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THE ROLE OF HIF-1ALPHA IN EPIGENETIC REGULATION OF TRANSCRIPTION

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The role of HIF-1alpha in epigenetic regulation of transcription
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“The significant problems we have cannot be solved at the same level of thinking with which we created them”.
- Albert Einstein

ABSTRACT

The oxygen level inside cells, determine the amount of HIF protein. By directly being involved in HIF protein turnover rates, through a mechanism, by which, oxygen is utilized as a co-factor, for the PHD enzymes, regulating HIF protein stability. This allows for rapid stabilization of the HIFs and subsequent gene activation during low oxygen tensions inside cells.

I investigated the role of HIF specific epigenetic effects, in cancer cell line models, as well as primary mouse $CD8^+$ T-lymphocytes. The data in Paper I illustrates how HIF has the ability to modulate chromatin through a HIF-1α dependent chromatin remodeling event, in hypoxia responsive gene promoters. We were able to show how, during hypoxic incubations of cancer cell line models HepG2 and SK-N-Be(2)c, hypoxia target gene promoters were remodeled, removing auxiliary factors bound to DNA. Possibly giving access to transcription factor binding. We identified two separate nucleosome free regions (NFRs) in hypoxia target genes, inducible NFRs and constitutive NFRs. The latter, were characterized by no initial remodeling event during hypoxia, conversely, the inducible NFRs required HIF-1α for remodeling to occur.

In Paper II we sought to identify the isoform specific effects of HIF-1α and HIF-2α in mouse primary CD8+ T-lymphocytes. We observed that HIF-1α was necessary for proper T-cell activation and cytotoxic function, by using mouse tumor xenograft models, showing decreased tumor size control in HIF-1α knockout T-cells. In contrast, HIF-2α knockout T-cells, activated properly, and controlled tumor size as well as control cells. VEGFA, a known HIF-1α target gene, conditional knockout in T-lymphocytes, showed increased tumor growth, in xenograft studies, as well, increased chemotherapeutic response. This finding, illustrates the complex nature of the tumor microenvironment, the role of the HIFα isoforms, as well as, VEGFA´s role in modulating tumor vessels and the ability for chemotherapeutics to be successfully delivered.

It has been shown that T-lymphocytes accumulated 2-hydroxyglutaric acid stereoisomers R/S-2HG in a HIF-1α dependent fashion. Were S-2HG, have important biological roles for $CD8^+$ T-cell function. In Paper III we identified the epigenetic effects of S-2HG during T-cell activation *ex vivo*, by following epigenetic modification changes through time. Our findings, illustrate how S-2HG metabolite decreases the overall acetylation of histone tails, at the histone 3 lysine 9 mark. The acetylation of histone proteins, has been widely studied, and is associated with increases in gene transcription. The global decreases in histone acetylation, observed by exogenous administration of S-2HG, might be a plausible mechanism for how, T-lymphocytes change their epigenome during activation.

LIST OF SCIENTIFIC PAPERS

I. ***HIF-Dependent And Reversible Nucleosome Disassembly In Hypoxia-Inducible Gene Promoters***. Norio Suzuki, **Nikola Vojnovic**, Kian-Leong Lee, Henry Yang, Katarina Gradin and Lorenz Poellinger. *Experimental Cell Research, 366, 181–191*

II. ***An HIF-1a/VEGF-A Axis in Cytotoxic T Cells Regulates Tumor Progression.*** Asis Palazon, Petros A. Tyrakis, David Macias, Pedro Velica, Helene Rundqvist, Susan Fitzpatrick, **Nikola Vojnovic**, Anthony T. Phan,Niklas Loman, Ingrid Hedenfalk, Thomas Hatschek, John Lovrot, Theodoros Foukakis, Ananda W. Goldrath, Jonas Bergh, and Randall S. Johnson. *Cancer Cell 32, 669–683*

III. ***Epigenetic effects of S-2HG in primary CD8$^+$ T-lymphocyte differentiation.*** **Nikola Vojnovic**, Pedro Velica, Pedro Cunha, Helene Rundqvist and Randall S Johnson. *Manuscript*

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LIST OF ABBREVIATIONS

ADM	Adrenomedullin
ALDH2	Aldehyde Dehydrogenase 2 Family Member
APC	antigen presenting cells
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
BAF170	Brahma associated factor 170
BAF57	Brahma associated factor 57
BRG1	Brahma-related gene 1
CM	central memory
CTL	cytolytic T-lymphocytes
Dhfr	Dihydrofolate Reductase
DNA	deoxyribonucleic acid
EHMT2 (G9a)	Euchromatic Histone Lysine Methyltransferase 2
EM	effector memory
EPO	erythropoietin
FIH	factor inhibiting HIF-1
GzmB	granzyme B
H3K4me	Histone 3 lysine 4 methylation
H3K9me	Histone 3 lysine 9 methylation
HAT	histone acetyltransferase
HIF	hypoxia inducible factor
Hk1	Hexokinase 1
Hk2	Hexokinase 2
HLA	Human Leukocyte Antigen
HP1α	heterochromatin binding protein 1α
HRE	hypoxia response element
LcK	Lymphocyte protein tyrosine kinase
LCMV	lymphocytic choriomeningitis virus
LDHA	Lactate Dehydrogenase A
LSD1	Lysine Demethylase 1A
LZIP	leucine zipper
MHC I	major histocompatibility complex class I
Mlh1	MutL Homolog 1
mRNA	messenger ribonucleic acid
mTORC1	mammalian target of rapamycin complex I
NFRs	nucleosome free regions
NLS	Nuclear localization signal
O_2	molecular oxygen
ODD	oxygen dependent degradation domain
PAS	PER-ARNT-SIM
PBMC	peripheral blood mononuclear cell
PD-1	programmed cell death 1
R-2HG	R-2-Hydroxyglutaric acid

