-2alpha expression with angiogenesis factors expression and prognosis in non-small cell lung cancer*. Chin Med J (Engl), 2011. **124**(1): p. 11-8.
45. Sun, J.C., et al., *High expression of HIF-2alpha and its anti-radiotherapy effect in lung cancer stem cells*. Genet Mol Res, 2015. **14**(4): p. 18110-20.
46. Ran, F.A., et al., *Genome engineering using the CRISPR-Cas9 system*. Nat Protoc, 2013. **8**(11): p. 2281-2308.
47. Das, K.C., *Hyperoxia decreases glycolytic capacity, glycolytic reserve and oxidative phosphorylation in MLE-12 cells and inhibits complex I and II function, but not complex IV in isolated mouse lung mitochondria*. PLoS One, 2013. **8**(9): p. e73358.
48. Greenspan, P., E.P. Mayer, and S.D. Fowler, *Nile red: a selective fluorescent stain for intracellular lipid droplets*. J Cell Biol, 1985. **100**(3): p. 965-73.
49. Brustugun, O.T., *Hypoxia as a cause of treatment failure in non-small cell carcinoma of the lung*. Semin Radiat Oncol, 2015. **25**(2): p. 87-92.
50. Salem, A., et al., *Targeting Hypoxia to Improve Non-Small Cell Lung Cancer Outcome*. J Natl Cancer Inst, 2018. **110**(1).
51. Isono, T., et al., *Hydroxyl-HIF2-alpha is potential therapeutic target for renal cell carcinomas*. Am J Cancer Res, 2016. **6**(10): p. 2263-2276.
52. Anelli, V., et al., *Sphingosine kinase 1 is up-regulated during hypoxia in U87MG glioma cells. Role of hypoxia-inducible factors 1 and 2*. J Biol Chem, 2008. **283**(6): p. 3365-75.

53. Schulz, K., et al., *HIF-1 α protein is upregulated in HIF-2 α depleted cells via enhanced translation*. FEBS Lett, 2012. **586**(11): p. 1652-7.
54. Sowter, H.M., et al., *Predominant role of hypoxia-inducible transcription factor (Hif)-1 α versus Hif-2 α in regulation of the transcriptional response to hypoxia*. Cancer Res, 2003. **63**(19): p. 6130-4.
55. Tang, N., et al., *Loss of HIF-1 α in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis*. Cancer Cell, 2004. **6**(5): p. 485-95.
56. Covello, K.L., et al., *HIF-2 α regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth*. Genes Dev, 2006. **20**(5): p. 557-70.
57. Adams, J.M., et al., *HIF-1: a key mediator in hypoxia*. Acta Physiol Hung, 2009. **96**(1): p. 19-28.
58. Goda, N., et al., *Hypoxia-inducible factor 1 α is essential for cell cycle arrest during hypoxia*. Mol Cell Biol, 2003. **23**(1): p. 359-69.
59. Piret, J.P., et al., *Hypoxia and CoCl₂ protect HepG2 cells against serum deprivation- and t-BHP-induced apoptosis: a possible anti-apoptotic role for HIF-1*. Exp Cell Res, 2004. **295**(2): p. 340-9.
60. Carmeliet, P., et al., *Role of HIF-1 α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis*. Nature, 1998. **394**(6692): p. 485-90.
61. Semenza, G.L., et al., *Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1*. J Biol Chem, 1994. **269**(38): p. 23757-63.
62. Koukourakis, M.I., et al., *Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer*. Int J Radiat Oncol Biol Phys, 2002. **53**(5): p. 1192-202.
63. Bhatt, R.S., et al., *Hypoxia-inducible factor-2 α : effect on radiation sensitivity and differential regulation by an mTOR inhibitor*. BJU Int, 2008. **102**(3): p. 358-63.
64. Pietras, A., et al., *Osteopontin-CD44 signaling in the glioma perivascular niche enhances cancer stem cell phenotypes and promotes aggressive tumor growth*. Cell Stem Cell, 2014. **14**(3): p. 357-69.
65. Bertout, J.A., et al., *HIF2 α inhibition promotes p53 pathway activity, tumor cell death, and radiation responses*. Proc Natl Acad Sci U S A, 2009. **106**(34): p. 14391-6.
66. Cam, H., et al., *mTORC1 signaling under hypoxic conditions is controlled by ATM-dependent phosphorylation of HIF-1 α* . Mol Cell, 2010. **40**(4): p. 509-20.
67. Kastan, M.B. and D.S. Lim, *The many substrates and functions of ATM*. Nat Rev Mol Cell Biol, 2000. **1**(3): p. 179-86.
68. Reinhardt, H.C., et al., *p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage*. Cancer Cell, 2007. **11**(2): p. 175-89.
69. Walenta, S. and W.F. Mueller-Klieser, *Lactate: mirror and motor of tumor malignancy*. Semin Radiat Oncol, 2004. **14**(3): p. 267-74.
70. Tarnawski, R., et al., *1H-MRS in vivo predicts the early treatment outcome of postoperative radiotherapy for malignant gliomas*. Int J Radiat Oncol Biol Phys, 2002. **52**(5): p. 1271-6.

71. Romero-Garcia, S., et al., *Lactate Contribution to the Tumor Microenvironment: Mechanisms, Effects on Immune Cells and Therapeutic Relevance*. Front Immunol, 2016. **7**: p. 52.
72. Leung, E., et al., *Metabolic targeting of HIF-dependent glycolysis reduces lactate, increases oxygen consumption and enhances response to high-dose single-fraction radiotherapy in hypoxic solid tumors*. BMC Cancer, 2017. **17**(1): p. 418.
73. Draoui, N., et al., *Antitumor activity of 7-aminocarboxycoumarin derivatives, a new class of potent inhibitors of lactate influx but not efflux*. Mol Cancer Ther, 2014. **13**(6): p. 1410-8.
74. Xie, J., et al., *Beyond Warburg effect--dual metabolic nature of cancer cells*. Sci Rep, 2014. **4**: p. 4927.
75. Rankin, E.B., et al., *Hypoxia-inducible factor 2 regulates hepatic lipid metabolism*. Mol Cell Biol, 2009. **29**(16): p. 4527-38.
76. Liu, Y., et al., *HIF-1 α and HIF-2 α are critically involved in hypoxia-induced lipid accumulation in hepatocytes through reducing PGC-1 α -mediated fatty acid beta-oxidation*. Toxicol Lett, 2014. **226**(2): p. 117-23.
77. Bensaad, K., et al., *Fatty acid uptake and lipid storage induced by HIF-1 α contribute to cell growth and survival after hypoxia-reoxygenation*. Cell Rep, 2014. **9**(1): p. 349-365.

Figure and Tables

FIGURE 1: (A) Western blot of HIF-1 α , HIF-2 α and HIF-1 β expression in H1299 cells under normoxic (21%) and hypoxic (0.2%) conditions. Lamin A/C was used as loading control. (B) mRNA expression of HIF target genes CAIX, GLUT1, CITED2 and TWIST1 after 24h hypoxia incubation. (C) Automated cell counting of H1299 cells under normoxia and hypoxia at 24h and 48h after seeding. (D) Cell viability was measured by crystal violet staining assay.

FIGURE 2: Clonogenic cell survival of H1299 cells irradiated under (A) normoxia (21% O₂) and (B) hypoxia (0.2% O₂). Surviving fraction was normalized to vehicle control. Average $\pm$ SD of three independent biological repeats is shown. Asterisks indicate statistical significance (*p<0.05; ***p<0.001; ****p<0.0001).

FIGURE 3: Representative merged fluorescent images of γ -H2AX foci (red) and nuclei (blue) upon irradiation under (A) normoxia and (B) hypoxia in H1299 cells. Quantification of γ -H2AX staining in cells. Average $\pm$ SD of three independent biological repeats is shown. Asterisks indicate statistical significance (** $p < 0.01$; *** $p < 0.001$).

FIGURE 4: Western blot of pATM, pCHK2 and CHK2 expression in H1299 cells irradiated with 2Gy under normoxic (21%) and hypoxic (0.2%) conditions. Lamin A/C was used as loading control.

FIGURE 5: Flow cytometry analysis of cell cycle distribution of H1299 cells after radiation under normoxic (21%) and hypoxic (0.2%) conditions. Fraction of cells in G1, S and G2-M phase in non-irradiated cells under normoxia (A) and hypoxia (C) and 4hr post-irradiation (2Gy) in normoxic (B) and hypoxic (D) cells. Average $\pm$ SD of three independent biological repeats is shown. Asterisks indicate statistical significance (* p <0.05; ** p <0.01).

FIGURE 6: (A-B) Measurement of extracellular pH and (C-D) extracellular lactic acid accumulation upon normoxia or hypoxia. mRNA expression of HIF target genes MCT1(E) and MCT4 (F) after hypoxia incubation. Average $\pm$ SD of three independent biological repeats is shown. Asterisks indicate statistical significance (* p <0.05; ** p <0.01; *** p <0.001).

FIGURE 7: Basal glycolytic function of H1299 cells under normoxia without (A) or with DFO (B) was quantified by measuring extracellular acidification rate (ECAR). Stacked plot of glycolysis and glycolytic reserve under normoxia without (C) or with DFO 100nM (D). Average $\pm$ SD of three independent biological repeats is shown. Asterisks indicate statistical significance (* p <0.05; ** p <0.01).

FIGURE 8: LD - Nile Red staining in H1299 cells in (A) non-treated cells, (B) hypoxia (0.2% O₂) / anoxia (0.0% O₂) incubated cells and irradiated cells (C). Average $\pm$ SD of three independent biological repeats is shown. Asterisks indicate statistical significance (* p <0.05; ** p <0.01; *** p <0.001).

SUPPLEMENTARY FIGURE 1: Schematic representation of HIF-1 α and HIF-2 α genes encoded by exons 1-15 and 1-7 respectively and the localization of the guide RNAs (sgRNA) used to target both genes.

SUPPLEMENTARY FIGURE 2: The PCR amplified DNA fragments containing the HIF-1 α and HIF-2 α sgRNA target site from positive clones was analysed by DNA sequencing. Example of sequence chromatogram is shown.

SUPPLEMENTARY FIGURE 3: mRNA expression of HIF-2 α after 24h hypoxia incubation.

SUPPLEMENTARY FIGURE 4: Clonogenic cell survival of H1KO and H2KO independent clones (IC) compared to wt cells irradiated under (A-C) normoxia (21% O₂) and (B-D) hypoxia (0.2% O₂). Surviving fraction was normalized to vehicle control.

SUPPLEMENTARY FIGURE 5: Western blot of pCHK2 expression in H1299 cells irradiated under normoxic (21%) and hypoxic (0.2%) conditions. Lamin A/C was used as loading control.

SUPPLEMENTARY FIGURE 6: Basal respiration expressed as oxygen consumption rate (OCR) under normoxia without (A) or with DFO (B).

SUPPLEMENTARY TABLE 1: Antibodies used in this study

Antibody	Dilution	Vendor
HIF-1 α	1:500	BD Biosciences
HIF-2 α	1:1000	Novus Biologicals
HIF-1 β	1:1000	BD Biosciences
Lamin-A	1:1000	Sigma-Aldrich
pATM	1:1000	Abcam
pCHK2	1:1000	Bioke
CHK2	1:1000	Bioke

SUPPLEMENTARY TABLE 2: Primers used in this study

Genes	Primers	Sequence
CAIX	Forward	CATCCTAGCCCTGGTTTTTGG
CAIX	Reverse	GCTCACACCCCCTTTGGTT
GLUT1	Forward	GATTGGCTCCTTCTCTGTGG
GLUT1	Reverse	TCAAAGGACTTGCCCAGTTT
CITED2	Forward	ACGCCTTCAACGCCCTAATGG
CITED2	Reverse	ATGCCTGATGCCGCTCGTG
TWIST1	Forward	GGAGTCCGCAGTCTTACGAG
TWIST1	Reverse	TCTGGAGGACCTGGTAGAGG
MCT1	Forward	TATGGTGGAGGTCCTATCAGC

MCT1	Reverse	TGTACGGTGTTACAGAAAGAAGC
MCT4	Forward	TTTGTGCTTTACGCCGTGG
MCT4	Reverse	CTGAAGAGGTAGACGGAGTAGG
HIF-2 α	Forward	TGCTCCCACGGCCTGTAC
HIF-2 α	Reverse	TTGTCACACCTATGGCATATCACA
HPRT	Forward	TATTGTAATGACCAGTCAACAG
HPRT	Reverse	GGTCCTTTTCACCAGCAAG

CHAPTER 4

NOTCH blockade in combination with radiotherapy increases the number of lung stem cells in differentiated cultures

Lorena Giuranno, Eloy Moreno Roig, Raffaella Iannone, Carolien Wansleebe, Lydie M.O.
Barbeau, Ludwig J. Dubois and Marc Vooijs*

Department of Radiotherapy (MAASTRO), GROW – School for Oncology and
Developmental Biology, Maastricht University, Maastricht, Netherlands

* Corresponding author: Marc Vooijs, Department of Radiotherapy (MAASTRO), GROW –
School for Oncology and Developmental Biology, Maastricht University, Universiteitssingel
50, 6229 ER Maastricht, The Netherlands; E-mail: marc.vooijs@maastrichtuniversity.nl

Manuscript in preparation

Abstract

Radiotherapy alone and in combination with other agents, such as chemotherapy, is the standard of care treatment for most lung cancer patients. Nevertheless, both may cause severe side effects, which limits tumor control and quality of life because of treatment induced lung toxicity. Radiation-related toxicity occurs in up to 30% of patients and may be short-term or permanent. The Notch signaling pathway is important in cell fate determination in the lung and regulates self-renewal and differentiation of epithelial progenitors in the adult upper airway epithelium. In this study, we assessed whether Notch signaling is involved in the response of the lung epithelium to radiation-induced injury. We found that pharmacological inhibition of the NOTCH signaling pathway by the γ -secretase inhibitor dibenzazepine (DBZ) increased survival of irradiated progenitor cells in organotypic air-liquid interface (ALI) culture of patient-derived primary bronchial epithelial cells (PBECS). NOTCH inhibition resulted in the induction of ciliated cell fate and increased the amount of progenitor cells, whereas the number of secretory cells was reduced. Radiation treatment alone reduced the survival of progenitor cells at early time points, whereas the combination with NOTCH inhibition induced a radioprotective effect. Mechanistically, NOTCH blockade increased the DNA damage response (DDR) and reduced double-strand break (DSB) formation in lung primary cells after irradiation. Our findings reveal a novel role of NOTCH signaling pathway in irradiation-induced lung toxicity and suggest a new application of Notch inhibitors in promoting progenitor cell survival when used in combination with radiotherapy.

Introduction

A major factor that limits long-term survival and quality of life of lung cancer patients is the maximum tolerated dose of radiation to the thorax [1]. Up to 30% of patients develop radiation-induced toxicity, which requires treatment de-intensification or termination negatively affecting tumor control probability (TCP) and quality of life. Exposure of the thorax to ionizing radiation during cancer treatment results in DNA damage, cell loss, tissue-repair and remodeling [2]. Long-term irreversible effects of thoracic irradiation include esophagitis and irreversible remodeling including lung fibrosis and the formation of fistulae [3]. The probability of developing radiation-induced lung damage (RILD) is determined by both physical factors (i.e. dose, irradiated volume) as well as biological factors [4, 5]. These side-effects are further exacerbated by combination treatments with chemotherapy and targeted therapies [6]. At present, radiomitigators and radioprotectors are possible strategies that lessen radiation-related damage to the normal lung epithelium in the treatment of malignancies [7]. Most of these agents have been tested in preclinical models and are currently being tested in several clinical trials [8]. Because the lung is highly sensitive to radiation damage and its resulting long-term adverse effects, there is an urgent need to reduce radiation damage to prevent delay or reverse early and late toxicities.

The human bronchial airway epithelium is composed of a luminal layer containing the differentiated mucous producing secretory cells, ciliated cells and a basal compartment containing self-renewing progenitors [9]. Progenitor cell dysfunction impairs lung repair and regeneration upon chemical injury [10, 11]. During normal bronchial epithelial homeostasis, the basal cells give rise to both ciliated and secretory cells which are responsible for the muco-ciliary clearance. Exposure of the lung to irradiation inhibits proliferation of progenitor cells placing a proliferative demand among the remaining progenitors to replace the damaged tissue [12]. Clonal capacity of progenitor cells is limited, which underlines a functional impairment and inability of the remaining stem cells to repair the damaged tissue [12]. The remaining basal stem cells have the ability to repair DNA damage upon injury using the error prone non-homologous end joining pathway. Thus, subsets of airway progenitors may accumulate DNA damage upon irradiation. Such cells are more likely to contribute to pulmonary disease and have potential to convert into malignant cells [13]. Furthermore, the molecular pathways involved in the damage response and repair after irradiation in basal cells of the airway epithelium are not clear. It has been demonstrated that irradiation induces EGFR

phosphorylation and increases interaction with caveolin involved in EGFR nuclear transport [14]. A better understanding of the signaling pathways contributing to RILD may provide opportunities for intervention.

The NOTCH signaling pathway is important in cell fate determination in many tissues during development and in adults including the lung. NOTCH receptors are transmembrane glycoproteins that interact with membrane bound ligands on adjacent cells. Mammalian cells encode for four NOTCH receptors (N1-4) and five ligands of the Delta (1-3)-Jagged (1-2) family (DSL) [15-17]. NOTCH inhibition alters the balance between basal, ciliated, neuroendocrine and secretory cell fates in the upper airway epithelium [18, 19] indicating that the NOTCH pathway promotes cell fate decisions. In the postnatal lung, NOTCH restricts basal cells to the secretory cell fate suppressing ciliated differentiation [20]. Furthermore, NOTCH signaling is also required to maintain the differentiated state of secretory cells in the upper airways and blocking JAGGED1/2-NOTCH signaling leads to transdifferentiation of club cells into ciliated cells [21]. NOTCH2 regulates differentiation of lineage-restricted progenitors into bronchial club cells and ciliated cells as well as contributes to alveolar morphogenesis and integrity of epithelial and smooth muscle layers of airways [17, 22]. NOTCH1/3 contributes additively to regulate pulmonary neuroendocrine cell fate [22] while NOTCH1 is dispensable for airway epithelial development upon lung epithelial injury [23]. Previous studies have shown that the NOTCH signaling pathway regulates basal stem cell self-renewal upon chemical injury [24] suggesting a key role of the NOTCH pathway in survival upon genotoxic damage. Similar results have been reported in lung cancer patients where deregulated NOTCH signaling was associated with worse disease-free survival [25, 26], treatment resistance [27, 28] and radio-resistance in non-small cell like cancer (NSCLC) tumor-bearing mice [29, 30].

NOTCH signaling has recently been shown to directly regulate the DNA damage response by competing with FOXO3 for binding to ATM kinase and thereby suppressing its phosphorylation. Inactivation of ATM/FOXO interaction by NOTCH expression is crucial for proliferation of T cell leukaemia with mutated NOTCH [31, 32]. Moreover, radiation has been shown to induce NOTCH signaling in breast cancer cells [33] which may be mediated via activation by the DNA repair BRCA1/p63 complex [34]. Given the fact that radiation selects for radiation resistant sub-populations of airway progenitor cells [35] that may require NOTCH signaling for their survival we assessed whether the combined effects of irradiation

and NOTCH pathway inhibition alters homeostasis of the bronchial epithelium in the upper airways. In the present study we therefore investigated the effect of NOTCH inhibition on proliferation and differentiation of irradiated primary human bronchial epithelium using an organotypic air-liquid interface culture system.

Materials and methods

Primary bronchial epithelial cells

PBECs were kindly provided by the Primary LUnG Culture (PLUC) facility MUMC+, Maastricht, The Netherlands. Lung tissue used for the isolation of PBECs was obtained from the Maastricht Pathology Tissue Collection (MPTC) and originated from tissue resected during lobectomies or pneumonectomies of patients who underwent surgery for lung cancer (3 different donors PUL 112, 119, 109). Collection, storage and use of tissue and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (<http://www.fmwv.nl>). The scientific board of the MPTC approved the use of materials for this study under MPTC2010-019. PBEC cells-passage 1 were grown in KSFM medium supplemented with 1mM isoproterenol, mycozap, 2.5ng EGF and BPE. Soybean Trypsin inhibitors from Glycine max were used to resuspend the cells after trypsinization. Cells were collected by centrifugation, 5 minutes at 150 RCF and counted with an automatic counter (Beckman Coulter).

Airway Epithelium Differentiation in Air-liquid Interface (ALI) Culture

Isolated PBECs were seeded onto 12mm transwell with 0.4µm pore polyester membrane inserts (Corning Incorporated, Corning, NY) (90.000 cells/transwell in 500 µL) using the stimulation medium. Stimulation medium consisted of Bronchial Epithelial Cell Growth Medium (BEGM) (Lonza ee-3171) and Dulbecco's Modified Eagle Medium (no glucose) (DMEM, no glucose) (Gibco 11366-025), supplemented with pen/strep, Hepes, BEGM SingleQuot Kit (except the gentamycin) and BSA. PBECs were submerged by adding 500 µL of cells in the insert and 1.5 mL stimulation medium in the bottom. PBECs were cultured in stimulation medium at 37°C in 5% CO₂ humidified incubator. Stimulation medium was replaced every 2 days until cells reached confluence. After cells reached confluence, medium was removed from the insert and only supplied in the basal chamber. Retinoic Acid in a final concentration of 50nM was supplemented to the medium. Cells received ALI treatment by only adding stimulation medium (+RA) to the bottom of each well (1ml/well).