RAD51	RAD51 Recombinase
RCC	renal clear cell carcinomas
S-2HG	S-2-Hydroxyglutaric acid
SAM	S-adenosylmethionine
SEM	stem cell effector
SLC2A1 (Glut1)	Solute Carrier Family 2 Member 1
SWI/SNF	switching sucrose non-fermenting
TAM	tumor associated macrophages
T_{CM}	T-cell central memory
TCR	T-cell receptor
VEGFA	Vascular Endothelial Growth Factor A
VHL	von Hippel-Lindau

1 INTRODUCTION

To generate the necessary energy demanded for an organism, molecular oxygen (O_2) has been utilized as the primary fuel for adenosine triphosphate (ATP) production in the respiratory chain of the mitochondrion. When levels of O_2 are in sufficient amounts for chemical reactions to occur, cells and tissues will function properly, however, when the supply of O_2 is less than the demand, a state of hypoxia will occur. At normal physiology, oxygenation in tissues will vary, for example during exercise skeletal muscle oxygen tension will decrease (Lundby, Calbet and Robach, 2009), and lactic acid will be produces as a consequence.

Numerous pathologies are associated with a state of hypoxia, such as stroke, ischemia, and cancer (Semenza, 2014; Schito and Semenza, 2016). Lack of oxygen in these tissues and cells leads to a more severe outcome. Tumors are often characterized by hypoxia, an impaired vasculature, and aerobic glycolysis (Hanahan and Weinberg, 2011). Tumors are also very heterogenous and patient specific, which leads to an even more complex depiction of the role tumor hypoxia has within a given tumor. In hepatocellular carcinoma (HCC), one of the most common liver cancers with a low 5 year survival rate (Siegel, Miller and Jemal, 2018), hypoxic tumors are correlated to a less favorable outcome, by being involved in angiogenesis, metabolic deregulation, sustaining proliferation, and activating metastatic growth. (Chen *et al.*, 2017). In childhood tumors like neuroblastoma, hypoxia has been shown to initiate a genetical program towards a more immature phenotype (Jogi *et al.*, 2002; Axelson *et al.*, 2005). In neuroblastoma tumor xenografts, hypoxia induced gene upregulation of sympathetic neural progenitor genes, such as c-kit and Notch-1, while down-regulated neuronal neuroendocrine genes. As with most solid tumors, the less differentiated a tumor is, the more malignant it can become (Oskarsson, Batlle and Massagué, 2014).

Tissue hypoxia has been shown to occur in inflamed tissues such as inflammatory bowel disease (IBD) (Taylor and Colgan, 2017). Furthermore, the link between T lymphocytes and hypoxia, as well as components of the hypoxia pathway is growing ever clearer. For instance, *VHL* deletions in early thymocytes, resulted in smaller thymus due to a severe reduction in CD4/CD8-double positive thymocytes, due to apoptosis (Biju *et al.*, 2004). Interestingly, further deletion of HIF-1α in the VHL knockout background, restored the thymocyte development and prevented excessive apoptosis (Biju *et al.*, 2004), suggesting a role for the hypoxia pathway in thymocyte development. The cellular response to hypoxia is mainly mediated through two transcription factors, hypoxia inducible factor 1 alpha (HIF-1α) and HIF-2α encoded by the genes *HIF1A* and *EPAS1* respectively. The aforementioned physiological or pathological hypoxic conditions are either directly or indirectly affecting HIFα or mediated through HIFα protein function.

HIFA PROTEIN STABILITY

Cellular response to hypoxia is driven by a family of transcription factors, HIF-1α, HIF-2α and HIF-3α, they belong to the PER-ARNT-SIM (PAS) family (Wang *et al.*, 1995) of the basic helix-loop-helix (bHLH) transcription factors, illustrated in Fig. 1. HIFα is continuously expressed at the mRNA level, however the protein products are tightly regulated in the presence of O_2 through post-translational hydroxylation and polyubiquitination of target residues present in HIFα proteins. A family of prolyl hydroxylase domain containing proteins (PHD-1-3), hydroxylate HIF-1α on proline residue 402 and 564 (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001), while HIF-2α is hydroxylated at proline residues 402 and 531. Hydroxylation of these key residues mediates binding of protein von Hippel-Lindau (pVHL), an E3 ubiquitin ligase, targeting HIFα for proteasomal degradation by the 26s proteasome (Huang *et al.*, 1998; Kallio *et al.*, 1999; Maxwell *et al.*, 1999) in the presence of O_2. Additional hydroxylation takes place on the C-terminal transactivation domain of HIFα proteins by factor inhibiting HIF-1 (FIH-1). This asparaginyl hydroxylation is added on asparagine 803 and 847 on HIF-1α and HIF-2α respectively (Lando, Peet, Whelan, *et al.*, 2002). The effect off FIH-1 hydroxylation during physiological oxygen tension has been shown to affect HIFα protein transactivation activity by inhibiting the interaction of p300/CBP binding proteins (Mahon, Hirota and Semenza, 2001; Lando, Peet, Gorman, *et al.*, 2002). Collectively the protein domain where hydroxylation as well as ubiquitination (Maxwell *et al.*, 1999), occurs on HIFα proteins has been appropriately termed oxygen dependent degradation domain (ODD), highlighting the oxygen sensitive protein turnover of the HIFα proteins.

During hypoxia, the PHD enzymes are limited in substrate availability of oxygen, leading to stabilization of HIFα protein, nuclear translocation and heterodimerization with the oxygen insensitive binding partner aryl hydrocarbon receptor nuclear translocator (ARNT) or HIF-1β (Jiang *et al.*, 1996). HIFα/HIFβ heterodimers bind to their cognate DNA binding sequence, hypoxia response element (HRE), in the nucleus, 5´- R/C-GTG -´3, were R is either adenine or guanine DNA bases, and induce gene transcription of target genes.

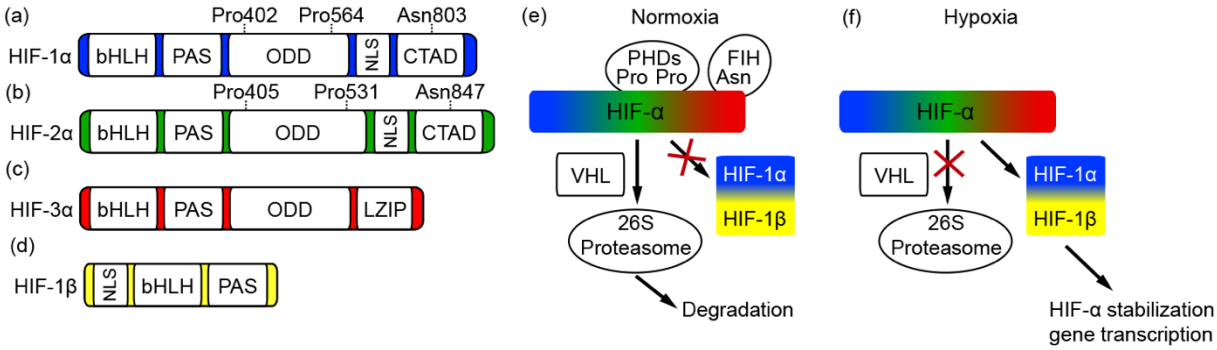


Fig 1. Schematic representation of human HIFα protein structures. (A) HIF-1α protein schematic, illustrated are protein domains of interest. Basic helix loop helix (bHLH) DNA binding domain, Per-Arnt-Sim (PAS), dimerization domain with binding partners. Oxygen degradation domain (ODD), herein lies the necessary proline residues which are hydroxylated by PHD enzymes for degradation. Nuclear localization signal (NLS), necessary for nuclear import. C-terminal transactivation domain (CTAD), transactivation domain for HIF-1α protein, as well as, FIH asparagine hydroxylation site. (B) HIF-2α protein schematic, illustrating the protein domains of interest. (C) HIF-3α protein schematic, multiple HIF-3α isoforms exist, chosen here is the HIF-3α-1 protein schematic containing the leucine zipper (LZIP) domain. (D) ARNT or HIF-1β protein schematic, illustrating the important protein domains, of interest, HIF-1β has no ODD protein domain, therefore is thought of as being oxygen insensitive. (E) Regulation of HIFα through PHD and FIH hydroxylation, as well as, VHL polyubiquitination. Degradation occurs at the 26s proteasome. (F) During low oxygen conditions, oxygen is limited, therefore, PHDs and FIH, will hydroxylate at lower rates, which enables stabilization of HIFα proteins, nuclear translocation, binding to HIF-1β with subsequent DNA transactivation.

1.1 HIFA GENE REGULATION

HIFα target gene regulation has been extensively studied in the past two decades. Pioneering work from Wang,GL et al., (Wang and Semenza, 1993), described how in Hep3B cells, a hepatoma cancer cell line, HIF-1α during hypoxia was stabilized and bound to the 3´enhancer of the erythropoietin (EPO) gene. The messenger ribonucleic acid (mRNA) of both *HIF-1α* and *EPO* genes were elevated during hypoxia, which gave insights into the notion that HIF-1α stability and *de novo* transcription of the *HIF1A* gene itself were required for optimal HIF response.

HIFα target genes can be characterized into two groups, the glycolytic genes (GLUT1, HK1, HK2, PGK1, ALDH2 and LDHA), and the angiogenesis genes (ADM1, VEGFA and EPO) (Ryan *et al.*, 1998; Hu *et al.*, 2003; Keith, Johnson and Simon, 2011), the upregulation of these genes is usually ascribed to HIF-1α function, although both HIF-1α and HIF-2α bind the same HRE sequences, shown through ChIP-seq data in cell line model MCF-7. Were HIF-1α and HIF-2α bound DNA is pulled down, together with HIF-1β antibody. And true HIFα peaks were determined only when sufficient amounts of HIF-1α/β or HIF-2α/β peaks were overlapping. (Schödel *et al.*, 2011). The authors could show that both HIFα isoforms, bind to the core motif, RCGTG, and no discernable difference could be shown.

The difference in target gene specificity could partially be explained by the fact that *EPAS1* is not expressed as widely as *HIF-1α* (Tian, McKnight and Russell, 1997). However, upon closer examination, *EPAS1* mRNA and protein could be detected in several rat organs after hypoxic incubation (WIESENER *et al.*, 2002). Elucidating which gene is regulated by which isoform is therefore more complex than the mere presence of the protein product. The biological evidence of HIFα isoform non-redundancy is highlighted by the different knockout mice models that have been generated. HIF-1α deficient mice die at embryonic day 10.5 displaying cardiac malformations, vascular regression and impaired erythropoiesis (Iyer, Leung and Semenza, 1998), however HIF-2α knockout mice survive until embryonic day 9.5 to 12.5 with severe vascular phenotypes, suggesting a more vascular role for HIF-2α protein function (Peng *et al.*, 2000).

The third HIFα isoform HIF-3α, is regulated by oxygen availability inside the cells through the PHD-pVHL system (Maynard *et al.*, 2003), and expressed in a multitude of organs such as, kidney, heart, thymus, lung and brain (Gu *et al.*, 1998). HIF-3α has been shown to act as a negative regulator of HIF-1α through posttranscriptional splice variants generating inhibitory pas protein (IPAS) (Makino *et al.*, 2001). HIF-3α splice variants have been shown to be HIF-1α regulated during hypoxia and might have important hypoxic modulatory roles in different tissues (Tanaka *et al.*, 2009).