NOTCH blockade and radiation therapy

The gamma secretase inhibitor dibenzazepine (DBZ) (Syncom, The Netherlands) at 1µM was used as NOTCH inhibitor. DMSO was added to samples as a vehicle control. PBECs at airlift

were irradiated with a single 2-4 Gy dose using a MCN 225 X-ray tube (Phillips) operated at 225 keV and 10 mA. Samples were collected 1h, 24h or 2 weeks after irradiation and processed for RNA isolation, immunofluorescent stainings or western blotting.

Western blotting

Western blot was performed according to standard protocol. RIPA buffer was used for protein extraction. Protein concentrations were determined with Bradford Protein Assay (Bio-Rad). Proteins (30 ug) were separated on a 7.5% SDS-PAGE and transferred to Nitrocellulose or PVDF membranes. Membranes were blocked with 5% non-fat dry bovine milk and subsequently incubated (O/N, 4°C) with primary antibodies (Supplementary table 1) and visualized using HRP-linked secondary antibodies (goat anti mouse and goat anti-rabbit, 1:2500, Cell-Signaling) and Super ECL Luminescence.

Whole mount immunostaining and confocal microscopy

PBECs were fixed in 4 % paraformaldehyde for 10 minutes, permeabilized with PBS-triton100 0,3% for 3 hours and blocked with PBS triton100 0,1%, BSA 3 % and normal goat serum 10 % (Bio-Connect, Cat# s1000) for 2 hours. Primary antibodies MUC5AC (Abcam, Cat# ab3649), Acetylated tubulin (Sigma-Aldrich, Cat# T7451-200UL), p63 (Abcam, Cat# ab124762), Cytokeratin 5 (Abcam, Cat# ab24647) and 53BP1 (BD Biosciences, Cat# 612522) were incubated overnight at 4 degrees. Goat anti-mouse Alexa 488 (Thermo fisher 1:500) and goat anti-rabbit Alexa Fluor 555 fluorescent secondary antibody (Thermo fisher 1:500) were used for 1 hour at room temperature. DAPI was used to stain the cellular nuclei. Stacks of 2 um were acquired for each sample using Confocal microscope (Leica Spe Confocal).

Click-it Edu Alexa Fluor 555 Imaging kit

Click-it Edu Imaging kit (Molecular Probes Cat# 1790497) was used to measure cell proliferation. Samples were incubated with Edu 2 hours prior to be harvested, washed, fixed and permeabilized as previously described (REF) and incubated for 30 minutes with 1x Click-it 10 X buffer, Alexa fluor azide, CuSO₄ reaction buffer 1x provided by the kit. DAPI was used to stain nuclei. Images were acquired used confocal microscopy.

Image analysis

Quantification of p63 and Edu stainings was performed by determining the number of positive nuclei as a proportion of total number of nuclei in 5 representative fields of each sample. Image J 6.4 was used for images quantification.

RNA isolation and quantitative PCR analysis

mRNA was extracted using the NucleoSpin RNA II kit (Bioke) and cDNA conversion was performed using the iScript cDNA Synthesis kit (BioRad) according to the manufacturers' instructions. Quantitative PCR was performed on the CFX96 (BioRad). The expression of *Hes1* was detected with SYBR Green I (Eurogentec). Values for *Hes1* (F-CGTGTCTCCTCCTCCCAT; R-GGCCTCTATATATATCTGGGAC) expression were normalized to *HPRT* expression levels. (F-TATTGTAATGACCAGTCAACAG; R-GGTCCTTTTCACCAGCAAG).

Results

To characterise NOTCH signalling in normal lung cells, we first analysed the total NOTCH cleavage as well as the expression of its target *Hes1* in primary bronchial epithelial cells (PBECs). We also examined the effect of pharmacological inhibition of NOTCH using the γ -secretase inhibitor dibenzazepine (DBZ) on NOTCH cleavage and mRNA target gene expression in PBECs. Culturing the cells on Dll4-coated plates increased the mRNA expression of NOTCH1-target gene *HES1*, as determined by real-time qPCR. Furthermore, DBZ inhibited Dll4 induced NOTCH1 cleavage (Fig. 1A). Western blot confirmed that NOTCH signaling is activated and that DBZ was effective in blocking the Val1744 cleaved activated form of NOTCH1 and the expression of the NOTCH target gene *HES1* from 3 independent patient samples (Fig. 1B). We used the Air-liquid interface (ALI) system to study proliferation and differentiation of primary bronchial epithelial progenitors (Fig. 1C). The PBEC population, mostly comprised of progenitor, goblet and ciliated cells, was strictly regulated by the activation of NOTCH. The undifferentiated basal cells express p63, a member of the p53 family crucial during the development of several organs and tissues [36]. Basal stem cells, which express the p63 marker, generate secretory and ciliated lineages, positive for MUC1 and LhS28 respectively. Specifically, continuous treatment with DBZ for 4 weeks resulted in an increase in the amount of progenitor and ciliated cells, which consequently leads to a reduction in goblet cells (Fig. 1D). Taken together, our results support the model depicted in Fig. 1E. According to this model, multipotent p63⁺ cells have the ability to renew and besides divide symmetrically to generate secretory and ciliated lineages in a NOTCH-dependent process.

The effect of continuous PBEC culturing on the emerging cell populations from basal cells differentiation at ALI was analysed by western blot (Fig. 2A). For this, cells on transwell membranes were collected at various time points to assess the expression of relevant cellular markers. We also quantified lung-specific cells by manually counting the positivity of different markers on confocal images of immunostained-tissue sections from the ALI (Fig. 2B). At the start of ALI culture approximately 70% of PBECs were positive for p63 but decreased over time until reaching a percentage of 40% at day 28, indicating that cells might be differentiating into other cell types. We next determined the number of ciliated cells emerging over time using the acetylated tubulin (AcTub) antibody, which stains the cilia of full-grown ciliated cells. After 28 days of ALI culture, approximately 30% of cells were ciliated. Additionally, the fraction of MUC5AC positive cells (a marker for goblet cells),

reached a peak of about 30% at day 15. Subsequently, a gradual drop to about 10% was observed at day 21 followed by an increase to about 20% at day 28. This pattern was consistent for three independent donors. Proliferation, as determined by Edu incorporation, progressively decreased until day 21 when proliferative capacity completely ceased. All proliferating cells were p63⁺ and CK5⁺ cells. Overall, these data suggest that PBECs cultured in the ALI system are mostly progenitor cells that eventually undergo differentiation into ciliated or goblet cells. To determine whether NOTCH signaling was implicated in determining cell fate decisions in differentiated PBECs (day 21 after ALI culture); we assessed the expression of p63, AcTub and MUC5A at 1, 3 and 7 days after DMSO or DBZ treatment. We observed a strong increase of p63 and KRT5 protein levels in ALI cultures treated with DBZ compared to vehicle-treated cells. Interestingly, we also saw an immediate decrease of MUC1A cell number that was consistent overtime, whereas the increase in ciliated cells was minor for all the time points tested (Fig.3A). NOTCH inhibition significantly increased the number of proliferating cells and the absolute number of p63⁺ cells. Proliferation remained stable in cultures where NOTCH signalling was not blocked. To note, Edu⁺ cells were exclusively p63⁺ positive. (Fig.3B).

To address the role of the NOTCH signalling in cell fate decisions in response to ionizing radiation, we investigated the expression of the different cell type specific markers on protein level at different time point after irradiation of differentiated ALI cultures. For this purpose, we designed a set of different treatment plans consisting of DBZ treatment in combination with irradiation using 2 or 4 Gy. The first treatment plan consisted of the addition of the drug vehicle DMSO or DBZ for two weeks and irradiation 24h prior cell collection (Fig.4A). NOTCH inhibition increased p63⁺ progenitor cells and reduced MUC5A expressing cells in cultures within 24h after treatment with DBZ. No differences were observed for the expression of ciliated cells. We also noticed that the number of mucous cells further decreased when NOTCH inhibition treatment was combined with the highest dose of radiation (4Gy). Increasing doses of radiation also resulted in reduction of p63⁺ progenitor cells suggesting that NOTCH inhibition is protective for p63⁺ progenitors. The findings of protein expression for p63 were corroborated with immunofluorescent staining for p63⁺ expression and quantitation. Here we also observed a dose-dependent increase in p63⁺ expressing cells amounting to an absolute increase of 30% in the number of p63⁺ cells (Fig 4.B). Therefore, we show that antagonizing NOTCH signalling in cells confers radiation resistance that subsequently is translated into a proliferative advantage.

Next, we assessed the long-term consequences of inhibiting NOTCH after irradiation in PBEC ALI's (Fig 5). We observed a strong increase in p63⁺ in both non-irradiated and irradiated cells upon NOTCH inhibition when analysed 14 days after irradiation at day 28. Ciliated cells at the highest doses were slightly affected but mucous cells were strongly reduced upon irradiation in both GSI and vehicle treated cultures at day 28 compared to non-treated ALI (Fig 5A). Confocal imaging of KRT5 positive cells in whole mount ALI cultures was performed at day 28 and showed that while in control conditions basal cells were located closest to the membrane, upon irradiation, the number of KRT5⁺ cells was increased, and they appeared redistributed to higher layers in the ALI culture. Moreover, when combined with NOTCH inhibition, KRT5⁺ further expanded and NOTCH blockade further enhanced KRT5⁺ cell numbers (Fig. 5B).

Radiation triggers activation of the DNA damage response (DDR) which can provoke growth arrest and cell death. Activation of the DNA damage response through phosphorylation of ATM triggers the phosphorylation of γ -H2AX and 53BP1, which both are recruited to the double-strand breaks in the DNA. We observed that irradiation of PBEC cultures after 14 days in cultures led to an immediate induction of phosphorylated ATM kinase. This increase in pATM was further increased when cells were cultured in the presence of DBZ (Fig.6A). We next determined the induction of γ -H2AX at two different time points after radiation treatment and noted that the strongest induction of positive foci was at 1h whereas no signal was found after 24h. Compared with DMSO-treated samples, NOTCH inhibition decreased the amount of γ -H2AX for both radiation doses tested (Fig. 6B). We also analysed 53BP1 foci as another measure of persistent DNA damage. Confocal imaging of positive 53BP1 marker in the nucleus demonstrated that there was a dose dependent increase in nuclear 53BP1 foci in p63⁺ progenitor cell and that -consistent with the γ -H2AX staining- this was reduced upon NOTCH inhibition (Fig. 6C).

Finally, we investigated if radiation itself altered the expression or response of NOTCH signalling. We observed that there was a dose-dependent decrease in Val1744 cleaved NOTCH1 in cultures 24h after irradiation. The reduction in cleaved NICD coincided with a reduction in the expression of Hes1, a canonical NOTCH target gene (Fig. 7A, B).

Discussion

In the present study we used patient-derived primary lung epithelial organotypic cultures to study the role of NOTCH signaling in the response of stem cells and differentiated bronchial epithelium to irradiation. Our results show that NOTCH signaling in primary bronchial epithelium suppresses the DNA damage response and the proliferative capacity of irradiated lung progenitors.

The importance of investigating the effects of NOTCH inhibitors on lung model systems arises from the need to reduce adverse effects in lung cancer patients treated with radiotherapy [2]. Conventional treatments for lung cancer include chemo-radiotherapy and targeted therapies [37]. There is mounting evidence that the NOTCH pathway is deregulated in various types of cancers, including lung cancer [26]. Moreover, NOTCH signaling influences treatment outcome of NSCLC patients treated with radiotherapy [29]. Therefore, its inhibition represents a potential candidate to be used in the clinic. So far clinical studies using Notch inhibitors have been disappointing in terms of tumor control and adverse effects in normal tissues [38, 39]. Radiation-related toxicity is also a strong dose-limiting factor in terms of long-term survival and quality of life in patients receiving thoracic irradiation as part of their cancer treatment [3]. In addition, lung cancer patients often suffer from co-morbidities such as COPD, which further reduce their tolerance to treatment [40]. Several studies have shown that the NOTCH signaling pathway is required for secretory cell differentiation from basal cell progenitors in the lung epithelium, whereas it triggers the proliferative capacity of NSCLC tumor cells. Indeed, we previously showed that NSCLC xenograft tumors with activated NOTCH signaling grow faster than non-Notch expressing controls and are more radiation resistant. Furthermore, we reported that genetic inactivation of NOTCH in this model blocks tumor growth [29]. Our findings indicate that pharmacological inhibition of NOTCH may simultaneously protect normal tissue from the detrimental effects of irradiation while promoting sensitivity towards radiotherapy in NSCLC patients.

Interestingly, we observed a progressive decrease in the relative amount of progenitor cells which was compensated by increased differentiation towards a mucous and ciliated cell fate. NOTCH is a well-known stem cell regulator that plays a key role controlling the turnover and adult tissue homeostasis of multiple tissues including the intestine [41, 42]. The NOTCH pathway is essential at multiple stages of development and controls stem cell numbers and activity in context of age-related tissue regeneration and repair [43]. Inactivation of NOTCH

in the intestinal epithelium of mice results in the loss of the proliferative crypt compartment through inhibition of *Bmi1*. NOTCH and *Bmi1* contribute to proliferation and self-renewal of local progenitor cells by regulating the cell cycle progression via the p16^{INK4a}/ p19^{ARF} pathway [44]. In our study, we found that anti-NOTCH therapy administered in differentiated PBECs showed important consequences in cell fate decision by increasing the percentage of p63+ and ciliated cells while reducing the number of secretory -mucous producing- cells. This is interesting as in the intestine Notch blockade increases secretory cell differentiation [41], a phenotype dependent of KLF4, which induces goblet cell differentiation [45, 46]. In line with our study, others have observed a similar role of NOTCH in regulating stem cell function and hierarchy. Specifically, genetically inhibition of NOTCH increased mammary stem cell activity, leading to anomalies in the mammary tissue such as the formation of aberrant end buds, suggesting a role of endogenous NOTCH signaling in restricting mammary stem cell expansion [47].

Prior studies have shown that expansion of progenitor/stem cell pools in tissues can play an important role as a mechanism to prevent damage by radiation [48]. Although the lung is a relatively quiescent organ, it shows great ability to proliferate and to repair after external exposure to DNA damage agents such as inhaled pollutants, suggesting the existence of highly active lung stem cells [49]. Immediately after injury, progenitor/stem cells are capable to restore normal tissue architecture by inducing the generation of the different cell types within a tissue. Following sulfur dioxide (SO₂) exposure, loss of differentiated luminal cells prompt the remaining basal cells to proliferate and differentiate into the lung-specific cell types in order to restore the airway epithelium. NOTCH pathway inhibition disrupted the normal pattern of lineage segregation upon injury [50]. During lung endoderm development, the NOTCH signaling pathway promotes the secretory cell phenotype over the ciliated cell phenotype, which resembles to the response of basal cells after airway injury [51, 18]. Upon SO₂-mediated airway epithelial injury, the secretory cell lineage is stimulated via NOTCH signalling, which induces progenitor cell self-renewal and differentiation [23, 52]. Other studies have seen that reactive oxygen species (ROS) trigger the NOTCH signalling pathway by activating Nrf2. Therefore, ROS and NOTCH together mediate progenitor cell self-renewal through regulating their proliferative capacity [24]. To our surprise, we observed that the loss of p63 basal stem cells induced by a single dose of radiation was counteracted by NOTCH inhibition. Radiotherapy, unlike damage by SO₂, mostly targets dividing cells, such as the progenitor/basal cells along the upper airway epithelium. To our knowledge, this is the

first study to test the consequences of administering NOTCH inhibition therapy on irradiated normal lung tissue. As regeneration upon irradiation ensues, we show that PBECs following anti-NOTCH treatment produce massive amounts of progenitor cells, whereas the other cell types remain more stable. Thus, we suggest that basal cells contribute to regeneration and repair of irradiated normal lung tissue via regulation of the NOTCH pathway.

Human lungs constantly deal with insults from inhaled pollutants which cause DNA damage. Therefore, lung stem cells need to efficiently respond to endogenous and exogenous DNA damage for proper repair of the epithelial airway after injury. Cigarette smoke contains over 4000 compounds that induce various types of DNA damage to exposed cells, which ultimately require the full DDR machinery including nucleotide excision repair (NER), base excision repair (BER), homologous recombination (HR) and non-homologous end joining (NHEJ) to repair these lesions [53]. In response to ionizing radiation, lung basal stem cells repair induced DNA DSBs by activating the error-prone NHEJ pathway, allowing basal cells to proliferate after injury while avoiding apoptosis [13]. In line with our data, Weeden, et al. found that the γ H2AX DSB marker, which leads to NHEJ activation, was strongly induced 1h after radiation exposure; however DSBs were resolved 24 hours post irradiation. Thereby, residual 53BP1 foci were quantified to analyze the DNA damage induced by radiation at latter time points. Our γ H2AX and 53BP1 labeling demonstrated that DBZ-treated PBECs are more proficient than control cells in repairing radiation-induced DSBs. The apparent association between γ -H2AX/53BP1 activation with DNA repair suggested a model whereby DSB-repair efficiency is normally limited by NOTCH activity in progenitor cells. In contradiction with this model, we observed an increased amount of phosphorylated-ATM, which mediates γ -H2AX/53BP1 activation, in cells treated with DBZ. Recent investigations have found that NOTCH suppresses the activation of the DDR by blocking the activation of ATM. Furthermore, NOTCH blockade promoted ATM-dependent apoptosis in irradiated leukemia cells that rely on the NOTCH signaling for their survival [32]. Further investigations will be required to unravel the specific role of NOTCH on regulating the DDR responsible for the protective effects of adding DBZ to irradiated-PBECs.

Acknowledgements

This work was supported by a grant from the European Research Council (ERC-CoG, 617060) and Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 642623

References

1. Prasanna, P.G., et al., *Normal tissue protection for improving radiotherapy: Where are the Gaps?* Translational cancer research, 2012. **1**(1): p. 35-48.
2. Wirsdorfer, F. and V. Jendrossek, *Modeling DNA damage-induced pneumopathy in mice: insight from danger signaling cascades.* Radiation oncology, 2017. **12**(1): p. 142.
3. Verma, V., C.B. Simone, 2nd, and M. Werner-Wasik, *Acute and Late Toxicities of Concurrent Chemoradiotherapy for Locally-Advanced Non-Small Cell Lung Cancer.* Cancers, 2017. **9**(9).
4. Vogelius, I.R. and S.M. Bentzen, *A literature-based meta-analysis of clinical risk factors for development of radiation induced pneumonitis.* Acta oncologica, 2012. **51**(8): p. 975-83.
5. Rodemann, H.P. and M. Bamberg, *Cellular basis of radiation-induced fibrosis.* Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 1995. **35**(2): p. 83-90.
6. Ryter, S.W., et al., *Mechanisms of cell death in oxidative stress.* Antioxidants & redox signaling, 2007. **9**(1): p. 49-89.
7. Rosen, E.M., R. Day, and V.K. Singh, *New approaches to radiation protection.* Frontiers in oncology, 2014. **4**: p. 381.
8. Prasanna, P.G., et al., *Radioprotectors and Radiomitigators for Improving Radiation Therapy: The Small Business Innovation Research (SBIR) Gateway for Accelerating Clinical Translation.* Radiat Res, 2015. **184**(3): p. 235-48.
9. Kotton, D.N. and E.E. Morrissey, *Lung regeneration: mechanisms, applications and emerging stem cell populations.* Nature medicine, 2014. **20**(8): p. 822-32.
10. Yokoyama, T., et al., *Depletion of club cells attenuates bleomycin-induced lung injury and fibrosis in mice.* Journal of inflammation, 2017. **14**: p. 20.
11. Reynolds, S.D., et al., *Airway injury in lung disease pathophysiology: selective depletion of airway stem and progenitor cell pools potentiates lung inflammation and alveolar dysfunction.* American journal of physiology. Lung cellular and molecular physiology, 2004. **287**(6): p. L1256-65.
12. Farin, A.M., et al., *Low- and high-LET radiation drives clonal expansion of lung progenitor cells in vivo.* Radiation research, 2015. **183**(1): p. 124-32.
13. Weeden, C.E., et al., *Lung Basal Stem Cells Rapidly Repair DNA Damage Using the Error-Prone Nonhomologous End-Joining Pathway.* PLoS biology, 2017. **15**(1): p. e2000731.
14. Kim, S.B., et al., *Mitigation of radiation-induced damage by targeting EGFR in noncancerous human epithelial cells.* Radiation research, 2013. **180**(3): p. 259-67.
15. Kopan, R. and M.X. Ilagan, *The canonical Notch signaling pathway: unfolding the activation mechanism.* Cell, 2009. **137**(2): p. 216-33.
16. Kong, Y., et al., *Functional diversity of notch family genes in fetal lung development.* American journal of physiology. Lung cellular and molecular physiology, 2004. **286**(5): p. L1075-83.