1.1.1 Epigenetic regulation of transcription

It is arguably one of the most important features of a cell to be able to accurately increase certain protein products at demand to maximize survival advantage. The process of transcription which involves a complex machinery of protein-complexes, can be initiated through mitotic signals, as well as cytoplasmic/nuclear signaling. All cells have the ability to respond to their environment, although not all signals will lead to gene activation, however, during hypoxic insults, cells will rapidly stabilize HIFα which has the capacity to initiate gene transcription.

Despite HIFα´s propensity to bind DNA, it is not the only determinant of successful transcription initiation, like all other transcription factors it faces the accessibility problem. The DNA in eukaryotic cells is wrapped around 4 core histone proteins as shown in Fig. 2. Namely, histone 2A (H2A), histone 2B (H2B), histone 3 (H3) and histone 4 (H4), they form what is called the nuclear core particle (NCP), which was successfully crystalized two decades ago (Luger *et al.*, 1997). The core proteins are bound together as tetramers H2A-H2B and H3-H4, with two tetramers connecting together to form the nucleosome. DNA is wrapped around the NCP at around 146 base pairs (bp) (Talbert and Henikoff, 2017), generating what is called the beads on a string chromatin. In between nucleosomes lays histone 1 (H1), which is even called the linker histone. The function of H1 has been proposed to range from chromatin compaction, to transcription efficiency (Bell *et al.*, 2011; Rhee *et al.*, 2014). DNA compaction is one of the ways cells modulate transcriptional output, the best-known examples of transcriptional regulation at the packaging level is described as euchromatin and heterochromatin. Heterochromatin is divided into two forms, constitutive heterochromatin are regions in the genome that are transcriptionally silent, such as the centromere and telomere, the silent rRNA genes and the majority of the transposable elements (Politz, Scalzo and Groudine, 2013), facultative heterochromatin are silent genomic regions that are gene rich. Which have the ability to re-activate transcription (Chen and Dent, 2014). These heterochromatic areas are often found to have H3 lysine 9 mono, di, and tri methylation (H3K9me1/2/3) modifications as well as heavy DNA methylation (Swygert and Peterson, 2014). The unpacked, transcriptionally active genome is termed euchromatin, which has been associated with acetylation modifications on histone N-terminal tails on lysine 9 and 27 of H3 (H3K9/27Ac), methylation modifications on lysine 4 and 36, phosphorylation on serine 10 on H3 as well as many other modifications (Venkatesh and Workman, 2015).

The post-translational modifications (PTMs) of histone N-terminal tails are another mechanism regulating transcription initiation, from yeast to human cells, these highly conserved PTMs, with some species differences, have been extensively studied (Waterborg, 2012; Woo and Li, 2012). An emerging hypothesis trying to explain how the addition or removal of methyl or acetyl-groups on histone proteins could have effects on such a complex mechanism as transcription, is that these modifications are read by other proteins, that recognize these modifications and bind to them (Braun *et al.*, 2017). Methylation recognizing protein motifs are termed chromodomains first described 2001 (Bannister *et al.*, 2001), acetylation recognizing motifs are termed bromodomains (Dhalluin *et al.*, 1999). The chromodomain of heterochromatin binding protein 1α (HP1α) was shown to be specifically binding H3K9me and was shown to not have as high affinity towards H3K4me. The binding of HP1α is thought to sustain heterochromatin regions. Analogously to the chromodomain, the bromodomain of Gcn5, an HAT acetylating H3K9/14, was shown

to have site specificity as well (Cieniewicz *et al.*, 2014). These motifs are found in many different protein complexes, with different affinities for said modification. HIFα has been shown to regulate both JMJD1a as well as G9a, two opposing histone protein PTM erasers and writers of H3K9me2 respectively. Germ cell derived tumors, such as seminomas, yolk sac tumors and embryonal carcinomas, were shown to downregulate JMJD1a, which was unsurprisingly not necessary for stem-cell renewal, however served as a tumor suppressor, acting as a counter balance to the hypoxia driven expression of G9a (Ueda *et al.*, 2014). Loss of JMJD1a in this context, generated larger tumors, while pharmacological inhibition of G9a generated reduced tumor size in nude mice supporting the notion that G9a expression through HIFα could be oncogenic.

HAT enzymes use acetyl-CoA as their substrate for acetylation of histone tails, conversely, histone methyltransferases (HMTs) use S-adenosylmethionine (SAM) as the major methyl donor. The link between the cells metabolic output and epigenetic function is beginning to emerge (Li *et al.*, 2018). To date, 550 histone PTMs have been found through mass spectrometry and increased refinement of these techniques (Andrews, Strahl and Kutateladze, 2016), however the vast majority of these modifications have not yet been ascribed to any biological process. In the nucleus, the DNA is positioned according to activity, being closer to the nuclear periphery in highly compacted states as heterochromatin anchored through lamin-associated domains (LADs) (Towbin *et al.*, 2012). These subnuclear DNA rearrangements were ascribed to different methylation states of H3K9me through stepwise addition of methylated lysine residues, in Caenorhabditis elegans embryos (Towbin *et al.*, 2012). The physical movement of DNA is also occurring on the nucleosomal level, by a family of nucleosome remodelers termed SWI/SNF (in humans BAF). These complexes have been implicated in numerous physiological processes, cell cycle progression (Dunaief *et al.*, 1994), T-cell $CD4^+/CD8^+$ reciprocal regulation (Chi *et al.*, 2002), neural development (Tang, Yoo and Crabtree, 2013) and cancer (Wang, Haswell and Roberts, 2014). Encompassing a whooping 30 genes in the BAF family, with the ability to assemble in multiple combinations generates many a complexes with cell type specific expression patterns, creating tissue specific BAFs (Lessard and Crabtree, 2010). These complexes use the energy of ATP hydrolysis to evict nucleosomes, reshuffle them as well reposition them during transcription, interacting with transcription factors in a cell type specific manner (Ho *et al.*, 2011). The study of nucleosome eviction, mapping regulatory sites in the genome, such as nucleosome free regions (NFRs) has been aided with the help of enzymes that have the ability to shred or cut DNA. DNase I has been widely used to map regulatory sites in the genome and coupled with next generation sequencing enable full genome wide identification of DNase hypersensitivity sites (DHS) (John *et al.*, 2013).

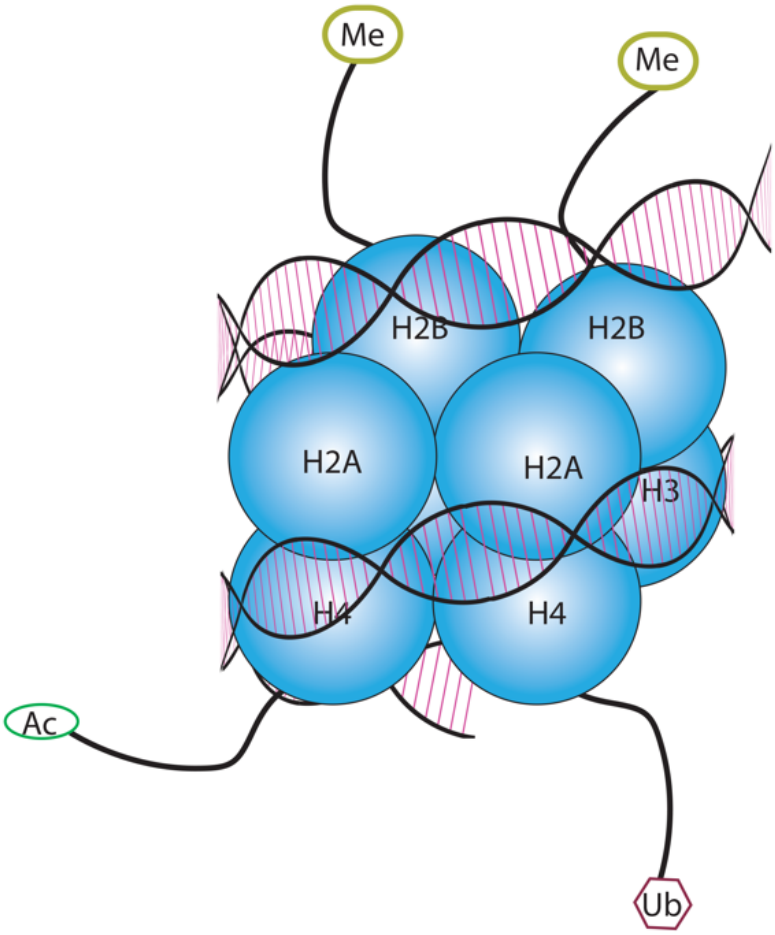


Fig 2. Schematic representation of the NCP with DNA wrapped around. Highlighted PTM modifications. The nuclear core particle (NCP) contains the 4 canonical histone proteins, namely H2A-H2B, H3 and H4, bound together as tetramers, to form the octamer NCP. Wrapped around and in between the histones, is roughly 146 base pairs of DNA. Histone post-translation modifications are added covalently to their respective N-terminal protruding tails, by numerous enzyme families.

1.2 HIFA EPIGENETIC REGULATION

The core activation complex of HIFα/HIFβ is augmented by its co-activator p300/CBP (Arany *et al.*, 1996; Kallio *et al.*, 1998). The p300/CBP complex functions as a histone acetyltransferase (HAT), adding acetylation moieties to histone lysine N-terminal tails. The acetylation of histones has long been associated with increased transcriptional activity (Sabari *et al.*, 2016). For over a decade, HIFα has been shown to bind to HRE sequences at target gene promoters that are affecting epigenetical readouts in a cell dependent manner. For instance, the histone deacetylase 7 (HDAC-7) protein has been shown to act as a binding partner of HIF-1α (Kato, Tamamizu-Kato and Shibasaki, 2004), even under normoxic conditions in HEK293 cells. During hypoxia, HDAC-7 would translocate to the nucleus, and form a complex with HIF-1α and p300, increasing target gene expression (Kato, Tamamizu-Kato and Shibasaki, 2004). Moreover, histone methylation of histone 3 lysine 9 (H3K9me), mediated through G9a/GLP enzyme, was shown to increase in hypoxia, in several cell line models (Chen *et al.*, 2006). H3K9me2, which is highly correlated to low transcriptional output, was increased in hypoxia, at Mlh1 and Dhfr genes (Chen *et al.*, 2006), as well as global levels of H3K9me2 was observed to increase during hypoxia, which was abrogated in $G9a^{-/-}$ mouse embryonic stem cells. The converse reaction of removing methylation from histones, mediated by histone demethylases, has been shown to be HIF-1α dependent (Beyer *et al.*, 2008). Through hypoxia incubations of cell line models, JMJD1a and JMJD2a mRNA were increased in a hypoxia dependent fashion. Furthermore, the authors could show by ChIP-qPCR analysis, how HIF-1α was associated with HRE sequences present in the proximal promoters of these gene loci (Beyer *et al.*, 2008). Furthermore, JMJD1a expression was also shown to be hypoxia regulated *in vivo*, through hypoxia incubation of rats (Wellmann *et al.*, 2008). The authors could show, increased JMJD1a mRNA expression in hypoxia, in brain, heart, lung, testis, and kidney.