17. Tsao, P.N., et al., *Epithelial Notch signaling regulates lung alveolar morphogenesis and airway epithelial integrity*. Proceedings of the National Academy of Sciences of the United States of America, 2016. **113**(29): p. 8242-7.
18. Tsao, P.N., et al., *Notch signaling controls the balance of ciliated and secretory cell fates in developing airways*. Development, 2009. **136**(13): p. 2297-307.
19. Morimoto, M., et al., *Canonical Notch signaling in the developing lung is required for determination of arterial smooth muscle cells and selection of Clara versus ciliated cell fate*. Journal of cell science, 2010. **123**(Pt 2): p. 213-24.
20. Rock, J.R., et al., *Notch-dependent differentiation of adult airway basal stem cells*. Cell stem cell, 2011. **8**(6): p. 639-48.
21. Lafkas, D., et al., *Therapeutic antibodies reveal Notch control of transdifferentiation in the adult lung*. Nature, 2015. **528**(7580): p. 127-31.
22. Morimoto, M., et al., *Different assemblies of Notch receptors coordinate the distribution of the major bronchial Clara, ciliated and neuroendocrine cells*. Development, 2012. **139**(23): p. 4365-73.
23. Xing, Y., et al., *NOTCH1 is required for regeneration of Clara cells during repair of airway injury*. Stem cells, 2012. **30**(5): p. 946-55.
24. Paul, M.K., et al., *Dynamic changes in intracellular ROS levels regulate airway basal stem cell homeostasis through Nrf2-dependent Notch signaling*. Cell stem cell, 2014. **15**(2): p. 199-214.
25. Aster, J.C., W.S. Pear, and S.C. Blacklow, *The Varied Roles of Notch in Cancer*. Annual review of pathology, 2017. **12**: p. 245-275.
26. Westhoff, B., et al., *Alterations of the Notch pathway in lung cancer*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(52): p. 22293-8.
27. Hassan, K.A., et al., *Notch pathway activity identifies cells with cancer stem cell-like properties and correlates with worse survival in lung adenocarcinoma*. Clinical cancer research : an official journal of the American Association for Cancer Research, 2013. **19**(8): p. 1972-80.
28. Xiong, J., et al., *Prognostic roles of mRNA expression of notch receptors in non-small cell lung cancer*. Oncotarget, 2017. **8**(8): p. 13157-13165.
29. Theys, J., et al., *High NOTCH activity induces radiation resistance in non small cell lung cancer*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2013. **108**(3): p. 440-445.
30. Mizugaki, H., et al., *gamma-Secretase inhibitor enhances antitumour effect of radiation in Notch-expressing lung cancer*. British journal of cancer, 2012. **106**(12): p. 1953-9.
31. Adamowicz, M., J. Vermezovic, and F. d'Adda di Fagagna, *NOTCH1 Inhibits Activation of ATM by Impairing the Formation of an ATM-FOXO3a-KAT5/Tip60 Complex*. Cell reports, 2016. **16**(8): p. 2068-2076.
32. Vermezovic, J., et al., *Notch is a direct negative regulator of the DNA-damage response*. Nature structural & molecular biology, 2015. **22**(5): p. 417-24.
33. Lagadec, C., et al., *Radiation-induced Notch signaling in breast cancer stem cells*. International journal of radiation oncology, biology, physics, 2013. **87**(3): p. 609-18.

34. Deng, X., et al., *Targeting Homologous Recombination in Notch-Driven C. elegans Stem Cell and Human Tumors*. PloS one, 2015. **10**(6): p. e0127862.
35. McConnell, A.M., et al., *Distal airway epithelial progenitor cells are radiosensitive to High-LET radiation*. Scientific reports, 2016. **6**: p. 33455.
36. Danilova, N., K.M. Sakamoto, and S. Lin, *p53 family in development*. Mech Dev, 2008. **125**(11-12): p. 919-31.
37. Zappa, C. and S.A. Mousa, *Non-small cell lung cancer: current treatment and future advances*. Transl Lung Cancer Res, 2016. **5**(3): p. 288-300.
38. Yahyanejad, S., J. Theys, and M. Vooijs, *Targeting Notch to overcome radiation resistance*. Oncotarget, 2016. **7**(7): p. 7610-28.
39. Andersson, E.R. and U. Lendahl, *Therapeutic modulation of Notch signalling--are we there yet?* Nature reviews. Drug discovery, 2014. **13**(5): p. 357-78.
40. Raviv, S., et al., *Lung cancer in chronic obstructive pulmonary disease: enhancing surgical options and outcomes*. Am J Respir Crit Care Med, 2011. **183**(9): p. 1138-46.
41. Vooijs, M., et al., *Mapping the consequence of Notch1 proteolysis in vivo with NIP-CRE*. Development, 2007. **134**(3): p. 535-44.
42. Riccio, O., et al., *Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors p27Kip1 and p57Kip2*. EMBO reports, 2008. **9**(4): p. 377-83.
43. Liu, J., et al., *Notch signaling in the regulation of stem cell self-renewal and differentiation*. Curr Top Dev Biol, 2010. **92**: p. 367-409.
44. Lopez-Arribillaga, E., et al., *Bmi1 regulates murine intestinal stem cell proliferation and self-renewal downstream of Notch*. Development, 2015. **142**(1): p. 41-50.
45. Real, P.J. and A.A. Ferrando, *NOTCH inhibition and glucocorticoid therapy in T-cell acute lymphoblastic leukemia*. Leukemia, 2009. **23**(8): p. 1374-7.
46. Zheng, H., et al., *KLF4 gene expression is inhibited by the notch signaling pathway that controls goblet cell differentiation in mouse gastrointestinal tract*. Am J Physiol Gastrointest Liver Physiol, 2009. **296**(3): p. G490-8.
47. Bouras, T., et al., *Notch signaling regulates mammary stem cell function and luminal cell-fate commitment*. Cell Stem Cell, 2008. **3**(4): p. 429-41.
48. Lombaert, I.M., et al., *Keratinocyte growth factor prevents radiation damage to salivary glands by expansion of the stem/progenitor pool*. Stem Cells, 2008. **26**(10): p. 2595-601.
49. Weeden, C.E. and M.L. Asselin-Labat, *Mechanisms of DNA damage repair in adult stem cells and implications for cancer formation*. Biochim Biophys Acta, 2018. **1864**(1): p. 89-101.
50. Pardo-Saganta, A., et al., *Injury induces direct lineage segregation of functionally distinct airway basal stem/progenitor cell subpopulations*. Cell stem cell, 2015. **16**(2): p. 184-97.
51. Guseh, J.S., et al., *Notch signaling promotes airway mucous metaplasia and inhibits alveolar development*. Development, 2009. **136**(10): p. 1751-9.
52. Rock, J.R., et al., *Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition*. Proc Natl Acad Sci U S A, 2011. **108**(52): p. E1475-83.

53. Hang, B., *Formation and repair of tobacco carcinogen-derived bulky DNA adducts*. J Nucleic Acids, 2010. **2010**: p. 709521.

Figures and Tables

FIGURE 1: (A) mRNA expression of NOTCH target gene Hes1 upon DMSO/DBZ treatment on uncoated or Dll4-coated plates. (B) Western blot of cleaved NOTCH1 (Val1744) and Hes1 in 3 independent PBECs samples treated with DMSO/DBZ. Tubulin was used as loading control. (C) Representative overview of PBEC culture in the air-liquid interface (ALI) system. (D) Protein expression profiles of epithelial lung cell markers p63 (basal stem cells), LhS28 (ciliated cells) and MUC1 (mucous cells) were analyzed after 28 days of continuous DMSO/DBZ treatment. Tubulin was used as loading control.

FIGURE 2: PBECs were grown for 28 days in ALI. (A) Western Blot of basal stem cell markers (p63 and KRT5) and differentiated cellular markers Acetylated Tubulin (ciliated cells) and MUC5AC (mucous cells) at different time points. Lamin A/C was used as loading control. (B) Confocal staining of PBECs at day 0 and day 28 stained for p63 and MUC5A; Acetylated Tubulin; p63 and Edu with the relative quantification graphs.

FIGURE 3: PBECs were grown for 28 days in ALI and treated with DMSO/DBZ from day 21 onwards. (A) Western blot of basal stem cell markers (p63 and KRT5) and differentiated cellular markers Acetylated Tubulin and MUC5AC were assessed at days 22, 24 and 28. Lamin A/C was used as loading control. (B) Confocal staining of PBECs at day 22, 24 and 28 stained for p63 and Edu with the relative quantification graphs.

FIGURE 4: (A) PBECs were grown for 15 days in ALI under DMSO/DBZ conditions following 4Gy irradiation at day 14. Cell collection for western blotting was performed 24 hours after irradiation. Protein expression profiles of epithelial lung cell markers p63, LhS28 and MUC1 were analyzed 15 days after continuous DMSO/DBZ treatment. Lamin A/C was used as loading control. (B) PBECs cultured for 17 days in ALI under DMSO/DBZ conditions following 4Gy irradiation at day 14. Quantification of confocal images for p63 positive cells in relation with DAPI.

FIGURE 5: PBECS were grown for 28 days in ALI. DMSO/DBZ treatment started on day 14 which followed 4Gy radiation exposure. Cell collection for western blotting was performed 2 weeks after irradiation (A) Protein expression profiles of epithelial lung cell markers p63, LhS28 and MUC1 were analyzed 14 days after continuous DMSO/DBZ treatment. Tubulin was used as loading control. (B) Confocal staining of PBECS at day 28 stained for KRT5 and DAPI with the relative quantification graphs.

FIGURE 6: PBECS were grown for 15 days in ALI under DMSO/DBZ conditions following 4Gy irradiation at day 14. Cell collection for western blotting was performed 1 and 24 hours after irradiation. (A) Protein expression profile of DDR marker ATM and its active form pATM were analyzed 24 hours after radiation. Tubulin was used as loading control. (B) Protein expression profile of DSB marker γ -H2AX was analyzed 1 and 24 hours after radiation. Tubulin was used as loading control. (C) Quantification of confocal images for 53BP1 positive foci in p63 positive cells was analysed 24 hours after radiation.

FIGURE 7: PBECs were grown for 15 days in ALI under DMSO/DBZ conditions following 4Gy irradiation at day 14. (A) Western blot of cleaved NOTCH1 (Val1744) was performed in samples collected 1 and 24 hours after radiation. Lamin A/C was used as loading control. (B) Western blot of Hes1 was performed in samples collected 1 hour after radiation. Tubulin was used as loading control.

SUPPLEMENTARY TABLE 1: List of antibodies used for Western Blot analysis

Antibodies	Dilutions	Vendor
anti-cleaved-Notch1 (Val1744)	1:1000	Bioke
anti-Hes1	1:1000	Aviva systems biology
Rabbit anti-P63	1:1000	Abcam
Rabbit anti-KRT5	1:1000	Dako
Mouse anti-Acetylated tubulin	1:1000	Sigma Aldrich
anti-LhS28	1:1000	Abcam
Rabbit anti-Muc1	1:1000	Abcam
Mouse anti-Muc5A	1:1000	Abcam
anti-ATM	1:10000	Sigma Aldrich
Rabbit anti-p-ATM	1:1000	Cell Signaling
anti- γ -H2AX	1:500	Millipore
Rabbit anti-Lamin A/C	1:1000	Sigma Aldrich
Rabbit anti-Tubulin	1:1000	Sigma Aldrich

CHAPTER 5

γ -secretase complex composition differentially regulates Notch1 wild type and oncogenic pathways

Eloy Moreno Roig¹, Roger A.J. Habets¹, Lutgarde Serneels², Lydie M.O. Barbeau¹, Arjan J. Groot¹ and Marc Vooijs^{1#}

¹ Department of Radiotherapy (MAASTRO)/GROW – School for Developmental Biology & Oncology, Maastricht University, Maastricht, The Netherlands

² Center for the Biology of Disease, Flemish Institute for Biology (VIB), Leuven, Belgium

Corresponding author: Marc Vooijs, Department of Radiotherapy (MAASTRO)/GROW – School for Developmental Biology & Oncology, Maastricht University, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands, Tel: +31 (0)433882912; E-mail: marc.vooijs@maastrichtuniversity.nl

Keywords: Notch, γ -secretase complex, presenilin, APH1, cleavage

Running title: γ -secretase complex regulating the Notch pathway

Patent pending - to be submitted

Abstract

γ -secretase (GS) is a multicomponent enzyme complex required for processing the amyloid precursor protein (APP), causal to Alzheimer's disease, and the Notch family proteins, frequently deregulated and mutated in cancers. However, clinical use of γ -secretase inhibitors (GSIs) for both diseases has been hampered due to adverse side effects, caused by the inhibition of physiological Notch signaling. The γ -secretase enzyme complex consists of four subunits, NICASTRIN, PEN-2, PRESENILIN (PSEN) and APH1. Furthermore, there are two homologs of both PRESENILIN (1 and 2) and APH1 (A and B), of which only one is present in every complex. Mammalian cells can therefore contain up to four GS complexes but how these contribute to substrate specificity and enzyme activity is unknown. We show using loss and gain of function approaches that both *Aph* genes are necessary and sufficient for Notch proteolysis but that different γ -secretase complexes produce characteristic Notch cleavage profiles and have distinct effects on target gene activation. Specifically, Psen2:Aph1b γ -secretase complexes efficiently process oncogenic ligand-independent Notch1 receptors, but are attenuated in their ability to cleave wild type Notch1 receptors at the physiological relevant Valine1744 cleavage site. These qualitative and quantitative differences in Notch processing by different γ -secretase complexes could be exploited to advance drug development by specific targeting of pathological substrates.

Introduction

γ -secretases (GS) are multicomponent integral membrane proteases which cleave single pass type I transmembrane proteins. The clinically most important substrates are Notch and APP for which cleavage results in the liberation of the cytoplasmic signaling domains that have important biological functions during development and in adult tissues and are associated with cancer and Alzheimer's disease, respectively [1]. All GS substrates have in common that they require shedding of the ectodomain preceding intramembrane proteolysis. This process of sequential cleavage of membrane signaling molecules is termed regulated intramembrane proteolysis (RIP) [2]. The sheddases for Notch proteins belong to the ADAM family of membrane metalloproteases [3]. Notch receptors interact with adjacent ligand expressing cells to transmit short-range signals. Upon ligand binding, Notch receptors are subject to ligand-induced ectodomain cleavage (called S2) by ADAM10 [4-6]. ADAM cleavage generates an intermediate substrate which is rapidly converted into the S3 cleaved Notch Intracellular Domain (NICD) by the γ -secretase complex [7]. There are four mammalian Notch proteins (Notch 1 – 4) that follow the RIP paradigm in response to ligand [6]. The best-studied receptor is Notch1 and γ -secretase cleavage of Notch1 occurs at valine1744 (also known as S3) and is essential for most of its activity [8, 9].

The γ -secretase complex consists of four subunits: Nicastrin (NCT), Presenilin (PSEN), anterior pharynx defective 1 (APH1) and presenilin enhancer 2 (PEN-2) [10]. Both PSEN and APH1 have two different homologs in humans, which are encoded by two separate genes. The stoichiometry of the γ -secretase complex is 1:1:1:1 and therefore different subunit combinations can result in four different γ -secretase complexes [11, 12], which all contain NCT and PEN-2 in combination with either PSEN1 or 2 and APH1A or B. Presenilins are aspartyl proteases and their catalytic activity is required for Notch and APP S3 cleavage [13, 14]. Cells and mice lacking *Psen1* resemble the hematopoietic and neuronal embryonic lethal phenotype of Notch1 loss of function, indicating Notch1 is a crucial *Psen1* substrate [15, 16]. In contrast, *Psen2* deficient mice are viable and show only mild pulmonary changes, but no clear Notch phenotype [16]. However, in the absence of PSEN1, PSEN2 is sufficient for Notch 1-3 receptor proteolysis in response to ligand [6]. Mice lacking *Aph1a* are embryonic lethal, with phenotypes resembling full γ -secretase (*Psen1/2*) KO in the yolk-sac and brain and a milder somitogenesis phenotype resembling a Notch1 processing deficient hypomorph allele [8, 17]. Mice lacking *Aph1bc* do not have a Notch phenotype but display memory defects and altered APP processing consistent with non-overlapping expression patterns of

Notch1 and Aph1b in the brain [18, 19]. Nicastrin^{-/-} mice display a full Notch1 loss of function phenotype [20]. Biochemical analysis has shown that all four subunits are required and sufficient for robust γ -secretase activity [11, 16, 21, 22]. Taken together, these findings indicate heterogeneity in substrate processing that is determined by combinations of PSEN and APH1 subunits.

There are more than 150 mutations known in PSEN1 and 2, which alter the processing of APP underlying the formation of pathogenic A β 42 amyloid plaques, associated with early-onset familial Alzheimer's disease (AD) [23-25]. As such, γ -secretase inhibitors (GSI) are widely explored as therapeutics for targeting AD. GSIs are also investigated as a strategy to block oncogenic Notch signaling in T cell acute lymphocytic leukaemia [26] and many solid cancers [27]. Unfortunately, the clinical implementation of GSI has been hampered by dose-limiting toxicities caused in normal tissues, most notably the skin, thymus, gut and brain in mice [28, 29] and humans [30, 31]. The intestinal toxicities are largely related to loss of function of Notch1 and Notch2 receptor signaling [32-34]. While the contributions of the Psen and Aph1 subunits for γ -secretase activity towards APP has been reported [35], little is still understood how different γ -secretase complexes affect Notch processing and activity. A better understanding of these specificities may aid in the design of Notch sparing γ -secretase inhibitors for the treatment of Alzheimer's disease [31] or GSIs that are specific for mutated Notch receptors in cancers while sparing wild type signaling and overcoming normal tissue side effects.

Here, we show that all four γ -secretase complexes in mammalian cells regulate ligand-dependent Notch1 processing and transcriptional activation. We find there is no direct correlation between total γ -secretase or epitope-specific Notch1 Val1744 processing and target gene expression. We show selectivity of the Psen2:Aph1b complex towards the processing of ligand-independent mutant Notch1 substrates compared to ligand induced physiological signaling. We exploited these differences by showing that targeting intracellular vesicles using chloroquine caused selective inhibition of oncogenic Notch1 signaling.

Experimental procedures

Cell lines and chemicals

Psen1/2 double knockout/*Aph1abc* triple Knockout (Quintuple Knockout, QKO) mouse embryonic fibroblasts (MEFs) and QKO reconstituted cells with *Psen1:Aph1a* (1A), *Psen1:Aph1b* (1B), *Psen2:Aph1a* (2A), *Psen2:Aph1b* (1B) were generated as described previously [35] and maintained in DMEM/F12 + 10% FCS + 0.1%PEN/STREP. Cells were treated for 24 hours with Dibenzazepine (DBZ) 0.2 μ M (Syncom, Groningen, The Netherlands) or DMSO unless stated otherwise. 1 μ g/ml of rDll4-Cf (R&D Systems) or rDll1 (kind gift from I. Bernstein), 0.2% gelatin and 0.1% BSA in PBS solution was used to coat 24-well plates overnight at 4 degrees, rinsed once with PBS before cells were plated.

Plasmids, transfections and infections

Murine Δ EGF-*Notch1*-L1594P-6xMYC (LNR) [4] was subcloned into the pBabe-Puro vector and was transfected together with pCMV-VSVG and pVPack-GP into HEK293FT cells to produce pseudo-lenti viruses to infect: QKO, 1A, 1B, 2A and 2B cells [6]. Cells transduced with Δ EGF-*Notch*-L1594P construct were maintained with 1 μ g/ml of puromycin. The pcDNA3 hNOTCH1 full-length construct [36] was used as a template to generate the L1594P mutant construct with primers forward LN1PF: 5'-CCTTCCACTTCCCCCGGGAGCTCAGCCGCG-3' and reverse LN1PR 5'-CGCGGCTGAGCTCCCGGGGAAGTGGAAGG-3' by QuickChange site directed mutagenesis according to manufacturer's instructions (Stratagene). Firefly luciferase encoding reporter plasmids containing synthetic CSL binding sites (pGL4.24-12xCSL) was used to monitor Notch activity, using pGL4.74 TK-hRL (Promega) as transfection control. 12XCSL Gaussia was generated by subcloning a fragment of the synthetic 12xCSL with minimal CMV promoter into pGLuc-Basic Vector (New England Biolabs). A CMV-driven Firefly luciferase-GFP construct was used as transfection control [37]. Transfections were performed using Fugene HD transfection reagent (Promega).

Ligand stimulation assay

Cells were plated on either rDll4-Cf or rDll1 coated plates and treated with either DMSO or DBZ for 24 hours. Co-culture with ligand was used to activate endogenous Notch1 in MEFs.

Reporter assays

Cells were plated 24 hours before transfecting with two reporter genes. 1 μ g of the primary reporter was used to record the effect of specific stimuli and 0.1 μ g of the control reporter for normalization. Cells were seeded 24 hours post-transfection into 24-well plates and treated as indicated for 24 hours. Cells were washed, lysed and luciferase was measured, according to the manufacturer's guidelines (dual-glo luciferase reporter assay system Promega) on a Fluostar Omega plate reader (BMG Labtech). For Gal4-luciferase assay, various concentrations of the NOTCH receptor plasmid were transfected and complemented with empty vector. A plasmid containing synthetic Gal4 binding sites (5xUAS FR-luc) was used together with a thymidine kinase driven Renilla luciferase plasmid to normalize the data. For Gaussia-Luc assay, medium was collected at different time points to monitor 12xCSL activation and measured with the BioLux® Gaussia Luciferase Assay Kit according to manufacturer's instructions (NEB). All values are expressed as *relative light units* (RLU).