DNA methylation which is strongly associated with heterochromatin formation, i.e., closed non transcriptionally active chromatin, has also been shown to be affected by hypoxia (Shahrzad *et al.*, 2007). The authors used cancer cell line models, and tumor xenografts, to correlate the level of DNA methylation to tumor hypoxia. They could observe a hypomethylated phenotype in tumor xenografts studies, by correlating hypoxyprobe staining to DNA methylation staining in xenografted tumors in SCID mice (Shahrzad *et al.*, 2007).

Finally the chromatin remodeling factors mating type switching sucrose non-fermenting (SWI/SNF) complexes have also been shown to be important for HIF-1α transactivation activity (Kenneth *et al.*, 2009). Through overexpression of SWI/SNF subunit proteins, BRG1, BAF170 and BAF57, HRE luciferase activity was increased in hypoxia incubated U2OS cells, suggesting how SWI/SNF complexes potentiate the hypoxia response. Furthermore, by siRNA depletion of the aforementioned subunits in U2OS cells, hypoxia target gene Glut1 protein levels were abrogated at 4 hours of hypoxia incubation compared to control (Kenneth *et al.*, 2009). Indicating that HIF-1α transactivation requires some chromatin remodeling to occur, at hypoxia target genes.

Epigenetic regulation of components in the HIFα pathway has been shown, in a study of human patients with multiple myeloma, 33% of patients had increased DNA

methylation in the *VHL* locus (Hatzimichael *et al.*, 2009), functionally decreasing the overall levels of pVHL leading to uncontrolled HIF-1α stability, which was correlated to a more severe outcome. Another study focusing on DNA methylation showed how *EGLN3* (PHD3) locus was hypermethylated in B-cell lymphomas and multiple myelomas (Hatzimichael *et al.*, 2010), correlating yet again to a more unfavorable outcome. DNA methylation enzyme DNMT3a has also been implicated in the regulation of *EPAS1* gene transcription. By comparing non-transformed and tumor cell lines, the authors could show how *EPAS1* mRNA and protein was regulated in the presence of DNMT3a (Lachance *et al.*, 2014), furthermore by performing short hairpin RNA (shRNA) knockdown targeting DNMT3a, cells would reactivate the expression and protein accumulation of EPAS1, leading to increased hypoxic cell survival and increased tumor size xenografts.

A more recent study has elucidated the aspects of epigenetic regulation and HIFα binding, with the chromosome conformation capture (3C) technique, (Platt *et al.*, 2016), could show HIFα DNA binding sites are established even in physiological oxygen tension. By using the 3C technique, coupled to ChIP-seq data of HIFα binding, to both proximal and distal sites in the MCF-7 cell genome, the authors could show RNA-polymerase II (RNAPII) occupancy was already present at these HIFα sites and distal enhancers, even under physiological oxygen conditions. The release and activation of RNAPII, during hypoxia is therefore correlated to hypoxia target gene expression, observed solely during hypoxia incubation of MCF-7 cells. Which suggests that not all HIFα binding sites are established during hypoxic conditions, furthermore, this might explain the rapid mRNA expression profiles seen in short term hypoxia exposures.

1.2.1 The effect of low oxygen tension in tumors

Cancer, the disease of uncontrolled cell proliferation. Is the second leading cause of mortality in the United States although estimated new cancer cases are projected to be higher in females then males, the estimated deaths are projected to be higher in males (Siegel, Miller and Jemal, 2018).

As with all cells, O_2 is a vital component of their sustainable survival, the organisms blood vessels are fine tuned to deliver the proper amount of oxygen throughout the entire lifespan. When cells initiate uncontrolled cell proliferation, the limits of oxygen diffusion from nearby blood vessels will eventually lead to hypoxic cells, which in turn stabilize HIFα, concomitantly induce gene expression of target genes involved in angiogenesis, the process of sprouting new blood vessels from pre-existing ones (Rey and Semenza, 2010). HIF-1α induces expression of VEGFA which in turn initiates the signal transduction cascade by binding to VEGFR2, a receptor tyrosine kinase pivotal for angiogenesis located on the surface of endothelial cells, pericytes and vascular smooth muscle cells (Rey and Semenza, 2010).

Through successive cell cycle progressions, cancer cells will acquire somatic mutations, the mutation load seems to be cell type specific, for example breast and ovarian cancers have a high mutation rate in the breast cancer type 1 (*BRCA1*) gene, which has been shown to be epigenetically regulated via hypoxia (Lu *et al.*, 2011). Through ChIP-qPCR analysis, the authors could show how the *BRCA1* and *RAD51* locus in MCF-7 cells, acquired H3K9me2/3 modifications during hypoxia, with decreases in H3K4 methylation. Furthermore, acetylation of H3K9 was decreased

during hypoxia at these loci, which could be reversed upon HDAC inhibitor treatment with TSA. Finally, the authors could show that lysine specific histone demethylase 1 (LSD1), is one of the factors, that mediates the loss of H3K4 methylation during hypoxia in MCF-7 cells (Lu *et al.*, 2011), through shRNA stable transfection of MCF-7 cells with LSD1 specific shRNA constructs. Demonstrating how hypoxia dependent epigenetic effects, can influence genetic stability through inhibition of *BRCA1* and *RAD51* gene loci.

Approximately 50-75 % of renal clear cell carcinomas (RCCs) have inactivating mutations in the tumor suppressor gene *VHL* (Banks *et al.*, 2006). Generating increased HIFα stabilization irrespective of O_2 tension (Shen and Kaelin, 2013). HIFα overexpression in RCC has been shown to have isoform specific effects, HIF-1α seems to act as a tumor suppressor, located on chromosome 14q frequently deleted in RCC tumors (Shen *et al.*, 2011), whereas HIF-2α seems to be necessary and sufficient for RCC mouse xenograft engraftment (Shen and Kaelin, 2013).

As previously mentioned, HIFα proteins have been shown to modulate several epigenetic writers, erasers and readers, it is therefore not improbable to assume that hypoxic tumors will exhibit deregulated epigenetic effects. Several solid tumors have been shown to have hypoxic cores with strong HIFα staining, like the palisading necrotic cells of glioblastoma tumor biopsies (Schito and Semenza, 2016). For instance, in human tubular epithelial cells, HIF-1α was shown to induce the expression of Polycomb Group RING Finger Protein 4 (BMI1), a key component of the Polycomb complex 1 (PRC1) epigenetic modifier (Du *et al.*, 2014). Enhancing the epithelial to mesenchymal (EMT) transition, which is associated to increased metastasis. HIF-1α was also shown to regulate SNAIL1 in Hepatocellular carcinoma cells (Zhang *et al.*, 2013), another EMT related marker. Lastly, using several cancer cell lines and *in vivo* models, (Wu *et al.*, 2011), could show how hypoxia drives HDAC3 expression, in turn mediating WDR5 dependent histone methylation on H3K4, upregulating mesenchymal gene programs contributing to EMT transitions. Hypoxia and the HIFα proteins modulate the epigenome through various mechanisms, potentiating cancer progression in a cell type dependent manner.

Intratumoral hypoxia has been associated with many of the steps in tumor progression, interaction between the tumor microenvironment (TME) and surrounding tissues, has been shown to play a major role in tumor progression. Stromal cells that are part of the TME have been shown to promote metastasis through a variety of mechanisms (Hanahan and Coussens, 2012; Qiu *et al.*, 2017). The TME is composed of several cell types, tumor associated macrophages (TAMs), cancer associated fibroblast (CAFs), cells of the innate immune system like macrophages as well as NK cells, and the adaptive immune system $CD8^+$ T-lymphocytes (Rankin, Nam and Giaccia, 2016). In a recent study performed on human patients with metastatic melanoma (Hugo *et al.*, 2016), administration of anti-programmed cell death 1 (PD-1) antibodies were delivered to these patients which acts as a checkpoint blockade regime increasing the potential survival of T-cells. Whole exome sequencing of the patient's tumors revealed a transcriptomic signature which evaded immune surveillance and the anti-PD-1 regime, furthermore, the transcriptome was closely related to mesenchymal transition, hypoxia, wound healing and angiogenesis (Hugo *et al.*, 2016), linking hypoxia signatures to immune evasion.

1.2.2 T-cell activation and the epigenetic control of cell fate

Immune cells, such as B-cells and T-cells are born in the bone marrow, through successive differentiation of the hematopoietic stem cell. T-lymphocytes migrate out from the bone marrow, subsequently ending up in the Thymus, a small organ responsible for the correct environment for T-cell differentiation. T-lymphocytes in the Thymus will undergo positive and negative selection, ensuring proper function of the cells and either express the surface marker $CD4^+$ or $CD8^+$. After which they will migrate out from the Thymus and depending on the chemokine receptors they express, either continue circulating in the blood or migrate to the lymph nodes or other organs.

T-lymphocytes express the T-cell receptor (TCR), which is composed of variable α and β chains, upon encounter with antigen presenting cells (APCs) which display the antigen (Ag) through major histocompatibility complex class I (MHC I in mice and HLA in humans), $CD8^+$ T-lymphocytes will activate the TCR upon Ag recognition which engages complementary receptor CD3 and CD28 (Van Der Merwe and Dushek, 2011) initiating a complex phosphorylation cascade dependent on Akt, mammalian target of rapamycin complex I and II (mTORC I/II), and LcK (Smith-Garvin and Koretzky, 2009). Subsequently leading to gene expression programs that will expand the $CD8^+$ T-cells, increase their size, change their metabolism and differentiate towards specific cell fates (Chisolm and Weinmann, 2015).

A naïve $CD8^+$ T-cell has not yet encountered any foreign antigen while outside the thymus, during which it primarily uses fatty acid synthesis for energy production (Pearce *et al.*, 2009). However during TCR triggering, a rapid metabolic shift takes place, which induces glycolytic genes and metabolic output towards glycolysis (van der Windt and Pearce, 2012). mTORC1 and pyruvate dehydrogenase kinase 1 (PDK1) have been shown to be part of this regulation, shifting to a more glycolytic metabolism. HIF-1α has been shown to be induced during CD3 mediated *ex vivo* TCR triggering from human PBMCs (Nakamura *et al.*, 2005), as stated previously, HIF-1α is known to regulate several glycolytic genes, it is not entirely improbable to assume a role for HIF-1α in changing the metabolic phenotype of $CD8^+$ T-cells after activation. This assertion is suggested from the observation of the sibling cells $CD4^+$ T-cells, were HIF-1α has been implicated in the glycolytic shift from naïve to T_H17 and Treg cells (Dang *et al.*, 2011; Shi *et al.*, 2011). As we could see in Paper II, HIF-1α knockout $CD8^+$ T-cells, displayed improper activation, compared to wild type littermates, these effects, had functional consequences. For instance, in increased tumor volume and growth in several tumor xenograft models. The cell intrinsic effects of loss of HIF-1α in $CD8^+$ cells during tumor progression is still not clear. Further studies will be needed.

After the initial TCR trigger, naïve $CD8^+$ T-cells will differentiate towards one of several cell types, effector (EFF), stem cell effector (SEM), central memory (CM) and effector memory (EM) (Henning, Roychoudhuri and Restifo, 2018). Two models have been proposed which differ slightly in their mode of action. The linear model stipulates that the strength of the antigen signal drives the $CD8^+$ T-cells to T_{CM} (Opferman, Ober and Ashton-Rickardt, 1999). T_{CM} cells have the capacity to recall antigen response in adoptive transfer experiments in antigen-free recipient mice 10 weeks post initial infection (Opferman, Ober and Ashton-Rickardt, 1999). This model places the T_{CM} as an intermediate cell type in the differentiation cascade, further evidence for the placement of T_{CM} as an intermediate in the linear model, comes

from transcriptional analysis of $CD8^+$ T-cell clones, were T_{CM} cells are similar in the transcriptional readouts of naïve cells as well as T_{EFF} cells (Holmes *et al.*, 2005; Roychoudhuri *et al.*, 2015).