Western blotting and antibodies

Cells were directly lysed in Laemmli buffer (6x) and samples were boiled prior to resolving by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and blocked for 1 hour in 5% skim milk in TBS, 0.05% Tween-20 (TBS-T). Membranes were probed overnight at 4°C with primary antibodies and bound antibodies were visualized using HRP-linked secondary antibodies (Cell-Signaling) and ECL Luminescence (Pierce Biotechnology). Anti-Lamin A/C (1:1000) and anti-beta-tubulin III (1:1500) were purchased from Sigma-Aldrich, anti-Notch1 S3-Val1744 (1:1000) and secondary anti-mouse and anti-rabbit (1:2500) were from Cell Signaling. Anti-Myc 9E10 (1:5000) and anti-Notch1 C20 (1:1000) were from Santa Cruz Biotechnology.

qRT-PCR

RNA isolation was performed according to the manufacturer's instructions (Nucleospin RNA isolation, Macherey Nagel) and cDNA synthesis was performed using I-script reverse transcriptase (Bio-RAD). mRNA expression was normalized to 18S. Primers used are described in supplementary Table 1.

Statistical analysis

GraphPad Prism software (version 5.01 for Windows) was used to perform statistical analyses. An unpaired Student's *t*-test test was used to determine the statistical significance of differences between two independent groups of variables. For all tests, a $P < 0.05$ was considered significant.

Results

Psen and Aph1 subunits produce different Notch1 cleavage profiles and activity

To directly investigate the contribution of different γ -secretase complex subunits on Notch signaling activity we compared QKO MEFs with these cells reconstituted with 4 different combinations of human *Aph1* cDNA (*Aph1A* or *Aph1B*) and *Psen* homologues (*Psen1* or *Psen2*) as described and characterized previously [35]. QKO and reconstituted cells were grown on plates with bound recombinant Dll4-Cf, a potent inducer of Notch1 cleavage and transcriptional activity [6] to monitor the effects on Notch1 cleavage. We used a C-terminal antibody (C-20), as well as the Val1744 neo-epitope antibody, which detects γ -secretase cleaved Notch1 at valine1744. Reconstitution with both *Psen* and *Aph1* resulted in Notch1 γ -secretase cleavage for all combinations in response to Dll4-Cf stimulation, albeit to a different extent. Consistently in QKO, no NOTCH1 cleavage at Val1744 was observed. A robust Val1744 cleavage was found for 1B and 2A cells and less for 1A cells. The lowest cleavage was noticed in 2B cells (Figure 1A). The extent of Notch receptor processing by the different γ -secretase complexes did not depend on the type of ligand used for stimulation, as similar results were obtained with recombinant rDll1 stimulation (Supplementary figure 1). The C-20 antibody is not sensitive enough to detect NICD fragments and therefore we can only indirectly estimate S3 cleavage by assessing S2 accumulation in the presence of the γ -secretase inhibitor DBZ. Only minor differences in S2 accumulation were observed, which did not correlate with the strong differences observed in Val1744 cleavage. These findings were replicated in an independently generated series of MEFs reconstituted with 1A, 1B, 2A and 2B (Supplementary figure 2).

Total NOTCH1 processing by γ -secretase does not correlate with cleavage at valine 1744.

To directly assess the effects of *Aph1* and *Psen* Notch1 γ -secretase cleavage irrespective of cleavage at neo-epitope Val1744 we transfected MEFs with NOTCH1-Gal4 fusion proteins [38] and co-cultured these with Dll4-Cf. Ligand induced γ -secretase of NOTCH1 will lead to the release of a Gal4-NICD fusion protein which can activate a reporter containing Gal4-DNA binding sites driving firefly luciferase expression. Thus, total γ -secretase cleavage of NOTCH1 (irrespective of cleavage site), leading to release of Gal4-NICD from the membrane is monitored in this assay. Upon Dll4-Cf stimulation, transcriptional activity was induced in all the four reconstituted cell lines, in a dose dependent manner (Figure 1B), while for QKO cells, no transcriptional activity was observed. Notably, the induction of Gal4 transcriptional

activity was highly dependent on the composition of the γ -secretase complex. Reporter activity was the highest in 2B MEFs, while lower in 1A, 1B and 2A MEFs. This finding suggests that NOTCH1-Val1744 cleavage assessed by immunoblotting does not correlate to total S3 cleavage after ligand stimulation. This unexpected finding suggests the existence of additional cleavage sites, in close proximity to the S3 cleavage site, capable of releasing a possibly transcriptionally competent NICD1.

Different γ -secretase complexes produce different transcriptional output

Based on the previous findings we hypothesized that different γ -secretase complexes producing different Notch1 cleavage profiles could also result in different transcriptional output and target gene activation. We observed that Dll4-Cf induced a γ -secretase complex dependent increase in NOTCH/RBP-J κ reporter luciferase activity, compared to the QKO cells (Figure 1C). The lowest Notch-dependent transcriptional activation was observed in cells reconstituted with Psen2, whereas activation in cells reconstituted with Psen1:Aph1B were several fold higher. DBZ treatment abolished transcriptional activity in all cell lines. Dynamic time-course experiments using a NOTCH transcriptional reporter expressing secreted Gaussia-Luc demonstrated a linear increase of NOTCH1 transcriptional activity in all cell line combinations excluding time-dependent differences (Figure 1D). We found a trend towards a higher Notch dependent transcriptional activation in cells reconstituted with Psen1:Aph1B in comparison with the other cell models.

To directly determine the effect of the different γ -secretase complexes on Dll4-Cf induced target gene activation, we assessed mRNA expression of two well-known Notch target genes *Hes1* and *Hey1*. We observed that Dll4-Cf stimulation induced similar levels of *Hes1* mRNA in all reconstituted cell lines. In contrast, *Hey1* mRNA expression was induced in 1A, 2A and 2B cells, but not in 1B cells after Dll4-Cf stimulation (Figure 1E). Thus, ligand-induced activation of endogenous Notch1 target genes in cells does not correlate qualitatively nor quantitatively with S3/ γ -secretase cleavage (Val1744) and differs dramatically with subunit composition.

Psen2:Aph1B γ -secretase complexes efficiently process oncogenic Notch receptor

Next, we questioned whether oncogenic mutant Notch1 receptors are also differentially processed depending on the γ -secretase complex composition. The oncogenic ligand-independent Notch mutant (Δ EGF-Notch1-L1594P-MYC) receptor was equally expressed in

all cell lines (anti-MYC) and processed by all four γ -secretase complexes (anti-Val1744-NICD1). NICD1 processing at Val744 cleavage was most strong in 1A and 2B cells and less pronounced in 1B and 2A cells (Figure 2A), which is opposite compared to Notch1 cleavage in wild type cells (Figure 1A). DBZ blocked NICD1-Val1744 irrespective of the GS complex. In the absence of GSI we observed accumulation of S2 cleaved and full-length unprocessed Notch1 indicative of blocked γ -secretase cleavage in 2A cells which further accumulated with GSI (Figure 2A). NOTCH1-L1594P expression induced Gal4 transcriptional activation in a dose dependent manner in 1A, 1B, 2A and 2B cells (Figure 2B), resembling Val1744 cleavage. To answer whether differences in Val1744 cleavage of oncogenic NOTCH1 by different γ -secretase complexes also results in varying transcriptional outputs we performed 12xCSL reporter assays in MEFs expressing Δ EGF-Notch1-L1594P-MYC. Transcriptional activation of 12xCSL was induced in all reconstituted cell lines expressing the mutant L1594P receptor. Markedly, γ -secretase dependent NOTCH1 cleavage was up to 10-fold higher in cells reconstituted with Psen2:Aph1B compared to cells expressing other GS complexes (Figure 2C). The cleavage and transcriptional induction of mutant NOTCH1 proteins by different γ -secretase complexes in cells was stable over time and was also reflected in the activation of *Hes1* and *Hey1* mRNA target genes (Figure 2D, E). Taken together these data demonstrate that different γ -secretase complexes that differently impact on physiological versus oncogenic Notch1 in cells.

Endosomal trafficking regulates the processing of oncogenic Notch1 receptor by Psen2:Aph1B γ -secretase complexes

The endocytic pathway has emerged as an alternative way to regulate the transport and processing of NOTCH receptors. Chloroquine (CQ), a lysosomotropic agent that alters lysosomal pH and blocks vesicular fusion, was used to investigate the contribution of this vesicular pathway to physiological and oncogenic Notch activity. We observed that regardless of the GS complex in cells, ligand induced wild type Notch1 signaling was not affected by the addition of CQ (Figure 3A). In cells expressing mutant Notch1 we observed that cells reconstituted with Psen2 were 10-fold more sensitive to CQ inhibition compared to Psen1 expressing cells (Figure 3B).

Discussion

Here we show for the first time γ -secretase complex selectivity based on Psen and Aph1 subunits that differentially affects cleavage and transcriptional activity of wild type and mutant Notch1 molecules in cells. γ -secretases are involved in a variety of physiological and pathological processes and this broad involvement explains the severe side effects when using broad-spectrum γ -secretase inhibitors [39]. However, most of the observed side-effects of γ -secretase inhibition are related to on target Notch toxicity in the intestine causing intestinal goblet cell metaplasia [32, 34, 40]. Previously, it was shown that the composition of the γ -secretase complexes, by expression of the different subunits, generated specific APP cleavage profiles [19, 35]. Here, we demonstrate heterogeneity in cleavage of the NOTCH1 receptor depending on the γ -secretase complex composition, resulting in different transcriptional activities and localization. Our results reveal an unexpected preference of the Psen2:Aph1B γ -secretase complex to process oncogenic mutant Notch1 receptors over wild type Notch receptors. Psen2-specific targeting could be exploited in cancer cells while sparing Psen1 substrates including Notch signaling in normal cells where Psen2 is non-essential.

Previously it has been shown that presenilin, APH1, PEN-2 and Nicastrin, in a 1:1:1:1 stoichiometry, are essential and sufficient for γ -secretase activity [11, 12, 22]. In line with this, we showed that Aph1-deficient cells are defective in ligand-induced proteolysis activation of Notch1 (Supplementary figure 3). However, it was shown that γ -secretase complexes lacking NCT retained minimal γ -secretase activity, although the complex appeared to be very unstable, suggesting that NCT provides a stable complex [41]. Interestingly mice deficient for the different γ -secretase complex subunits do not have similar phenotypes. Both *Psen1* and *Aph1a* knockout mice are shown to be lethal and exhibit phenotypes comparable to the *Notch1* knockout phenotype, albeit somewhat milder [15, 42]. *Aph1b/c* (*APH1B* in humans due to specific duplication of the gene in rodents) deficiency only causes mild behavioral changes, coincident with defective Neuregulin cleavage [18, 19], as well as a mild reduction in APP processing in some regions of the brain [17]. *Psen2* knockout mice show a mild pulmonary phenotype, but do enhance the *Psen1* knockout phenotype, indicating redundancy in Presenilin 1 and 2 cleavage of Notch *in vivo* [16, 43]. While PSEN1 seems to be dominant over PSEN2 in Notch receptor processing, as we confirm in our studies as well for ligand induced NOTCH signaling overexpression of PSEN2 in dKO cells clearly indicates that NOTCH1, 2 and 3 receptors can be cleaved by PSEN2 as well [6]. Specific targeting of Aph1B in an AD mouse model did lower A β 42 aggregation within the brain, without signs of

Notch related side effects. This can be explained by the fact that the Aph1B subunits produces longer A β peptides compared to Aph1A and that Aph1B, predominantly expressed in the hippocampus important for AD, does not show co-localization with Notch mRNA [18, 19, 35]. We confirm and extend these findings by showing that in cells lacking Psen1 and Aph1A (2B) Notch1 Val1744 cleavage is low compared to the other cells. Remarkably total cleavage of endogenous and transfected Notch1 as well as transcriptional activation is unaffected. These results show experimental evidence that different γ -secretase complexes can execute different cleavage sites in the Notch transmembrane, not all of which have similar activity. As was shown before, γ -secretase complexes are able to cleave both Notch and APP at the cell surface and within endosomes, and the location is important to determine the precision of the cleavage [44, 45]. Cleavage for Notch1 occurs at valine1744 (V-NICD) at the cell surface, whereas cleavage within the acidic endosomes results in cleavage at serine1747(S-NICD), which is less stable [45]. While our study does not address the fate of NICD molecules it highlights that Val1744 cleaved Notch1 molecules do not represent all cleaved NICD1 molecules in cells and the shows the existence of multiple NICD species with unknown activities and stability. NICD molecules with distinct N-termini (X-NICD) are subject to N-rule degradation [46] and recently shown to be a direct target for autophagy [47]. The selective inhibition of Psen2:Aph1B with chloroquine suggests that this GS-complex produces N-termini distinct from Psen1 that are subject to autophagosomal endosomal regulation and that this is specific for mutated Notch1 molecules. Significantly we recently demonstrated that Psen2 containing γ -secretase complex has a preferred intracellular vesicular location that would be selectively affected by chloroquine [48]. These findings are in line with the absence of Notch related phenotypes in *Aph1b* and *Psen2* KO mice [16, 19]. Whether NOTCH mutant molecules have a distinct subcellular location consistent with Psen2 location is not known.

Notch and APP processing by γ -secretase complexes is highly similar. Both undergo endoproteolytic (ϵ) cleavage, releasing the C-terminal NICD or AICD fragment respectively [49]. Next, the N-terminal fragments are trimmed by carboxypeptidase-like (γ) cleavage, resulting in A β or Notch β peptides which are secreted [50-53]. For APP processing, it has been shown that the Psen subunits predominantly regulate ϵ cleavage, whereas the Aph subunits predominantly regulate γ cleavage [35]. Here, we showed that both the Psen and the Aph subunits determine the endoproteolytic cleavage of Notch1. To date, there was no physiological evidence for a role for Aph1B in mutant Notch processing. To the best of our

knowledge our results show for the first time that Aph1B is sufficient to cleave Notch1 when a functional presenilin subunit is present.

By dissecting the role for γ -secretase complexes in physiological versus pathological processes selective inhibition may be accomplished reducing normal tissue toxicity. This has already led to subunit specific pharmacological targeting of γ -secretase complexes. For example, Psen2 sparing GSIs show lower A β aggregation in the brain of AD mice, without showing obvious Notch related toxicity, which resulted in clinical trials for PSEN2 sparing GSIs [54-56]. These results are in line with the reduced selectivity of Psen2/Aph complexes for ligand induced NOTCH signaling we observed here. These studies showed that PSEN2 can regulate Notch receptor processing in the absence of PSEN1 and are in line with the observation that PSEN1 is the major enzyme responsible for A β production in the brain, whereas PSEN2 only showed a minor contribution [57, 58]. However, a disadvantage of PSEN2 sparing GSIs is the increase in plasma A β levels due to the prominent role for PSEN2 in A β production in cells following to its release to the plasma [55, 59]. Unfortunately, there are currently no PSEN2-specific inhibitors available. Another interesting therapeutic strategy is the use of γ -secretase modulators (GSMs). These compounds specifically target γ -cleavage, while leaving ϵ cleavage unaltered, affecting A β 42, but not NICD generation [60]. First generations GSMs, nonsteroidal anti-inflammatory drugs (NSAIDs), specifically target APP whereas second generation GSMs target APP with a higher specificity than Notch β production, but do not affect NICD formation [61]. If the fate and behavior of secreted NOTCH β peptides is similar to A β 40/42 peptides is unknown. To date, no NOTCH-specific γ -secretase modulators are known. In addition, a recent study described the benefits of using γ -secretase stabilizing compounds (GSSC) as potential therapeutics for AD. The length of A β peptides, and consequently the production of “pathologic-like A β profiles, is determined by the stability of enzyme-substrate binding which is affected by e.g. temperature change [62]. Further research is needed to investigate the consequences of using GSSC for Notch processing.

Here, we show for the first time that targeting the Psen2:Aph1B γ -secretase complex might be a selective option to target mutated Notch proteins as found in some cancers [27]. To date Psen2 selective inhibitors have not been described. When available Psen2-selective inhibitors would not affect essential Psen1 substrates, such physiological Notch signaling, in normal

tissues enabling more tumor specific targeting of mutated Notch while sparing adverse effects in normal tissues.

Acknowledgments

We thank Bart de Strooper (VIB, Leuven) for providing Aph/Psen-deficient MEF. This work was supported by the European Research Council (ERC) under the European Community Seventh Framework Program (FP7/2007-2013) ERC starting Grant 208259.

References

1. De Strooper, B., T. Iwatsubo, and M.S. Wolfe, *Presenilins and gamma-secretase: structure, function, and role in Alzheimer Disease*. Cold Spring Harbor perspectives in medicine, 2012. **2**(1): p. a006304.
2. Brown, M.S., et al., *Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans*. Cell, 2000. **100**(4): p. 391-8.
3. Schlondorff, J. and C.P. Blobel, *Metalloprotease-disintegrins: modular proteins capable of promoting cell-cell interactions and triggering signals by protein-ectodomain shedding*. J Cell Sci, 1999. **112** (Pt 21): p. 3603-17.
4. van Tetering, G., et al., *Metalloprotease ADAM10 is required for Notch1 site 2 cleavage*. J Biol Chem, 2009. **284**(45): p. 31018-27.
5. Bozkulak, E.C. and G. Weinmaster, *Selective use of ADAM10 and ADAM17 in activation of Notch1 signaling*. Mol Cell Biol, 2009. **29**(21): p. 5679-95.
6. Groot, A.J., et al., *Regulated proteolysis of NOTCH2 and NOTCH3 receptors by ADAM10 and presenilins*. Mol Cell Biol, 2014. **34**(15): p. 2822-32.
7. Huppert, S., et al., *Embryonic Lethality in Mice Homozygous for a Processing Deficient Notch1 Allele*. Nature, 2000. **405**: p. 966-970.
8. Huppert, S.S., et al., *Embryonic lethality in mice homozygous for a processing-deficient allele of Notch1*. Nature, 2000. **405**(6789): p. 966-70.
9. Schroeter, E.H., J.A. Kisslinger, and R. Kopan, *Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain*. Nature, 1998. **393**(6683): p. 382-6.
10. De Strooper, B., *Aph-1, Pen-2, and Nicastrin with Presenilin generate an active gamma-Secretase complex*. Neuron, 2003. **38**(1): p. 9-12.
11. Takasugi, N., et al., *The role of presenilin cofactors in the gamma-secretase complex*. Nature, 2003. **422**(6930): p. 438-41.
12. Kimberly, W.T., et al., *Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2*. Proc Natl Acad Sci U S A, 2003. **100**(11): p. 6382-7.
13. Wolfe, M.S., et al., *Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity*. Nature, 1999. **398**(6727): p. 513-7.
14. Esler, W.P., et al., *Transition-state analogue inhibitors of gamma-secretase bind directly to presenilin-1*. Nat Cell Biol, 2000. **2**(7): p. 428-34.
15. Shen, J., et al., *Skeletal and CNS defects in Presenilin-1-deficient mice*. Cell, 1997. **89**(4): p. 629-39.
16. Herreman, A., et al., *Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency*. Proc Natl Acad Sci U S A, 1999. **96**(21): p. 11872-7.
17. Serneels, L., et al., *Differential contribution of the three Aph1 genes to gamma-secretase activity in vivo*. Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1719-24.
18. Dejaegere, T., et al., *Deficiency of Aph1B/C-gamma-secretase disturbs Nrg1 cleavage and sensorimotor gating that can be reversed with antipsychotic treatment*. Proc Natl Acad Sci U S A, 2008. **105**(28): p. 9775-80.

19. Serneels, L., et al., *gamma-Secretase heterogeneity in the Aph1 subunit: relevance for Alzheimer's disease*. Science, 2009. **324**(5927): p. 639-42.
20. Kimberly, W.T., et al., *Notch and the amyloid precursor protein are cleaved by similar gamma-secretase(s)*. Biochemistry, 2003. **42**(1): p. 137-144.
21. Kimberly, W.T., et al., *Notch and the amyloid precursor protein are cleaved by similar gamma-secretase(s)*. Biochemistry, 2003. **42**(1): p. 137-44.
22. Edbauer, D., et al., *Reconstitution of gamma-secretase activity*. Nat Cell Biol, 2003. **5**(5): p. 486-8.
23. Kumar-Singh, S., et al., *Mean age-of-onset of familial alzheimer disease caused by presenilin mutations correlates with both increased Abeta42 and decreased Abeta40*. Hum Mutat, 2006. **27**(7): p. 686-95.
24. Yoshiike, Y., et al., *Specific compositions of amyloid-beta peptides as the determinant of toxic beta-aggregation*. J Biol Chem, 2003. **278**(26): p. 23648-55.
25. Chavez-Gutierrez, L., et al., *The mechanism of gamma-Secretase dysfunction in familial Alzheimer disease*. EMBO J, 2012. **31**(10): p. 2261-74.
26. Weng, A.P., et al., *Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia*. Science, 2004. **306**(5694): p. 269-71.
27. Aster, J.C., W.S. Pear, and S.C. Blacklow, *The Varied Roles of Notch in Cancer*. Annual Review of Pathology: Mechanisms of Disease, 2017. **12**(1): p. 245-275.
28. Milano, J., et al., *Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation*. Toxicol Sci, 2004. **82**(1): p. 341-58.
29. Wong, G.T., et al., *Chronic treatment with the gamma-secretase inhibitor LY-411,575 inhibits beta-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation*. J Biol Chem, 2004. **279**(13): p. 12876-82.
30. Tolcher, A.W., et al., *Phase I study of RO4929097, a gamma secretase inhibitor of Notch signaling, in patients with refractory metastatic or locally advanced solid tumors*. J Clin Oncol, 2012. **30**(19): p. 2348-53.
31. Doody, R.S., et al., *A phase 3 trial of semagacestat for treatment of Alzheimer's disease*. N Engl J Med, 2013. **369**(4): p. 341-50.
32. van Es, J.H., et al., *Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells*. Nature, 2005. **435**(7044): p. 959-63.
33. Vooijs, M., et al., *Mapping the consequence of Notch1 proteolysis in vivo with NIP-CRE*. Development, 2007. **134**(3): p. 535-44.
34. Riccio, O., et al., *Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors p27Kip1 and p57Kip2*. EMBO Rep, 2008. **9**(4): p. 377-83.
35. Acx, H., et al., *Signature amyloid beta profiles are produced by different gamma-secretase complexes*. J Biol Chem, 2014. **289**(7): p. 4346-55.
36. Habets, R.A., et al., *Human NOTCH2 Is Resistant to Ligand-independent Activation by Metalloprotease Adam17*. J Biol Chem, 2015. **290**(23): p. 14705-16.
37. Konings, G.F., et al., *Blocking 17beta-hydroxysteroid dehydrogenase type 1 in endometrial cancer: a potential novel endocrine therapeutic approach*. J Pathol, 2018. **244**(2): p. 203-214.

38. Li, K., et al., *Modulation of Notch signaling by antibodies specific for the extracellular negative regulatory region of NOTCH3*. J Biol Chem, 2008. **283**(12): p. 8046-54.
39. De Strooper, B. and W. Annaert, *Novel research horizons for presenilins and gamma-secretases in cell biology and disease*. Annu Rev Cell Dev Biol, 2010. **26**: p. 235-60.
40. Searfoss, G.H., et al., *Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional gamma-secretase inhibitor*. J Biol Chem, 2003. **278**(46): p. 46107-16.
41. Zhao, G., et al., *Gamma-secretase composed of PS1/Pen2/Aph1a can cleave notch and amyloid precursor protein in the absence of nicastrin*. J Neurosci, 2010. **30**(5): p. 1648-56.
42. Wong, P.C., et al., *Presenilin 1 is required for Notch1 and D111 expression in the paraxial mesoderm*. Nature, 1997. **387**(6630): p. 288-92.
43. Donoviel, D.B., et al., *Mice lacking both presenilin genes exhibit early embryonic patterning defects*. Genes Dev, 1999. **13**(21): p. 2801-10.
44. Fukumori, T., et al., *Galectin-3 regulates mitochondrial stability and antiapoptotic function in response to anticancer drug in prostate cancer*. Cancer Res, 2006. **66**(6): p. 3114-9.
45. Tagami, S., et al., *Regulation of Notch signaling by dynamic changes in the precision of S3 cleavage of Notch-1*. Mol Cell Biol, 2008. **28**(1): p. 165-76.
46. Blat, Y., et al., *Mutations at the P1' position of Notch1 decrease intracellular domain stability rather than cleavage by gamma-secretase*. Biochem Biophys Res Commun, 2002. **299**(4): p. 569-73.
47. Cha-Molstad, H., et al., *Regulation of autophagic proteolysis by the N-recognin SQSTM1/p62 of the N-end rule pathway*. Autophagy, 2018: p. 1-3.
48. Sannerud, R., et al., *Restricted Location of PSEN2/gamma-Secretase Determines Substrate Specificity and Generates an Intracellular Abeta Pool*. Cell, 2016. **166**(1): p. 193-208.
49. Sastre, M., et al., *Presenilin-dependent gamma-secretase processing of beta-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch*. EMBO Rep, 2001. **2**(9): p. 835-41.
50. Qi-Takahara, Y., et al., *Longer forms of amyloid beta protein: implications for the mechanism of intramembrane cleavage by gamma-secretase*. J Neurosci, 2005. **25**(2): p. 436-45.
51. Takami, M., et al., *gamma-Secretase: successive tripeptide and tetrapeptide release from the transmembrane domain of beta-carboxyl terminal fragment*. J Neurosci, 2009. **29**(41): p. 13042-52.
52. Fukumori, A., et al., *Presenilin-dependent gamma-secretase on plasma membrane and endosomes is functionally distinct*. Biochemistry, 2006. **45**(15): p. 4907-14.
53. Okochi, M., et al., *Presenilins mediate a dual intramembraneous gamma-secretase cleavage of Notch-1*. EMBO J, 2002. **21**(20): p. 5408-16.
54. Wu, W.L., et al., *Discovery of SCH 900229, a Potent Presenilin 1 Selective gamma-Secretase Inhibitor for the Treatment of Alzheimer's Disease*. ACS Med Chem Lett, 2012. **3**(11): p. 892-6.

55. Borgegard, T., et al., *Alzheimer's disease: presenilin 2-sparing gamma-secretase inhibition is a tolerable Abeta peptide-lowering strategy*. J Neurosci, 2012. **32**(48): p. 17297-305.
56. Best, J.D., et al., *The novel gamma secretase inhibitor N-[cis-4-[(4-chlorophenyl)sulfonyl]-4-(2,5-difluorophenyl)cyclohexyl]-1,1,1-trifluoromethanesulfonamide (MRK-560) reduces amyloid plaque deposition without evidence of notch-related pathology in the Tg2576 mouse*. J Pharmacol Exp Ther, 2007. **320**(2): p. 552-8.
57. Franberg, J., et al., *Minor contribution of presenilin 2 for gamma-secretase activity in mouse embryonic fibroblasts and adult mouse brain*. Biochem Biophys Res Commun, 2011. **404**(1): p. 564-8.
58. De Strooper, B., et al., *Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein*. Nature, 1998. **391**(6665): p. 387-90.
59. Lanz, T.A., et al., *Concentration-dependent modulation of amyloid-beta in vivo and in vitro using the gamma-secretase inhibitor, LY-450139*. J Pharmacol Exp Ther, 2006. **319**(2): p. 924-33.
60. Weggen, S., et al., *A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity*. Nature, 2001. **414**(6860): p. 212-6.
61. Wanngren, J., et al., *Second generation gamma-secretase modulators exhibit different modulation of Notch beta and Abeta production*. J Biol Chem, 2012. **287**(39): p. 32640-50.
62. Szaruga, M., et al., *Alzheimer's-Causing Mutations Shift Abeta Length by Destabilizing gamma-Secretase-Abetan Interactions*. Cell, 2017. **170**(3): p. 443-456 e14.

Figures and Tables

FIGURE 1: γ -secretase complex composition regulates wild type Notch1 receptor cleavage and activation

(A) Western blot analysis of Notch1 in QKO cells and QKO cells reconstituted with different γ -secretase subunits, Psen1:Aph1A, Psen1:Aph1B, Psen2:AphA and Psen2:Aph1B after stimulation with Dll4-Cf in the presence or absence of DBZ. The Notch1 C-20 antibody is directed against the c-terminal 20 amino acids of Notch1, detecting all cleaved forms of the receptor, whereas the valine1744 NICD1 antibody only recognizes Notch1 fragments cleaved at Valine1744 (NICD1/S3). (B) Different concentrations of NOTCH1-Gal4 were introduced into QKO, 1A, 1B, 2A and 2B MEFs and Gal4 reporter assay was performed after Dll4-Cf stimulation. (C) NOTCH transcriptional activation measured by RBP-j κ /CSL-luciferase reporter gene activation in QKO, 1A, 1B, 2A and 2B cells stimulated by Dll4-Cf in the presence or absence of DBZ. (D) Notch transcriptional activation overtime measured by RBP-j κ /CSL-Gaussia reporter gene activation in QKO, 1A, 1B, 2A and 2B cells stimulated by Dll4-Cf. (E) *Hes1* and *Hey1* mRNA expression levels in QKO, 1A, 1B, 2A and 2B cells after stimulation with Dll4-Cf corrected with 18S. Lamin A/C and actin are used as loading controls. RLU; relative light units. Data are presented as mean $\pm$ SEM for n=3.

FIGURE 2: Psen2:Aph1B efficiently cleaves oncogenic mutant Notch1 receptor

(A) Western blot analysis of Δ EGF-Notch1-L1594P-MYC in QKO cells and QKO cells reconstituted with different γ -secretase subunits in the presence or absence of DBZ. (B) Different concentrations of NOTCH1-L1594P-Gal4 were introduced into QKO, 1A, 1B, 2A and 2B MEFs and Gal4 reporter assay was performed. (C) NOTCH transcriptional activation measured by RBP-j κ /CSL-luciferase reporter gene activation in QKO, 1A, 1B, 2A and 2B cells expressing Δ EGF-Notch1-L1594P-MYC in the presence or absence of DBZ. (D) Notch transcriptional activation overtime measured by RBP-j κ /CSL-Gaussia reporter gene activation in QKO, 1A, 1B, 2A and 2B cells. (E) *Hes1* and *Hey1* mRNA expression levels in QKO, 1A, 1B, 2A and 2B cells corrected with 18S. Lamin A/C is used as loading control. RLU; relative light units. Data are represented as mean $\pm$ SEM for n=3.

FIGURE 3: Differential 12XCSL activity inhibition in different Psen:Aph1 reconstituted cells upon CQ treatment

(A) Inhibitory effect by CQ treatment of NOTCH transcriptional activity measured by RBP-j κ /CSL-luciferase reporter gene activation in QKO, 1A, 1B, 2A and 2B cells stimulated by Dll4-Cf (B) Inhibitory effect by CQ treatment of NOTCH transcriptional activity measured by RBP-j κ /CSL-luciferase reporter gene activation in QKO, 1A, 1B, 2A and 2B cells expressing Δ EGF-Notch1-L1594P-MYC.

SUPPLEMENTARY FIGURE 1: Different γ -secretase complexes generate specific Notch receptor cleavage profiles upon rDll1 stimulation

Western blot analysis of valine 1744 cleaved Notch1 in QKO, 1A, 1B, 2A and 2B cells after stimulation with rDll1 in the presence or absence of DBZ.

SUPPLEMENTARY FIGURE 2: Different γ -secretase complexes generate specific Notch receptor cleavage profiles.

Western blot analysis of Notch1 cleaved at valine 1744 in QKO cells and an independently generated series of reconstituted 1A, 2A, 1B and 2B MEFs after stimulation with Dll4-Cf in the presence or absence of DBZ.

SUPPLEMENTARY FIGURE 3: APh1 is required to process Notch1 upon Dll4-Cf stimulation

Western blot analysis of Notch1 in MEF cells lacking Aph1A, Aph1B, Psen1 and Psen2 (QKO) and QKO cells with reconstituted PSEN1 after stimulation with Dll4-Cf either in the presence or absence of DBZ.

SUPPLEMENTARY TABLE 1: qRT-PCR Primers used in this study

mHes1 forward	5' TCCTAACGCAGTGTCACCTTCCAG 3'
mHes1 reverse	5' CCAAGTTCGTTTTAGTGTCCGTC 3'
mHey1 forward	5' CAGGAGGGAAAGGTTATTTGACG 3'
mHey1 reverse	5'TAGTTGTTGAGATGGGAGACCAGGCG3'
18S forward	5' AGTCCCTGCCCTTTGTACACA 3'
18S reverse	5' GATCCGAGGGCCTCACTAAAC 3'

CHAPTER 6

General discussion

Eloy Moreno Roig

Aiming for the right target

This thesis is the result of work on hypoxia and Notch signaling in relation with their roles as important factors mediating the response to therapy in cancer. Cellular adaptation to low levels of oxygen (hypoxia) is mainly mediated by the activity of the hypoxia-inducible factor alpha (HIF- α), which rapidly becomes activated under hypoxia [1]. The Notch signaling pathway is critical for different aspects related with the self-renewal and differentiation capacity of progenitor cell types, thus mediating cell fate determination in through local cell interactions [2]. It is thereby not surprising that a deregulation on any of these pathways confers cells with pathological capacities which promote aberrant cellular behavior. As hypoxic tumor areas, which often correlate with the location of Notch-expressing cancer stem cells (CSCs), contain most of the malignant cells, it is important that we understand the role of such pathways in keeping these cells alive and escape the effect of treatment [3]. However, the molecular mechanisms by which hypoxia and Notch signaling stimulate the self-renewal and treatment resistance in CSCs are poorly understood. In **chapter 1**, a comprehensive overview is given on all known HIF and Notch functions involving normal and pathological behavior in cancer. Furthermore, we also give some understanding to the mechanisms by which alteration in the hypoxic and Notch pathway result in poorer survival through resistance to therapy. To ensure better clinical translation of novel HIF- α and Notch interventions we have employed novel preclinical tumor models including HIF-deficient cell lines generated by CRISPR/CAS9, air-liquid interface (ALI) culture of primary bronchial epithelial cells (PBECS) and genetically-engineered mouse embryonic fibroblasts (MEFs) lacking the different subunits of the γ -secretase complex.

Targeting HIF- α for cancer therapy

HIF- α is a member of a family of transcription factors, called the basic helix-loop-helix (bHLH) family. HIF- α seems to have a general function in controlling the expression of various target genes involved in different cellular pathways, which are vital for the metabolic adaptation and tissue organization under low nutrient and oxygen conditions. For instance, HIF- α proteins upregulate certain proangiogenic factors promoting the formation of blood vessels, which are crucial for the development of the vascular system in embryos and for neoangiogenesis in tumors [4]. There are three known isoforms of HIF- α subunits, namely

HIF-1 α , HIF-2 α and HIF-3 α . However, mounting evidence has confirmed that HIF-1 α and HIF-2 α function as master regulators of the transcriptional response to hypoxia, whereas the role of HIF-3 α under hypoxia and its mode of action are far less clear [5]. Overall, HIF-1 α and HIF-2 α play both distinct and overlapping functions in normal tissues as well as in cancer.

Different strategies to target HIF- α -expressing cells in cancer include hypoxia-activated prodrugs (HAPs), inhibitors of HIF dimerization, mRNA/protein expression, promoter binding capacity and transcriptional activity. Other novel approaches such as gene therapy or the using of molecular inhibitors against relevant pathways for hypoxic cells such as mTOR and UPR are currently being investigated [6, 7]. Our laboratory is currently investigating the efficiency of HAPs in different tumor models. A prodrug is a non-active compound that via certain metabolic pathways is capable of being converted into a pharmacologically active drug which can selectively kill hypoxic tumor cells [8]. Many prodrugs have been extensively tested in clinical trials, but so far results have been disappointing [9, 10]. Some of the reasons for failure in clinical trials include the lack of “personalized” or precision approach by optimizing the potential therapeutic effect, thus stratifying patients based on tumor-specific predictive biomarker expression, which indicates sensitivity to a certain prodrug. Nonetheless, predictive biomarker selection for hypoxic prodrugs is yet poorly established. Furthermore, HAPs only target extremely low oxygen areas whereas HIF is already active at 3% O₂. Other strategies to target hypoxia include HIF targeting as well as targeting of downstream HIF-related genes. The using of HIF inhibitors provides of a wider range of O₂ targeting effect but may also have normal tissue side-effects (bone marrow, skin, etc.). A common example of the latter is the using of a monoclonal antibody targeting VEGF, named bevacizumab, which has shown impressive clinical benefit in advanced cancer [11]. Despite the success of bevacizumab, most of the clinical trials using drugs targeting HIF have either been terminated or withdrawn prior to public distribution due to lack of efficacy. Several pre-clinical studies have also evaluated the efficiency of independently targeting each HIF- α isoform. Instead, other investigators have followed a more global anti-HIF- α treatment approach by dual targeting both HIF-1 α and HIF-2 α . HIF-1 α and HIF-2 α are broadly expressed in many human cancers and as discussed in **chapter 2** and their expression frequently correlates with poor prognosis.

HIF- α targeting: Beneficial or detrimental?

Several meta-analysis have focused on the clinicopathological and prognostic significance of HIF-1 α many types of cancers [12, 13]. Moreover, the association between specific HIF-1 α gene polymorphisms and the risk of developing cancer has also been evaluated in different systematic reviews [14, 15]. However, many of these results should be treated with caution as the number of studies is limited depending on the research question. Our meta-analysis is the first proof demonstrating the prognostic value of HIF-2 α in a large pool of different types of cancer. We confirmed the negative impact that HIF-2 α expression confers to patients suffering from different types of cancer, excluding clear cell renal cell carcinoma (ccRCC) patients which showed a trend towards good prognosis for some of the endpoints tested. These observations go in line with previous investigations demonstrating a diverse regulatory mechanism of HIF- α in ccRCC by the mutation of the von Hippel-Lindau (VHL) protein, suggesting an alternative tumor-driven pathology by HIF-2 α in this specific type of cancer [16]. As HIF inhibitors are currently undergoing clinical evaluation as potential anti-cancer drugs, a more thorough understanding of the unique roles performed by HIF-1 α and HIF-2 α is needed.

As shown in **chapter 3**, specific inhibition of HIF-1 α and/or HIF-2 α contribute to relevant changes in several cellular properties which are crucial for the maintenance and regulation of many tumorigenic characteristics of non-small cell like cancer (NSCLC) cells. Importantly, our studies show that the consequences of inhibiting the HIF pathway in cells can be detrimental in response to irradiation. Specifically, we found that HIF-1 α -deficient (H1KO) cells displayed an enhanced radioprotective phenotype, lower glycolytic activity levels and an increased accumulation of extracellular lactate acid. It should be noted that H1KO cells exposed to low oxygen levels strongly upregulate the expression of HIF-2 α as a compensatory mechanism. In contrast, HIF-1 α is not upregulated in H2KO cells. The radioresistant phenotype and increased accumulation of extracellular lactate in H1KO cells was reversed in cells lacking both HIF-1 α and HIF-2 α , demonstrating that HIF-2 α protein is responsible for these effects. Previous data also demonstrated a key role of HIF-2 α in protecting against radiation-induced damage using a knock-out mouse model, whereas HIF-1 α had a lower impact [17].

Altogether, these data support the outcome of our meta-analysis pointing HIF-2 α as a crucial regulator of tumorigenic activities which eventually lead to bad prognosis in patients. We believe this research is of great importance for clinical trials, which aim to evaluate the effectiveness of using HIF-1 α inhibitors in cancer patients, since there is clear evidence that compensation by HIF-2 α might be counterproductive in the cancer progression and treatment outcome of those patients. We therefore suggest an alternative consisting of using dual-HIF-1/2 α in cancer patients. Pastorino, et al. show the benefits of downregulating both HIF-1 α and HIF-2 α in a neuroblastoma model by using an active metabolite of irinotecan (PEG-SN38) [18]. Furthermore, a phase I study of PEG-SN38 in neuroblastoma patients showed low toxicity and encouraged further study of this drug in these cancers [19]. Although our work argues that HIF-1 α -deficiency results in an improved response against radiation therapy by upregulating HIF-2 α , its exact molecular mechanism and ability to signal still remains undefined.

The era of CRISPR/CAS9

The discovery of the CRISPR/CAS9 microbial system and its ongoing development into a genome editing tool represents the effort of hundreds of researchers from around the world. The CRISPR locus, as such, was first reported in 1993 by Francisco Mojica, who later observed it comprised a series of sequences that shared a common set of features, also known as the hallmarks of CRISPR sequences [20]. The newly discovered CRISPR locus contained novel CAS genes, including one encoding a large protein with nuclease activity, currently known as CAS9 [21]. This discovery was crucial for later unraveling the role of the CRISPR/CAS9 system in specifically binding and cleaving target DNA. Specifically, Moineau and colleagues showed that CRISPR/CAS9 induces double-strand breaks in desired DNA sequences at precise positions, 3 nucleotides upstream the protospacer adjacent motif (PAM), which is required for target recognition [22]. However, it wasn't until the work from Feng Zhang in 2013 that the CRISPR/CAS9 was successfully adapted for genome editing in eukaryotic cells [23]. Nowadays, there is a great scientific and commercial interest in potential applications for human therapeutics and agricultural biotechnology. The CRISPR/CAS9 technology has become the most reliable commercially available tool to precisely and efficiently edit the genome of living eukaryotic cells.

The CRISPR/CAS9 gene-editing tool is an excellent way to evaluate the real consequences of deleting a certain region from the genome of living cells. The generation of HIF- α -deficient genetic models using the CRISPR/CAS9 system to compare the real contribution of both HIF- α proteins in radiation response has uniquely been performed in our study. Our model allows us to precisely describe the mechanisms behind several aspects of tumorigenesis and response to therapy, over other models in which HIF- α were knocked-down or knocked-out using alternative methods [24, 25]. Therefore, this is the first report in which the CRISPR/CAS9 system has been used to describe the real contribution of each HIF- α isoform (including HIF-1 α and HIF-2 α) in all the aspects taken into consideration in chapter 3, including radiation response and metabolic adaptation to hypoxia. The CRISPR/CAS9 system presents some advantages which stand out from other genome editing tools, such as TALEN or homologous recombination, and siRNA technologies, achieving a unique way to induce target-site mutagenesis in living cells. Above all, the most important advantages of using the CRISPR/CAS9 technology over other genome editing tools are its simplicity and efficiency. Furthermore, the CRISPR methodology entails a way to readily design and assemble guide RNAs (gRNAs) into the CAS9 nuclease, which direct the CAS9 protein to target any genomic region of interest. To date, this is the only available system that enables the generation of multiplexing mutations (up to 5 genes on both alleles) in vitro and in vivo. Nevertheless, this novel technology also involves a list of limitations as a genome-engineering technology, including a high chance of inducing off-target effects throughout the genome, a low target selection and recognition, limited by requirement for PAM sequence and suppression of p53 function to prevent cell cycle arrest after double-strand breaks occur [26, 27]. Accurate gene silencing by knocking-out our gene of interest is also more convenient when studying the real contribution of HIF- α proteins in cancer, in contrast with other studies which used knock-down cellular models generated by siRNA technology. To note, most of the studies unravelling the contribution of HIF-1 α and HIF-2 α to radiation response have been performed using knock-down models. Complete gene silencing can lead to unexpected but interesting phenotypes such as the strong upregulation of HIF-2 α seen in H1KO cells, which may not occur in a knock-down model due to possible traces of HIF-1 α . We now understand that the accuracy of our model is driving the striking radioresistant phenotype seen in HIF-1 α -deficient cells, in contrast with previous publications which repeatedly observed a radiosensitivity effect when pharmacologically and genetically inhibiting HIF-1 α from tumor cells [28-30]. Furthermore, such phenotype could potentially be a tumor-type depending

effect or be specific of the genetic background of H1299 cells. The same reasoning can be applied to the other phenotypes described in chapter 3 such as the crucial contribution of each HIF- α subunit to gene target activation or the impaired glycolytic profile observed in all the genetic models.

HIF takes it up a Notch

The Notch and HIF signaling pathways are highly conserved among metazoans in terms of protein sequence and function [31, 32]. Mukherjee et al. was the first to demonstrate that HIF and Notch regulate the cell fate of circulating blood cells in *Drosophila* through a non-canonical pathway [33]. The canonical Notch pathway determines cell fate decision through the interaction between the Notch receptor with its ligand located on the surface of an adjacent cell and subsequent release of a Notch-intracellular domain (NICD) capable of activating a set of Notch-related genes [34]. Mukherjee, et al. demonstrated that circulating myeloid cells in *Drosophila* produce nitric oxide to constitutively stabilize HIF- α , which in turn interacts with Notch after endocytosis and allows a dual HIF-Notch complex capable of transcriptionally regulating different target genes related with cell fate specification and maintenance [33]. Previous studies have shown that HIF interacts with NICD from vesicles and help to transcriptionally up-regulate Notch target genes responsible for maintaining the undifferentiated state of progenitor cells as well as their cell fate [35, 36].

The HIF and Notch signaling pathways are also associated with promoting self-renewal and resistance to therapy in CSCs, although the specific molecular mechanisms are yet to be understood. Although hypoxia often leads to reduced proliferative capacity via HIF-activation, in some cancers, proliferation is maintained through an increased activity of Notch, which promote proliferation and cell survival [37]. Moreover, the non-canonical hypoxic-Notch axis activates the acquisition of CSC-like characteristics in ovarian cancer cells by inducing SOX2 [3]. Further data suggested that HIF-1 α and HIF-2 α differently regulate Notch signaling through competitive interaction with the NICD located in intracellular vesicles in glioma stem cells. Both proteins interact with NICD competitively and regulate the transactivation of Notch depending on the oxygen levels. Also, mounting evidence exists for a role of HIF-2 α repressing while HIF-1 α enhancing the activation of Notch signaling [38]. Therefore, we speculate that individually blocking HIF-1 α or HIF-2 α

might have severe consequences on promoting a CSCs-related phenotype via regulating the Notch pathway. In support of this hypothesis, it has been reported that Notch1 inhibition in NSCLC result in apoptosis specifically under hypoxia [39]. Notch1 was shown to positively regulate Akt-1 and IGF-R, both important growth factors necessary for cells to survive under extreme conditions such as hypoxia [40]. These data provided evidence that Notch1 regulates pivotal pathways controlling growth and survival in the hypoxic tumor environment. In this regard, some caution is warranted in drug interventions aiming to specifically inhibit either HIF-1 α or HIF-2 α within the tumor as these might inadvertently overactivate Notch in the hypoxic tumor and consequently promote tumor survival and radioresistance.

Targeting Notch, a key pathway for lung stem cells

Standard first line treatment of locally advanced stage III NSCLC is concurrent chemotherapy with 60Gy of fractionated radiotherapy [41]. In **chapter 4**, we have described the evidence pertaining to a role of Notch signaling in cell fate decision and other radiobiological properties of primary bronchial epithelial cells (PBECS) isolated from human lung. As explained above, we propose Notch inhibition as a promising therapeutic strategy to eradicate the abnormal proliferative and survival properties of CSCs and thus improving tumor control. Indeed, there is evidence that when combined with chemotherapy [42] or radiotherapy [43], Notch inhibition prolongs tumor control. There is broad preclinical evidence for long lasting anti-tumor activity in lung cancer with γ -secretase inhibitors (GSI), which efficiently block Notch signaling at a membrane level [44]. Previous research from our team has demonstrated that Notch signaling also promotes radiation resistance [45], and that Notch inhibition is effective in combination treatments with chemo-radiotherapy [46]. Therefore, we propose Notch inhibition as a promising strategy to defeat lung cancer malignancy by targeting tumor resistant cells such as CSCs. Traditional therapies against cancer including chemo-radiotherapy, but also anti-Notch treatment to abrogate CSC malignancy can have multiple limitations that lead to treatment failure and cancer recurrence. These limitations are related to systemic and local toxicity due to the adverse effects in the normal tissue. One of the main functions of Notch is to control cell fate decisions in diverse group of cells within tissues including progenitor/stem cells. Therefore, when aiming against Notch there is an increased chance to induce an unbalance for many differentiated and undifferentiated cell types in tissues which highly rely on the Notch pathway. For instance, GSI treatment was believed to

have high anti-leukemic potential but did not complete translation into clinic until now. This is due to the apparent gastrointestinal toxicity caused by inhibition of Notch in basal intestinal cells, leading to differentiation of intestinal progenitor cells into secretory cells [47-50].

The airway epithelium plays a major role in the defense against respiratory viruses and functions by providing a physical barrier to the environment. It also maintains the balance of water and other molecules between the basolateral and apical surfaces and mediates the mucociliary clearance of the epithelial surface. Moreover, bronchial epithelial cells express a variety of genes that help sustain normal airway structure and function [51, 52]. The pseudostratified epithelial barrier that protects the airways against external agents is sustained by basal cell proliferation and subsequent differentiation to other cell types. Upon injury such as radiation exposure, basal cells play a crucial role in regenerating the tissue and provide with differentiated cells that will help maintain an organized and functional tissue architecture [53, 54]. In particular, our work has demonstrated that Notch signaling plays a crucial role in inducing mucous cell fate whereas its inhibition would lead to an increased stem cell self-renewal capacity together with enhanced ciliated differentiation. Under these conditions, the regenerative capacity of stem cells located in areas where radiation exposure has damaged the normal tissue might be affected. Thereby, in chapter 4 we developed an air-liquid interface (ALI) 3D model in which the basal cell surface is in contact with liquid culture medium, whereas the apical surface is exposed to air. Non-tumoral biopsies from non-COPD patients, but with different smoking pack-year habits, were used for the study, possibly contributing to the small variability among samples when assessing cell-fate decision outcome upon the different treatments. Isolation of progenitor cells within the patient biopsy is performed by following a conditional medium protocol as an approach to isolate cells with higher-proliferative capacity while discarding differentiated cell types and cellular debris. A common approach is to seed and expand cells onto the permeable membrane of a cell culture insert, which is initially supplied with specialized stem-cell culture media to both the apical and basal compartments. Approximately after a week confluence is reached and cells are subjected to “air-lift”, where the medium is supplied only to the basal-side of the well. This configuration is ideal to mimic the conditions seen in the human airway and to drive differentiation towards a mucociliary phenotype. Also, the trans-well technology allows us to readily add different compounds to the media and expose our cellular culture to radiation throughout the whole experiment. However, this model fails to mimic the complexity and heterogeneity of airway tissue, the organization and architecture, areas of limited-oxygen

diffusion, cell-cell interactions, and the interaction with the tumor microenvironment and the immune system.

Overcoming radiation-related toxicity in the normal tissue

Using the ALI model, we showed in chapter 4 that human primary bronchial epithelial cells (hPBECs) displayed different responses to radiotherapy with or without anti-Notch treatment. Traditionally, researchers have focused on pharmacological strategies to harness the adverse effects of radiation [55, 56]. However, stem cell therapies provide unique opportunities for restoring the structure and functionality of tissue damaged by radiation. We therefore aimed to characterize the therapeutic potential of Notch inhibitors in improving radiation-related damage by up-regulating the self-renewal capacity of progenitor cells [57]. Quantification of the two main differentiated cell types along the airway tissue consisting of ciliated and mucous cells suggest that the later are more radiosensitive at increased doses of radiation especially when Notch is inhibited. Increasing doses of radiation progressively reduced the amount of progenitor P63⁺ cells in the ALI culture 24 hours and 3 days after cells were exposed to radiation. Strikingly, the addition of DBZ (a potent γ -secretase inhibitor) 2 weeks prior radiation treatment resulted in a radioprotective effect and stimulated progenitor cell growth. In addition, such phenotype became more evident 2 weeks after radiation exposure, where progenitor cells covered with almost 100% of the cellular population. We thereby propose a model in which radiation exposure negatively affects the abundance of progenitor cells; however, surviving cells would stimulate their proliferative capacities in order to restore the tissue affected. Overall, we speculate an important role for Notch in the maintenance of lung stem cells which seems to be beneficial in improving radiation-related damage.

Further investigations aimed to uncover the mechanism(s) responsible for the radioprotective phenotype of PBECs treated with anti-Notch therapy. Recent evidence has shown that Notch receptor binds and inactivates ATM kinase, which is a key modulator of the DNA damage response (DDR) pathway in worms, flies and mammals. NOTCH does not impair the recruitment of ATM to DSBs upon DNA damage. Instead NOTCH prevents the binding of ATM to FOXO3A by actively competing with ATM. Inactivation of ATM by NOTCH abrogates a TP53 dependent cell cycle arrest that promotes a survival benefit of Notch1-

driven leukemia cells upon DNA damage [58, 59]. In line with these studies, our data provided evidence that Notch inhibition also regulates ATM activation in PBECs. As a result, the levels of two relevant double-strand breaks markers (γ -H2AX and 53BP1) were reduced in irradiated cells pre-treated with γ -secretase inhibitors. Collectively, these findings suggest a new promising use for Notch inhibitors as modulators of the response to DNA damage which in consequence would benefit the regenerative capacity of tissues subjected to irradiation. Further investigations are needed to validate the regenerative properties of DBZ in the normal lung tissue.

Once ascertained that Notch inhibition affects radiation sensitivity in PBECs, we wondered whether radiation treatment alone has the potential to interfere with the Notch signaling pathway. For that, we evaluated the expression of the cleaved form of Notch as well as the expression of one of its major target genes (Hes1) and observed that both are downregulated upon increasing doses of radiation. Such observation was seen at early time points (1 and 24 hours after radiation), suggesting that Notch signaling inhibition upon radiation treatment is based on an immediate molecular mechanism(s) rather than by changes in the reorganization of the different cell types within the tissue. Going forward, it will be of great interest and importance to investigate the specific molecular mechanism(s) behind such novel finding. Also, these findings highlight the fact that we need to keep investigating the regulation of the Notch pathway as well as potential alternative strategies for its inhibition.

The γ -secretase complex: Selecting the best combination to take Notch down

Normal tissue tolerance is the major impediment to traditional chemo-radiotherapy and other novel therapeutic strategies such as Notch inhibition which is normally focused on specifically regulating the activity of the γ -secretase complex. At the cellular membrane, two different metalloproteases mediate S2 cleavage of Notch, resulting in a short-lived fragment that is immediately S3 processed by the γ -secretase complex. In **chapter 5**, we performed biochemical studies showing that different subunit organization of the γ -secretase complex, constituted of different presenilin and Aph1 subunits, produce characteristic Notch cleavage profiles. Due to the important involvement of the Notch signaling in a variety of cancers and its role in inducing goblet metaplasia when inhibited, γ -secretase modulation is believed to be an interesting approach in the treatment of both cancer and reduction of normal tissue toxicity

[47, 60]. The γ -secretase complex consists of four essential subunits, the aspartyl protease presenilin (PSEN), Nicastrin (NCT), presenilin enhancer 2 (PEN-2) and anterior pharynx defective 1 (APH1). Furthermore, both PSEN and APH1 have two different subunits in humans, PSEN1, PSEN2, PH1A and APH1B, which are encoded by separate genes [61]. Thereby, the different subunits can result in at least four different γ -secretase complexes, since all the four different subunits are required in a stoichiometry of 1:1:1:1 [62-64]. In this project, we hypothesized that different γ -secretase complexes may display variable activities towards the NOTCH receptor, which we can use as a therapeutic advantage when treating cancer and reducing NOTCH-related side-effects. Understanding the involvement of the different γ -secretase subunit organization in Notch processing may help to develop novel GSIs specifically aiming to one disease-related substrate, while sparing the other physiological substrates. The differential involvement of the γ -secretase subunit organization in different substrates was first investigated in models for Alzheimer disease (AD). Previously, it was demonstrated that PSEN1-specific γ -secretase complexes were capable of producing more A β aggregates, whereas the contribution of PSEN2 was less obvious [65, 66]. In line with this, no Notch related toxicity in the brain or other organs was seen when using PSEN2 sparing GSIs to successfully lower the fraction of A β aggregates in the brain [67, 68]. In contrast, in clinical trials using γ -secretase inhibitors against Alzheimer's disease, severe side effects were noted, most probably due to inhibition of Notch [69,70]. Many PSEN2 sparing GSIs entered clinical trials but failed because of Notch related side effects, probably due to the high variety in preference for PSEN1 inhibition over PSEN2 [71]. It is therefore of great importance to characterize the real contribution of each γ -secretase complex subtype to Notch cleavage and its consequences in disease.

PSEN2:APH1B: The right candidate?

Biochemical studies addressing the intrinsic capability of the different γ -secretase isoforms in Notch processing showed that the PSEN1:APH1B containing complex indeed produced the highest amount of Notch1 intracellular domain (NICD) cleaved at valine 1744 compared to the other complexes. However, cells containing the PSEN2:APH1B produced the lowest amount of NICD also cleaved at valine 1744. Previous studies have shown that γ -secretase complexes can be active both at the cell surface and in endosomes, occurring the S3 cleavage

at different residues depending on the location of the enzyme [72, 73]. In the endosomes, Notch1 is cleaved at serine1747, resulting in a highly unstable but active NICD form. In line with these observations, we observed that total S3 cleavage by the different γ -secretase isoforms did not correlate with cleavage at residue valine1744, suggesting that an alternative cleavage site could be a potential substrate for the enzyme, such as the one described above.

Interestingly, the least active isoform PSEN2:APH1B in cleaving the wild type Notch1 receptor, showed high efficiency of valine1744 cleavage and transcriptional activation for oncogenic Notch1 receptors, which incorporate a L1594P mutation resulting in ligand-independent activation of Notch. Such mutated Notch proteins are commonly found in T-cell acute lymphoblastic leukemia (T-ALL), which requires high doses of the Notch signaling for oncogenic development [74]. These findings argue that specific targeting of the PSEN2:APH1B isoform might be a potential therapeutic option to specifically target oncogenic mutant Notch1 receptors, while sparing the processing of endogenous wild type receptors, for example mitigating gastrointestinal toxicity. To further investigate the mechanisms behind oncogenic Notch1 regulation, we tested the possibility of inhibiting Notch at different subcellular locations, while comparing the wild type with the oncogenic isoform. If true, this might explain the differences in cleavage at Valine1744 versus total cleavage of the receptor and potentially the heterogeneity in processing wild type versus oncogenic Notch receptors by the γ -secretase isoforms. Chloroquine (CQ) was used as an endocytic blocking agent to unravel the potential role of the endocytic pathway in an alternative cleavage in Notch1 by the γ -secretase complex. Chloroquine is a lysomotrophic agent which enters acidic endocytic vesicles and form insoluble aggregates preventing the fusion with the autophagosome and formation of autolysosomes. This results in the blocking of autophagy and degradation and turn-over of endocytic proteins. Interestingly, we found that endocytic inhibition mainly affected the cleavage of oncogenic Notch1 by complexes harboring PSEN2, especially the PSEN2:APH1B combination, whereas no effects were seen regarding wild type Notch1 cleavage. Altogether, we showed that oncogenic Notch1 signaling is differently processed by the γ -secretase complex, proposing PSEN2:APH1B as a potential candidate in order to effectively inhibit oncogenic signaling while sparing normal Notch activity. If our hypothesis is correct, the clinically approved drug CQ might be a potential application in the treatment of T-ALL. Moreover, the design and testing of novel PSEN1-sparing inhibitors to effectively target PSEN2 is warranted.

References

1. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci U S A* **92**, 5510-5514
2. Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999) Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776
3. Seo, E. J., Kim, D. K., Jang, I. H., Choi, E. J., Shin, S. H., Lee, S. I., Kwon, S. M., Kim, K. H., Suh, D. S., and Kim, J. H. (2016) Hypoxia-NOTCH1-SOX2 signaling is important for maintaining cancer stem cells in ovarian cancer. *Oncotarget* **7**, 55624-55638
4. Liu, Y., Cox, S. R., Morita, T., and Kourembanas, S. (1995) Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. *Circ Res* **77**, 638-643
5. Gu, Y. Z., Moran, S. M., Hogenesch, J. B., Wartman, L., and Bradfield, C. A. (1998) Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. *Gene Expr* **7**, 205-213
6. Wigerup, C., Pahlman, S., and Bexell, D. (2016) Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. *Pharmacol Ther* **164**, 152-169
7. Semenza, G. L. (2012) Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. *Trends Pharmacol Sci* **33**, 207-214
8. Denny, W. A. (2000) The role of hypoxia-activated prodrugs in cancer therapy. *Lancet Oncol* **1**, 25-29
9. Rischin, D., Peters, L. J., O'Sullivan, B., Giralt, J., Fisher, R., Yuen, K., Trotti, A., Bernier, J., Bourhis, J., Ringash, J., Henke, M., and Kenny, L. (2010) Tirapazamine, cisplatin, and radiation versus cisplatin and radiation for advanced squamous cell carcinoma of the head and neck (TROG 02.02, HeadSTART): a phase III trial of the Trans-Tasman Radiation Oncology Group. *J Clin Oncol* **28**, 2989-2995
10. DiSilvestro, P. A., Ali, S., Craighead, P. S., Lucci, J. A., Lee, Y. C., Cohn, D. E., Spirtos, N. M., Tewari, K. S., Muller, C., Gajewski, W. H., Steinhoff, M. M., and Monk, B. J. (2014) Phase III randomized trial of weekly cisplatin and irradiation versus cisplatin and tirapazamine and irradiation in stages IB2, IIA, IIB, IIIB, and IVA cervical carcinoma limited to the pelvis: a Gynecologic Oncology Group study. *J Clin Oncol* **32**, 458-464
11. Ellis, L. M., and Hicklin, D. J. (2008) VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat Rev Cancer* **8**, 579-591
12. Ren, H. Y., Zhang, Y. H., Li, H. Y., Xie, T., Sun, L. L., Zhu, T., Wang, S. D., and Ye, Z. M. (2016) Prognostic role of hypoxia-inducible factor-1 alpha expression in osteosarcoma: a meta-analysis. *Onco Targets Ther* **9**, 1477-1487

13. Yang, S. L., Ren, Q. G., Wen, L., and Hu, J. L. (2016) Clinicopathological and prognostic significance of hypoxia-inducible factor-1 alpha in lung cancer: a systematic review with meta-analysis. *J Huazhong Univ Sci Technolog Med Sci* **36**, 321-327
14. Li, D., Liu, J., Zhang, W., Ren, J., Yan, L., Liu, H., and Xu, Z. (2013) Association between HIF1A P582S and A588T polymorphisms and the risk of urinary cancers: a meta-analysis. *PLoS One* **8**, e63445
15. He, P., Han, Q., Liu, J., Liu, D., Zhao, X., Hu, T., Jiang, L., Dan, H., Zeng, X., Li, J., Wang, J., and Chen, Q. (2013) The association between hypoxia-inducible factor-1 alpha gene C1772T polymorphism and cancer risk: a meta-analysis of 37 case-control studies. *PLoS One* **8**, e83441
16. Banks, R. E., Tirukonda, P., Taylor, C., Hornigold, N., Astuti, D., Cohen, D., Maher, E. R., Stanley, A. J., Harnden, P., Joyce, A., Knowles, M., and Selby, P. J. (2006) Genetic and epigenetic analysis of von Hippel-Lindau (VHL) gene alterations and relationship with clinical variables in sporadic renal cancer. *Cancer Res* **66**, 2000-2011
17. Taniguchi, C. M., Miao, Y. R., Diep, A. N., Wu, C., Rankin, E. B., Atwood, T. F., Xing, L., and Giaccia, A. J. (2014) PHD inhibition mitigates and protects against radiation-induced gastrointestinal toxicity via HIF2. *Sci Transl Med* **6**, 236ra264
18. Pastorino, F., Loi, M., Sapra, P., Becherini, P., Cilli, M., Emionite, L., Ribatti, D., Greenberger, L. M., Horak, I. D., and Ponzoni, M. (2010) Tumor regression and curability of preclinical neuroblastoma models by PEGylated SN38 (EZN-2208), a novel topoisomerase I inhibitor. *Clin Cancer Res* **16**, 4809-4821
19. Norris, R. E., Shusterman, S., Gore, L., Muscal, J. A., Macy, M. E., Fox, E., Berkowitz, N., Buchbinder, A., and Bagatell, R. (2014) Phase 1 evaluation of EZN-2208, a polyethylene glycol conjugate of SN38, in children adolescents and young adults with relapsed or refractory solid tumors. *Pediatr Blood Cancer* **61**, 1792-1797
20. Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J., and Soria, E. (2005) Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol* **60**, 174-182
21. Bolotin, A., Quinkis, B., Sorokin, A., and Ehrlich, S. D. (2005) Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* **151**, 2551-2561
22. Garneau, J. E., Dupuis, M. E., Villion, M., Romero, D. A., Barrangou, R., Boyaval, P., Fremaux, C., Horvath, P., Magadan, A. H., and Moineau, S. (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**, 67-71
23. Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., and Zhang, F. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823

24. Bensellam, M., Duvillie, B., Rybachuk, G., Laybutt, D. R., Magnan, C., Guiot, Y., Pouyssegur, J., and Jonas, J. C. (2012) Glucose-induced O(2) consumption activates hypoxia inducible factors 1 and 2 in rat insulin-secreting pancreatic beta-cells. *PLoS One* **7**, e29807
25. Yang, X., Yang, S., Wang, C., and Kuang, S. (2017) The hypoxia-inducible factors HIF1alpha and HIF2alpha are dispensable for embryonic muscle development but essential for postnatal muscle regeneration. *J Biol Chem* **292**, 5981-5991
26. Walsh, R. M., and Hochedlinger, K. (2013) A variant CRISPR-Cas9 system adds versatility to genome engineering. *Proc Natl Acad Sci U S A* **110**, 15514-15515
27. Haapaniemi, E., Botla, S., Persson, J., Schmierer, B., and Taipale, J. (2018) CRISPR-Cas9 genome editing induces a p53-mediated DNA damage response. *Nature Medicine*
28. Staab, A., Loeffler, J., Said, H. M., Diehlmann, D., Katzer, A., Beyer, M., Fleischer, M., Schwab, F., Baier, K., Einsele, H., Flentje, M., and Vordermark, D. (2007) Effects of HIF-1 inhibition by chetomin on hypoxia-related transcription and radiosensitivity in HT 1080 human fibrosarcoma cells. *BMC Cancer* **7**, 213
29. Zhang, X., Kon, T., Wang, H., Li, F., Huang, Q., Rabbani, Z. N., Kirkpatrick, J. P., Vujaskovic, Z., Dewhirst, M. W., and Li, C. Y. (2004) Enhancement of hypoxia-induced tumor cell death in vitro and radiation therapy in vivo by use of small interfering RNA targeted to hypoxia-inducible factor-1alpha. *Cancer Res* **64**, 8139-8142
30. Moeller, B. J., Dreher, M. R., Rabbani, Z. N., Schroeder, T., Cao, Y., Li, C. Y., and Dewhirst, M. W. (2005) Pleiotropic effects of HIF-1 blockade on tumor radiosensitivity. *Cancer Cell* **8**, 99-110
31. Richards, G. S., and Degnan, B. M. (2009) The dawn of developmental signaling in the metazoa. *Cold Spring Harb Symp Quant Biol* **74**, 81-90
32. Taylor, C. T., and McElwain, J. C. (2010) Ancient atmospheres and the evolution of oxygen sensing via the hypoxia-inducible factor in metazoans. *Physiology (Bethesda)* **25**, 272-279
33. Mukherjee, T., Kim, W. S., Mandal, L., and Banerjee, U. (2011) Interaction between Notch and Hif-alpha in development and survival of Drosophila blood cells. *Science* **332**, 1210-1213
34. Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M. E. (1995) Notch signaling. *Science* **268**, 225-232
35. Gustafsson, M. V., Zheng, X., Pereira, T., Gradin, K., Jin, S., Lundkvist, J., Ruas, J. L., Poellinger, L., Lendahl, U., and Bondesson, M. (2005) Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Dev Cell* **9**, 617-628
36. Pistollato, F., Rampazzo, E., Persano, L., Abbadi, S., Frasson, C., Denaro, L., D'Avella, D., Panchision, D. M., Della Puppa, A., Scienza, R., and Basso, G. (2010)

Interaction of hypoxia-inducible factor-1alpha and Notch signaling regulates medulloblastoma precursor proliferation and fate. *Stem Cells* **28**, 1918-1929

37. Jundt, F., Anagnostopoulos, I., Forster, R., Mathas, S., Stein, H., and Dorken, B. (2002) Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. *Blood* **99**, 3398-3403

38. Hu, Y. Y., Fu, L. A., Li, S. Z., Chen, Y., Li, J. C., Han, J., Liang, L., Li, L., Ji, C. C., Zheng, M. H., and Han, H. (2014) Hif-1alpha and Hif-2alpha differentially regulate Notch signaling through competitive interaction with the intracellular domain of Notch receptors in glioma stem cells. *Cancer Lett* **349**, 67-76

39. Elias, S., Liang, S., Chen, Y., De Marco, M. A., Machek, O., Skucha, S., Miele, L., and Bocchetta, M. (2010) Notch-1 stimulates survival of lung adenocarcinoma cells during hypoxia by activating the IGF-1R pathway. *Oncogene* **29**, 2488-2498

40. Pollak, M. (2008) Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* **8**, 915-928

41. Auperin, A., Le Pechoux, C., Rolland, E., Curran, W. J., Furuse, K., Fournel, P., Belderbos, J., Clamon, G., Ulutin, H. C., Paulus, R., Yamanaka, T., Bozonnet, M. C., Uitterhoeve, A., Wang, X., Stewart, L., Arriagada, R., Burdett, S., and Pignon, J. P. (2010) Meta-analysis of concomitant versus sequential radiochemotherapy in locally advanced non-small-cell lung cancer. *J Clin Oncol* **28**, 2181-2190

42. Morgan, K. M., Fischer, B. S., Lee, F. Y., Shah, J. J., Bertino, J. R., Rosenfeld, J., Singh, A., Khiabani, H., and Pine, S. R. (2017) Gamma Secretase Inhibition by BMS-906024 Enhances Efficacy of Paclitaxel in Lung Adenocarcinoma. *Mol Cancer Ther* **16**, 2759-2769

43. Mizugaki, H., Sakakibara-Konishi, J., Ikezawa, Y., Kikuchi, J., Kikuchi, E., Oizumi, S., Dang, T. P., and Nishimura, M. (2012) gamma-Secretase inhibitor enhances antitumour effect of radiation in Notch-expressing lung cancer. *Br J Cancer* **106**, 1953-1959

44. van Tetering, G., and Vooijs, M. (2011) Proteolytic cleavage of Notch: "HIT and RUN". *Curr Mol Med* **11**, 255-269

45. Yahyanejad, S., Theys, J., and Vooijs, M. (2016) Targeting Notch to overcome radiation resistance. *Oncotarget* **7**, 7610-7628

46. Yahyanejad, S., King, H., Iglesias, V. S., Granton, P. V., Barbeau, L. M., van Hoof, S. J., Groot, A. J., Habets, R., Prickaerts, J., Chalmers, A. J., Eekers, D. B., Theys, J., Short, S. C., Verhaegen, F., and Vooijs, M. (2016) NOTCH blockade combined with radiation therapy and temozolomide prolongs survival of orthotopic glioblastoma. *Oncotarget* **7**, 41251-41264

47. van Es, J. H., van Gijn, M. E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D. J., Radtke, F., and Clevers, H. (2005) Notch/gamma-

secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* **435**, 959-963

48. Real, P. J., Tosello, V., Palomero, T., Castillo, M., Hernando, E., de Stanchina, E., Sulis, M. L., Barnes, K., Sawai, C., Homminga, I., Meijerink, J., Aifantis, I., Basso, G., Cordon-Cardo, C., Ai, W., and Ferrando, A. (2009) Gamma-secretase inhibitors reverse glucocorticoid resistance in T cell acute lymphoblastic leukemia. *Nat Med* **15**, 50-58
49. Zheng, H., Pritchard, D. M., Yang, X., Bennett, E., Liu, G., Liu, C., and Ai, W. (2009) KLF4 gene expression is inhibited by the notch signaling pathway that controls goblet cell differentiation in mouse gastrointestinal tract. *Am J Physiol Gastrointest Liver Physiol* **296**, G490-498
50. Kim, T. H., and Shivdasani, R. A. (2011) Genetic evidence that intestinal Notch functions vary regionally and operate through a common mechanism of Math1 repression. *J Biol Chem* **286**, 11427-11433
51. Knight, D. A., and Holgate, S. T. (2003) The airway epithelium: structural and functional properties in health and disease. *Respirology* **8**, 432-446
52. Thompson, A. B., Robbins, R. A., Romberger, D. J., Sisson, J. H., Spurzem, J. R., Teschler, H., and Rennard, S. I. (1995) Immunological functions of the pulmonary epithelium. *Eur Respir J* **8**, 127-149
53. Bishop, A. E. (2004) Pulmonary epithelial stem cells. *Cell Prolif* **37**, 89-96
54. Bowden, D. H. (1983) Cell turnover in the lung. *Am Rev Respir Dis* **128**, S46-48
55. Brizel, D. M. (2007) Pharmacologic approaches to radiation protection. *J Clin Oncol* **25**, 4084-4089
56. Prasanna, P. G., Stone, H. B., Wong, R. S., Capala, J., Bernhard, E. J., Vikram, B., and Coleman, C. N. (2012) Normal tissue protection for improving radiotherapy: Where are the Gaps? *Transl Cancer Res* **1**, 35-48
57. Benderitter, M., Caviggioli, F., Chapel, A., Coppes, R. P., Guha, C., Klinger, M., Malard, O., Stewart, F., Tamarat, R., Luijk, P. V., and Limoli, C. L. (2014) Stem cell therapies for the treatment of radiation-induced normal tissue side effects. *Antioxid Redox Signal* **21**, 338-355
58. Vermezovic, J., Adamowicz, M., Santarpia, L., Rustighi, A., Forcato, M., Lucano, C., Massimiliano, L., Costanzo, V., Biciato, S., Del Sal, G., and d'Adda di Fagagna, F. (2015) Notch is a direct negative regulator of the DNA-damage response. *Nat Struct Mol Biol* **22**, 417-424
59. Adamowicz, M., Vermezovic, J., and d'Adda di Fagagna, F. (2016) NOTCH1 Inhibits Activation of ATM by Impairing the Formation of an ATM-FOXO3a-KAT5/Tip60 Complex. *Cell Rep* **16**, 2068-2076

60. Milano, J., McKay, J., Dagenais, C., Foster-Brown, L., Pognan, F., Gadiant, R., Jacobs, R. T., Zacco, A., Greenberg, B., and Ciaccio, P. J. (2004) Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. *Toxicol Sci* **82**, 341-358
61. Gu, Y., Chen, F., Sanjo, N., Kawarai, T., Hasegawa, H., Duthie, M., Li, W., Ruan, X., Luthra, A., Mount, H. T., Tandon, A., Fraser, P. E., and St George-Hyslop, P. (2003) APH-1 interacts with mature and immature forms of presenilins and nicastrin and may play a role in maturation of presenilin.nicastrin complexes. *J Biol Chem* **278**, 7374-7380
62. Edbauer, D., Winkler, E., Regula, J. T., Pesold, B., Steiner, H., and Haass, C. (2003) Reconstitution of gamma-secretase activity. *Nat Cell Biol* **5**, 486-488
63. Kimberly, W. T., LaVoie, M. J., Ostaszewski, B. L., Ye, W., Wolfe, M. S., and Selkoe, D. J. (2003) Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2. *Proc Natl Acad Sci U S A* **100**, 6382-6387
64. Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) The role of presenilin cofactors in the gamma-secretase complex. *Nature* **422**, 438-441
65. De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., Von Figura, K., and Van Leuven, F. (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391**, 387-390
66. Franberg, J., Svensson, A. I., Winblad, B., Karlstrom, H., and Frykman, S. (2011) Minor contribution of presenilin 2 for gamma-secretase activity in mouse embryonic fibroblasts and adult mouse brain. *Biochem Biophys Res Commun* **404**, 564-568
67. Borgegard, T., Gustavsson, S., Nilsson, C., Parpal, S., Klinterberg, R., Berg, A. L., Rosqvist, S., Serneels, L., Svensson, S., Olsson, F., Jin, S., Yan, H., Wänggren, J., Jureus, A., Ridderstad-Wollberg, A., Wollberg, P., Stockling, K., Karlstrom, H., Malmberg, A., Lund, J., Arvidsson, P. I., De Strooper, B., Lendahl, U., and Lundkvist, J. (2012) Alzheimer's disease: presenilin 2-sparing gamma-secretase inhibition is a tolerable Abeta peptide-lowering strategy. *J Neurosci* **32**, 17297-17305
68. Best, J. D., Smith, D. W., Reilly, M. A., O'Donnell, R., Lewis, H. D., Ellis, S., Wilkie, N., Rosahl, T. W., Laroque, P. A., Boussiquet-Leroux, C., Churcher, I., Atack, J. R., Harrison, T., and Shearman, M. S. (2007) The novel gamma secretase inhibitor N-[cis-4-[(4-chlorophenyl)sulfonyl]-4-(2,5-difluorophenyl)cyclohexyl]-1,1,1-trifluoromethanesulfonamide (MRK-560) reduces amyloid plaque deposition without evidence of notch-related pathology in the Tg2576 mouse. *J Pharmacol Exp Ther* **320**, 552-558
69. Doody, R. S., Raman, R., Farlow, M., Iwatsubo, T., Vellas, B., Joffe, S., Kieburtz, K., He, F., Sun, X., Thomas, R. G., Aisen, P. S., Siemers, E., Sethuraman, G., and Mohs, R.

(2013) A Phase 3 Trial of Semagacestat for Treatment of Alzheimer's Disease. in *NEJM*

70. De Strooper, B. (2014) Lessons from a failed γ -secretase Alzheimer trial. *Cell* **159**, 721-726

71. Imbimbo, B. P., Panza, F., Frisardi, V., Solfrizzi, V., D'Onofrio, G., Logroscino, G., Seripa, D., and Pilotto, A. (2011) Therapeutic intervention for Alzheimer's disease with gamma-secretase inhibitors: still a viable option? *Expert Opin Investig Drugs* **20**, 325-341

72. Tagami, S., Okochi, M., Yanagida, K., Ikuta, A., Fukumori, A., Matsumoto, N., Ishizuka-Katsura, Y., Nakayama, T., Itoh, N., Jiang, J., Nishitomi, K., Kamino, K., Morihara, T., Hashimoto, R., Tanaka, T., Kudo, T., Chiba, S., and Takeda, M. (2008) Regulation of Notch signaling by dynamic changes in the precision of S3 cleavage of Notch-1. *Mol Cell Biol* **28**, 165-176

73. Fukumori, A., Okochi, M., Tagami, S., Jiang, J., Itoh, N., Nakayama, T., Yanagida, K., Ishizuka-Katsura, Y., Morihara, T., Kamino, K., Tanaka, T., Kudo, T., Tanii, H., Ikuta, A., Haass, C., and Takeda, M. (2006) Presenilin-dependent gamma-secretase on plasma membrane and endosomes is functionally distinct. *Biochemistry* **45**, 4907-4914

74. Weng, A. P., Ferrando, A. A., Lee, W., Morris, J. P. t., Silverman, L. B., Sanchez-Irizarry, C., Blacklow, S. C., Look, A. T., and Aster, J. C. (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* **306**, 269-271

Summary

Cancer is the leading cause of death worldwide according to the World Health Organization (WHO). Despite the major advances in our understanding of cancer biology, efficient diagnosis and improvement of anticancer therapies, the burden of cancer is increasing worldwide. Some tumors initially respond to treatment but ultimately acquire resistance to chemo-radiotherapy which leads to tumor recurrence. Normal cell renewal in adult tissues and adaptation to low oxygen levels are regulated by several signalling pathways that often are deregulated in cancer and contribute to treatment failure. In this thesis, we studied the effects of inhibiting two important signalling pathways in cancer, NOTCH and HIF, to overcome resistance to cancer therapy.

Hypoxia is a major feature in many solid tumors and arises due to an inadequate and immature vascular function resulting in a decreased delivery of oxygen in nutrients. Hypoxia areas within the tumors show resistance towards conventional treatment modalities such as radio-chemotherapy, causing a reduction in survival of cancer patients. One of the most recognized mechanism to adapt and survive hypoxic stress conditions is mediated via stabilization of the hypoxia-inducible factors (HIFs), mainly consisting of HIF-1 α and HIF-2 α . Under hypoxic conditions, HIFs- α dimerize with HIF-1 β , which forms a transcriptionally active dimer that binds to the hypoxia responsive elements (HRE) in the promoter regions of hundreds of genes to enhance to expression of many hypoxia-related genes. Direct target genes of HIFs have been shown to be functionally related with many tumor-associated characteristics such as energy metabolism, angiogenesis, tumor metastasis and resistance to therapy. HIF-1 α and HIF-2 α show unique but also overlapping roles in regulating many of the hypoxia-related features seen in hypoxic tumors.

One of the main aims of this thesis was to investigate the differential role of HIF-1 α and HIF-2 α in regulating tumor-associated characteristics in view of the possibility of implementing them in a personalized treatment approach and to use them as independent prognostic factors in cancer. Several clinical studies investigate the prognostic role of HIF-1 α in cancer, including many meta-analyses for all types of cancer and different HIF-1 α polymorphisms. Prognostic data on HIF-2 α can be found for some of the clinical studies included in these meta-analyses, which often differ from HIF-1 α data. However, many discrepancies are seen among the investigations that were performed during the last decades, in which HIF-2 α has been reported to be either a positive or negative prognostic factor in cancer. To expand the knowledge on the prognostic value of HIF-2 α in cancer, **chapter 2** describes a comprehensive overview the prognostic data of patients suffering from diverse types of cancer according to

their intratumoral HIF-2 α expression. Here, we demonstrate that HIF-2 α expression is a negative prognostic factor when evaluating 5 out of the 6 endpoints tested, including overall survival, disease-free survival, disease-specific survival, metastatic-free survival and progression-free survival. An inverse relationship found when evaluating renal cell carcinomas, in which HIF-2 α seems to behave as a positive prognostic factor depending on its subcellular localization. The results of this meta-analysis therefore support that development of a clinically applicable test to assess HIF-2 α expression to estimate patient prognosis.

The outcome of this meta-analysis also suggests its use as a target for anti-cancer therapy. Therefore we studied the consequences of genetically depleting both HIF-1 α and HIF-2 α in radiation sensitivity in cancer cells in **chapter 3**. To directly assess the unique and overlapping functions of HIF-1 α and HIF-2 α , we use CRISPR gene-editing to generate isogenic H1299 non-small cell lung carcinoma cells lacking HIF-1 α or HIF-2 α or both HIF1 and HIF2. When cultured under hypoxia HIF-1 α -deficient cells strongly upregulate the levels of HIF-2 α probably through enhanced protein stabilization or reduction of its degradation. In accordance with our published meta-analysis describing the negative impact of HIF-2 α in cancer, we observed that HIF-1 α -deficient cells, which overexpress HIF-2 α , are more radioresistant than wild type cells when irradiated under low-oxygen conditions (0.2% O₂). A Higher survival of irradiated HIF-1 α -deficient H1299 cells was associated with a reduced recruitment or accelerated repair of γ -H2AX foci directly after irradiation and not due to differences in proliferation. HIF-1 α -deficient cells were used as a cellular template to target HIF-2 α using the CRISPR/CAS9 system, thus generate a double-HIF-1/2 α defective cell line and confirm that HIF-2 α is responsible for the radioresistant phenotype. On the contrary, HIF-1/2 α -deficient cells conferred radiosensitivity to H1299 cells under both normoxic and hypoxic conditions. *Lactate* has been hypothesized to mediate *radioresistance by virtue of its antioxidant properties, mediate resistance to apoptosis and promote a stem cell phenotype*. *We thereby hypothesize that the increased extracellular lactate levels in HIF-1 α -deficient cells contribute to the induction of a radioresistant phenotype*. Furthermore, a strong reduction in basal glycolytic activity was seen in HIF-1/2 α -deficient cells possibly contributing to the higher radiation sensitivity phenotype. This suggests that there is an urgent need for better understanding the combined function of the HIF proteins in cancer and further translate it into clinical application in patients.

NOTCH performs activities that have key roles during embryonic development and also in tissue maintenance of adult tissues. NOTCH is also associated with cancer by modulating

resistance to standard treatment modalities such as radiotherapy and chemotherapy. Moreover, Notch is often deregulated in certain types of cancer, which makes it a promising targeting candidate by using γ -secretase inhibitors. However, many side-effects of γ -secretase inhibition were seen which are related to on target Notch toxicity in the intestine causing intestinal goblet cell metaplasia and other tissue-related toxicities. Previously, our group has shown the benefits of combining NOTCH inhibition with radiotherapy in a lung cancer model. No models are currently available to study the effect of radiotherapy and Notch inhibitors on normal tissue response in patients with lung cancer. Therefore, in **chapter 4** we developed a model to investigate the role of the Notch signalling in cell fate decision and the radiobiological properties of primary bronchial epithelial cells (PBEs) isolated from human lungs. In particular, our work has demonstrated that NOTCH signalling plays a crucial role in modulating cell fate decisions by modulating the differentiation and self-renewal capacity of PBEs. The capacity of modulating a specific cellular balance within the lung by blocking NOTCH is further enhanced by the effect of radiation. Based on the results from this chapter it can therefore be concluded that NOTCH inhibition increases the number of lung stem cells in irradiated PBEs cultures by enhancing the DNA damage response and thus reducing the amount of double-strand breaks.

In **chapter 5**, we investigated the activity of the different components of the γ -secretase complex on a mouse embryonic fibroblast (MEF) cellular model expressing wild type of oncogenic NOTCH1. Using this mouse model, we demonstrate that both NOTCH isoforms cleaved by the different components of the γ -secretase complex show characteristic cleavage and activation capacities that lead to a differential gene expression pattern. Our results reveal an unexpected preference of the Psen2:Aph1B γ -secretase complex to process oncogenic mutant NOTCH1 receptors over wild type NOTCH receptors. Psen2-specific targeting could be exploited in cancer cells while sparing Psen1 substrates including Notch signaling in normal cells where Psen2 is non-essential. We also used chloroquine as an inhibitor of the endocytic pathway and observed that oncogenic NOTCH1 protein processed by Psen2:Aph1B preferably relies on the endocytic pathway for its activation and latter function as a transcription factor.

Finally, **chapter 6** summarizes the findings of this thesis which describe some of the benefits and limitations of blocking HIF and NOTCH in cancer.

Valorization addendum

Tumor biomarker selection and targeting is crucial when treating cancer patients with the ultimate goal of overcoming resistance to conventional therapy. Over the last decades, a whole new research on biomarker discovery and validation is being tested in preclinical and clinical studies, demonstrating that selective biomarker targeting is a highly active field of research. Targeting resistant cancer cells remains the main barrier we face in improving outcome. The remarkable ability of cancer cells to adapt to the new environment conditions is the most frustrating characteristic of therapy resistance. However, the introduction of new targeting strategies such as the CRISPR/CAS9 technology or the discovery of novel oncogenic genes helps developing new treatment options for patients. The main aim of this thesis was to evaluate several tumorigenic aspects of different oncogenic proteins while also combining its inhibition with the effect of radiation.

Clinical relevance

In this thesis, the scientific relevance of modulating the HIF-1/2 α proteins and NOTCH in different cellular models and in the context of cancer treatment has been demonstrated. Scientific knowledge in cancer research allows us to better characterize the basic molecular mechanisms behind a tumorigenic phenotype but also to develop new technologies or treatment strategies that eventually benefit society. Nowadays, cancer disease is one of the foremost causes of death worldwide. The most common causes of cancer death worldwide include lung, liver, stomach and bowel cancer, accounting for almost half of all cancer deaths. Plenty of treatments are currently available to treat cancer, some of which are optimal in initially reducing tumor growth and even lead to cures, however, this positive outcome is not always long-lasting and some tumors may recur. There is a population of resistant cancer stem cells that we have aimed to tackle in this thesis by trying to inhibit the proteins that those cells express and thus improve the effect of radiation. Many biomarkers have been already identified to strategy patients for personalized treatment. Since many cancer patients receive radiation treatment, approaches that influence radiation therapy have a great impact on cancer lives.

HIF α proteins are transcription factors of which the stabilization and activity are often tumor-specific especially under hypoxic conditions and generally associated with worse outcome for cancer patients. Therefore, HIF α might be considered as a valuable biomarker for stratification of cancer patients, although the specific treatment based on this type of stratification needs further investigation. Evidence on the prognostic value of HIF-2 α protein

in cancer patients can be seen in this thesis in a meta-analysis collecting survival data from previously published studies. HIF α has become a potential target for developing novel cancer therapeutics since early 1990s. These inhibitors target the expression and/or function of HIF-1 α , HIF-2 α , or both, through direct and indirect mechanisms. A number of HIF α inhibitors have been developed and although some of them are under investigation, monotherapy treatments with HIF α inhibitors have not been successful in clinical trials and many have been halted. However, these inhibitors might be beneficial in order to sensitize resistant cells to conventional treatment modalities such as radiotherapy. Indeed, in this thesis we have shown that double- HIF1/2 α inhibition greatly improves radiation sensitivity. Nevertheless, we also showed that individually inhibiting HIF-1 α in hypoxic cells might confer an opposite phenotype due to a compensation mechanism mainly triggered by HIF-2 α overexpression. Potentially, this is applicable to other treatment modalities like chemotherapy or immunotherapy and thereby contribute to a more optimal treatment of patients.

NOTCH has been found to influence tumor sensitivity to therapy and normal tissue regenerative capacity. Both characteristics are essential to improve treatment tolerance in cancer patients by increasing the effectiveness of the therapy while sparing normal tissue toxicity. The using of NOTCH inhibitors seems to be a promising strategy to eliminate the resistant cancer stem cells of the tumor and has been tested in many clinical trials. Patients receiving NOTCH inhibition treatment however, show severe adverse effects which ultimately lead to the abrogation of clinical studies. In our thesis we described a stem cell proliferative advantage in primary bronchial epithelial cells upon Notch inhibition. Since radiotherapy damages the normal tissue, increasing the proliferative capacity of stem cells might benefit patients which undergo radiotherapy and thus broad the dose-effect in cancer cells. Of course, the effect on the tumor and its microenvironment still need to be carefully elucidated in patients when combined with radiotherapy. The toxic effects driven by NOTCH inhibition might also be reduced by targeting different components and combinations of the γ -secretase complex. Since NOTCH regulates various pathways necessary for proper tissue regeneration and homeostasis, assessing a more specific NOTCH treatment strategy is essential. This thesis demonstrated that it is utmost important to modulate the activity of NOTCH by targeting specific components of the γ -secretase complex.

Gain for society

Despite the novel advances on cancer therapeutics, prevalence of cancer and its mortality rate is still affecting human health while also influencing economic stability in the world. The long and sometimes frustrating process of drug development for treating cancer accounts for most of the costs of cancer in society. Preclinical data aims to identify a compound, describe its mechanism of action and eventually transfer it to the clinic for patient testing. Before entering the market most of the drugs are discarded due to elevated toxicity or lack of effects. This inefficient process of drug discovery for patient usage not only costs millions of dollars every year but also the people we love.

The observations made in this thesis could potentially benefit cancer patients and the society in general. First, a new way of stratifying patients based on HIF-2 α expression gives more insight into the prognosis of cancer patients, thus better predicting the survival rate of patients. Patients with lower HIF2 expression maybe selected for less aggressive treatment because they have a better prognostic outcome. This thesis also investigates the application of a novel way of biomarker targeting by using the CRISPR/CAS9 in human cells, a unique and elegant genetic-engineering tool that efficiently targets and removes specific genes from the genome. More studies are needed to better characterize the effects of using CRISPR/CAS9 in human cells in terms of a live organism and ultimately its application in cancer gene modification in patients. Differences in HIF1/2 α -dual targeting offers an opportunity to design more efficient HIF α -blockers and increase the radiation response in cancer patients. Also, our study found a compensation mechanism by HIF-2 α that should be considered when treating patients with HIF-1 α inhibitors. Moreover, previous investigations from our research group found a beneficial effect on targeting NOTCH in combination with conventional therapies in cancer, which in this thesis we also show it would help patients prevent toxic-effects in the normal tissue. To end, patients presenting oncogenic NOTCH might benefit from modulatory-NOTCH inhibition by using Psen2:Aph1B γ -secretase inhibitors while sparing total NOTCH inhibition-related effects. Together, these findings may result into more effective therapeutic ratio when using NOTCH inhibitors in cancer.

Road to the market

Although the discoveries of our work represent a potential clinical relevance with a gain for society and could eventually lead to an improved outcome for treatment of cancer patients, it's translation from bench to bedside for daily clinical practice needs further investigation. Our data indicates that HIF1/2 α inhibitors are unlikely to radiosensitize tumors alone, but that

they can significantly increase their effectiveness when combined. Consequently, several clinical trials using dual-HIF1/2 α inhibitors should be of interest to test how well these inhibitors are tolerated also when combined with chemotherapy and radiotherapy, and whether any added benefit is observed. For instance, clinical trials have reported that BAY 87-2243 was found to suppress the accumulation of both HIF1/2 α in non-small cell lung cancer cells; however, a phase I clinical trial (NCT01297530) using such compound was terminated due to safety reasons. Other HIF inhibitors have been selected to treat solid tumors. Unfortunately, up to now, no drugs directly inhibiting HIF1/2 α have been approved for treating cancer due to safety or limited therapeutic efficacy. Lack of patient selection might also contribute to clinical trial failure. When evaluating the efficacy of HIF inhibitors in clinical trials, patients with advanced solid tumors are recruited, regardless of HIF expression in tumor cells. In this case, HIF inhibitors might not show therapeutic effects for accounting with those with low HIF levels. Therefore, clinical trials should also be stratified for patients with different protein levels in order to improve the concept of personalized medicine in cancer treatment and reach a better therapeutic effect for a specific subpopulation of patients with elevated HIF levels. Also, advances in CRISPR/CAS9 in applied medicine might offer a new way of selectively and accurately targeting cancer cells in patients. In total, the process may take many years before this technology can be used in daily clinical practice.

Acknowledgements

Overall, these 4 years in Maastricht have been very intense for me. Being far from family and friends is never easy, but luckily this city offered me lots of amazing and unique people that made this PhD dissertation possible. I was also very fortunate to click very well with many of my colleagues, which during these years have become very close friends.

It all started while doing my second internship in Barcelona on a random day around March 2014. I was actively seeking for PhD or technician positions on the field of cancer research preferably in the Netherlands or the UK and soon realized it was not going to be an easy task. While searching for positions in the website “academic transfer” I came across an interesting technician’s position at Marc Vooijs lab. I quickly prepared a motivation letter hoping that perhaps at some point he would answer me, the project was really of my interest and for me that was all what mattered. Shortly after, I saw Marc was also offering a PhD position for the same project, which motivated me to apply for that position as well!

I always liked the idea of moving back to the Netherlands. During my bachelor’s degree I did an internship at the Netherlands Cancer Institute (Amsterdam) for one year, and for me that had been the best experience of my life. I enjoyed living among international people, working with experienced scientists and grow as a scientist in the cancer research field. I also got much experience working with different cell lines, testing a set of molecular inhibitors in combination with chemotherapy and radiotherapy. My year in Amsterdam passed with a lot of good experience and I just came to be in love with going back to the Netherlands and seeking for a PhD position there. Unexpectedly, the PhD position was in Maastricht, a pretty unknown city for me since I just knew it from one of my hitchhiking trips, but never had the chance to really visit it. However, my beloved friend Bea (who I met during my internship in Amsterdam), was working in Maastricht, which meant an extra reason to like the city.

My first interview with Marc was pretty scary. I expected something perhaps more formal but instead everything went pretty smooth. He did not talk into detail about the project but instead he wanted to know more about me, my personality and my lab experience. During the interview, I also clarified my preference for a PhD over the technician position. The second interview was more intense, this time Prof. Jan Theys was also behind the webcam and I woke up a bit sick. I do not remember many details about that interview but somehow after we finished the call I knew they were not going to select me for a next interview. To my surprise, short after they contact me and invited me for a final interview in Maastricht! I was so excited I had to call my entire family and some friends. I really liked the project and I

knew it was a great opportunity to expand my knowledge and start an academic career in cancer research. My first impression of Maastricht was pretty good! Not a big city but still with a lot of potential for meeting international people and living a good life. The night before the interview I slept one hour due to lots of stress and nerves. Right before the interview I drank a “monster” which helped me focus and allowed me to keep with the interview with all the members of the department. It was long, repetitive and a bit tedious. I mostly got the same questions from everybody regarding my personality and the way I work in the lab. Also, I had to give a couple of oral presentations which I was really afraid of. Luckily, Arjan was there to make his typical jokes and that made me feel more relaxed. I felt insecure, but at the same time I wanted to show how I really am: a very active and passionate guy. Well, it worked since they contacted me a few days after accepting me as a future PhD student in the department and I couldn't be happier. Shortly after, I got another job offer in Amsterdam Medical Centre, but I just had to refuse it since I really liked the topic in Maastricht and the team had been so lovely with me; even though living again in Amsterdam was a dream for me.

To my promotion team:

Marc, I would like to begin to acknowledge that without you I would not have lived this amazing experience and written this beautiful thesis. I am extremely thankful for giving me the opportunity to join your team. Thank you for all the meetings, advices, support but also the critiques. Altogether, I believe it was important for my professional and personal growing. Thank you for encouraging me to be curious while teaching me many aspects of basic and translational research. It was also very nice to get to know you better in different places outside the lab. Also, for your patience when I felt it was over for me.

Ludwig, thanks for your contribution during the last period of my PhD. I really appreciate those meetings on Friday late afternoon which were supposed to last for one hour but would always be extended for 2 hours or even longer. Your input and knowledge has been crucial for the developing of my thesis. Your daily supervision encouraged me to perform many experiments which currently are included in this thesis. Also, you have been my biggest support when continuing with my PhD was tough and I did not believe in myself, which I will never forget.

To my reading committee:

Thank you to the chairman Prof dr. de Windt, Prof dr. W. Annaert, Prof. P. van Diest, Prof. dr. P. Peters and Prof. dr. H. Schmidt for your time and effort to review my thesis.

To my colleagues in Maastricht lab:

Thanks to everyone in the department for being such nice people and for helping me improve as a scientist and human being.

Arjan, thank you for your guidance and help specially during the first period of my PhD. Your input was crucial for the content of many chapters included in my thesis. Thank you for the heated and sometimes funny discussions we had in your room, Marc's office or the coffee room. Also, for introducing me to my currently favourite beer: tripel karmeliet. I will never forget those crazy evenings and nights when we both had way too many tripel karmeliets and going back home (specially by bike) was a bit complicated. I hope the best for you and your lovely family.

Ala, thanks for all the help and our very efficient and interesting discussions in your office. Having you by my side has always been a safe feeling for me. I really appreciate all the effort you put on my thesis. I learned a lot from you and your extremely huge knowledge on many topics related to my thesis. Also, I appreciate you shared with me your human side in many situations like team buildings or while having a coffee. You are just an amazing person and I hope the best for you, your family and the coming baby.

Lorena (Lures), you have always been my biggest in the lab and outside the lab. Working with you has been a pleasure from the first day I met you picking up membranes from the ALI plates. We always understood each other and shared many experiences. Laughing, singing songs, dancing (Lorena likes to dance a lot), drinking, partying and also sharing food. Cooking for each other has been a thing since she brought the best tiramisu ever. We both share so many experiences we could perfectly write a book, a comedy-drama most likely. I have no words to describe how special you are to me and how much I will miss you. During these years, Lorena not only has become a colleague for me, but also a sister. Ti ringrazio per tutto e ti penso sempre.

Venus (Vinus), what a nice thing meeting you! You are such a special and lovely human being! All I can tell about you is good things, since your soul is one of the purest I have ever felt. You were my first confident and good friend in the lab. We shared lots of nice dinners and cool game nights. I hope you find your way and soon you find a job as a post-doc. And let's not forget the amazing mother you have. You are both lovely people.

Jonathan (Jeffrey), apart from being a great colleague and a good friend, I would like to thank you for not escaping the first time we hanged out together! We share amazing and very funny moments in the lab and outside. I hope you never lose your peculiar British accent and at some point, I learn how to say "four" the way you pronounce it. Thanks a lot for everything mate and see you in London! Remember to keep up with my 'kitifollen' in the lab, you are officially the heir.

Veronica (Viros), you are an incredibly sweet and funny person! I will never forget how much we have laughed together in the lab while singing or just making fun of ourselves. This experience has made us stronger and better people. Please never give up on yourself, you deserve a lot and I hope you know it. I will miss you heavens but listen to me! I will be in your isla de coco chilling and dancing bachata with you in a near future.

Damiene (Neni xulah), you are one of the funniest girls I have ever met! We clicked from the first beginning I met you. We had always wonderful chats and discussions about many topics (well it's also true that we both love talking). And also... rock and rolling! I just love how active, motivated and inspiring you are! Sharing office with you has been one of the best things of my PhD since because of that I really got a very good friend. There is still so much we have to do together! I invite you to one of my trips, also to your lovely mother. Thanks for being just the way you are, a beautiful and strong woman.

Alex and Maikel (tekila mates), you guys will drive me crazy one day! Meeting you on the last year of my PhD has just been so refreshing. I really admire your ability to bring joy and nice moments. In one year, you both became one of my biggest supports in this city. Haven't you nearby can be dangerous (I guess you both know why) but also an experience full of fun facts and amazing moments. Pity I have to leave when you guys just moved next to my apartment but maybe it is better this way!!!. Lots of love and good luck on your PhDs.

To Lures, Vinus, Jeffrey, Viros, Neni xulah and my tekila mates there is only one more thing I can say: kitifollen guys, you will always be in my heart and I love you so much.

Natasja and Rianne, I would like to thank you all the times you have helped me during my PhD. From the beginning, I felt very comfortable working and sharing department with you. We share amazing moments together not only in the lab but also while walking our dogs or drinking wine. I enjoyed many moments with you and also learned a lot from both of you. Thank you for teaching me how to use several techniques and also for your patience in the lab. Sometimes I think you both should receive a peace nobel price to really compensate the amazing work you are doing for each of the groups of the department. Your help will never be forgotten by me or the rest of our colleagues. By the way, I hope I will not receive many postcards for my dissertation (and I mean it) haha. You both have a special place in my heart which I will carry all my life.

Marike (Mariska), you are an absolutely funny and amazing person! Chatting with you and sharing experiences with you has always been very nice. Abba music put us together and maybe it will also separate us in my defence. In any case, thanks for all those moments lived together and for being such a nice colleague.

Nicolle (blonde), you are just so much fun. I loved the time spent with you in the lab and outside (salsa lessons). Wish you the best with your coming baby. I will never forget you!

Kranthi, you are such a sweet person. Thank you for all your support and the nice moments lived together.

Hi Tom! Lots of luck on your future mate! It was very nice meeting you and getting to know you better. I hope your PhD defence is a success and we can celebrate it soon! Wish you the best.

Lydie, you are a very kind person. Thank you for all your effort in helping me with my PhD. I wish you the best on your future as a PhD student, you will do it great I know it!

Judith, thanks for being a great colleague. You have always been so helpful and very supportive. Best of luck on your PhD!

Raymon, it was so nice to arrange with you the team building! At some point it was hard, but we really had a blast. It was also very nice to share with you movies and series! Man, thanks to you I discovered 'stranger things'! Best of luck on your future mate.

Marijke, Kim, Kasper, Jan, Alex, Evelien, Jolanda and Carla thanks for being great colleagues.

To all my former colleagues: Paolo, Tessa, Roger, Sanaz, Caroline, Linda, Simon, Sarah, Maud, Kim, Hanneke, I really thank the contribution you had to my thesis and the good moments we lived together in the lab.

To my former students Bjorn and Glenn for all the support and the contribution to this thesis.

To my friends and family:

First, I need to thank to my friends in Maastricht for being a very important support during these 4 years. I would not have made it without them. Second, thanks to my friends worldwide because even distance being hard, real friends always stay.

Now, I want to thank to my biggest support, my sister. You are the one I can always trust and share everything of my life. Thanks for always been there. Also, to my brother in law, cousins and aunties. Each one is a very important part of my life and I must say we are a great team: Els roig que estan boig. Also, to my grandmother which for many years was my second mother. Because without her support I would not be here.

Finally, to my mother. Because she is the reason why I decided to start a career on cancer research. Because she always believed in me and supported me.

Wherever you are, I hope you are proud of me. This book is fully dedicated to you.

Curriculum Vitae

Eloy Moreno Roig was born on October 18th, 1990 in Barcelona, Spain. In 2008 he graduated from Escola del Treball, Barcelona. After obtaining a bachelor's degree for Biomedicine at University of Barcelona in 2013 he continued with a master in Biomedicine and cancer research. During his bachelor he performed his first internship at the Netherlands Cancer Institute (NKI) in Amsterdam, the Netherlands. He studied the sensitivity of several molecular inhibitors in combination with the standard of care treatment in a series of GBM cell lines. His master's internship was performed at the Josep Carreras Leukaemia foundation in Barcelona, studying the depletion of cancer stem cells in leukaemia using Bromocriptine, a dopamine agonist used to treat pituitary tumors and other diseases such as Parkinson. In 2014 he received his master's degree and shortly thereafter he relocated to the Netherlands to commence a PhD track under the supervision of prof. Marc Vooijs at the department of radiation oncology, Maastricht University. His research involved basic and therapeutic aspects of the HIF and NOTCH signaling pathways for cancer treatment. The results obtained during his PhD are described in this thesis.

List of Publications

Moreno Roig, E., et al., *Prognostic Role of Hypoxia-Inducible Factor-2 α Tumor Cell Expression in Cancer Patients: A Meta-Analysis*. *Frontiers in Oncology*, 2018. **8**(224).

Eloy Moreno Roig, Arjan J. Groot*, Ala Yaromina*, Tessa C. Hendrickx, Lydie M.O. Barbeau, Lorena Giuranno, Glenn Dams, Jonathan Ient, Veronica Olivo Pimentel, Marike van Gisbergen, Ludwig Dubois and Marc Vooijs. *HIF-1 α and HIF-2 α differently regulate the radiation sensitivity of NSCLC cells, partly by impairing their metabolic profile*.
Submitted

Eloy Moreno Roig*, Roger A.J. Habets*, Lutgarde Serneels, Lydie M.O. Barbeau, Arjan J. Groot and Marc Vooijs. *γ -secretase complex composition differentially regulates Notch1 wild type and oncogenic pathways*.
Patent pending – to be submitted

Lorena Giuranno, **Eloy Moreno Roig**, Raffaella Iannone, Carolien Wansleebe, Lydie M.O. Barbeau, Ludwig J. Dubois and Marc Vooijs. *NOTCH blockade in combination with radiotherapy increases the number of lung stem cells in differentiated cultures*.
In preparation