The circular model of T-cell differentiation proposes that a proportion of T-cells differentiate from naïve cells to effector cells and finally to memory cells. Secondary antigen encounter is intercepted by T_{EM}, restarting the cycle anew with dedifferentiation and redifferentiation occurring throughout the different states (Akondy *et al.*, 2017; Youngblood *et al.*, 2017). These effects have been proposed to occur both transcriptionally and epigenetically (Henning, Roychoudhuri and Restifo, 2018).

The histone PTM H3K9me3 is catalyzed by suppressor of variegation 3-9 homolog 1 (Suv39h1), one of many histone methyltransferases which can perform this reaction. Through $CD8^+$ T-cell specific KO of Suv39h1, (Pace *et al.*, 2018) could show how stem cell genes are deregulated in the KO animals compared to wild type littermates through single cell RNA sequencing technology. After *Listeria monocytogenesis* infection in mice, Suv39h1 was important for peripheral effector differentiation versus memory T-cell differentiation. Therefore Suv39h1 seems to be important for the commitment of $CD8^+$ T-cells to memory cell differentiation (Pace *et al.*, 2018).

One important effector molecule which is expressed by $CD8^+$ T-cells, is the granzyme family, granzyme B (GzmB) expression is associated with cytolytic activity and is usually expressed in high amounts in terminally cytolytic T-lymphocytes (CTLs). Epigenetic regulation of the GzmB locus has been shown to occur in $CD8^+$ T-cells through acetylation of lysine 9, H3K9Ac. (Araki *et al.*, 2008) could show increased H3K9Ac levels in the GzmB locus in memory T-cells compared to naïve, which correlated to increased mRNA levels, conversely, decreased acetylation of H3K9Ac in the GzmB locus via HAT inhibition with curcumin, was associated with less GzmB expression (Araki *et al.*, 2008). This suggests that memory T-cells can rapidly switch on the expression of CTL effector molecules, through epigenetic mechanisms.

H3K4me3 and H3K27me3 are two opposing histone PTMs, H3K4me3 is strongly associated with expression of a genetic loci, however H3K27me3 is strongly associated with heterochromatin. Crompton J et. al generated global H3K4me3 and H3K27me3 ChIP-seq maps of $CD8^+$ T-cells during differentiation (Crompton *et al.*, 2016). The authors showed how the levels of H3K4me3 were changed between the different T-cell subtypes, for instance, H3K4me3 levels were high in the promoter of transcription factor 7 (Tcf7) in naïve cells and T_{SCM} subtypes, however the T_{CM} and T_{EM} subtypes progressively lost this modification. These data suggest as with the previous H3K9Ac data (Araki *et al.*, 2008), epigenetic modifiers such as methyltransferases and demethylases are important for the acquisition of cell fate during T-cell differentiation. As we could show in Paper III, exogenous administration of S-2HG during TCR activation for 3 days, showed global histone acetylation decreases, furthermore, ChIP-qPCR analysis of target gene promoters showed differential acetylation enrichment between vehicle and S-2HG treated $CD8^+$ T-cells, possibly linking H3K9Ac loss at target gene promoters to cell type differentiation.
During cell type differentiation from naïve to effector cell, $CD8^+$ T-cells have been shown to have differential methylation regions (DMRs) (Scharer *et al.*, 2013). During lymphocytic choriomeningitis virus (LCMV) infection in mice, antigen specific naïve

cells were compared to 8-day long effector CD8 cells, promoter DMRs were characterized between the naïve and effector cells. Unexpectedly, DMRs were negatively correlated to gene expression, having increased DNA methylation at naïve associated genes in effector cells. DNA methylation is thus one possible mechanism of cell type determination in T-cells. Altogether, many epigenetic mechanisms could impact the differentiation from naïve to effector $CD8^+$ T-cell, with multiple mechanisms acting in conjunction with one another, generating a complex image of probable outcomes.

1.2.3 R/S-2-Hydroxyglutaric acid production and its epigenetic effects

The Krebs cycle proposed by Adolf Krebs in the late 1930s, is the primary metabolic pathway during aerobic processes in cells and tissues. Generation of energy in the form of ATP as well as numerous co-factors for enzymes and the oxidation of carbohydrates, proteins and lipids (Akram, 2014) and illustrated in Fig.3. The isocitrate dehydrogenase enzymes 1 and 2 (IDH1/2) convert isocitrate to 2-oxoglutarate (α-ketoglutarate) which is further metabolized to succinyl-CoA by α-ketoglutarate dehydrogenase (Akram, 2014). Mutations in the TCA cycle were often thought of as detrimental to cells, however (Dang *et al.*, 2009), showed how a single point mutation in IDH1 (R132H) shifted the enzyme output towards generating R-2-Hydroxyglutaric acid (R-2HG). The study found that in 80% of grade II-III gliomas and secondary glioblastomas IDH1 R132H mutation was present, defining R-2HG as an oncogenic metabolite (Dang *et al.*, 2009). Following this study, (Figueroa *et al.*, 2010), showed how *IDH1/2* mutations in human acute myeloid leukemia (AML) patient cohort had mutually exclusive mutations of either *IDH1/2* or ten-elven translocation 2 or tet methylcytosine dioxygenase 2 (*TET2),* generating a DNA hypermethylation phenotype in these cancers. The TET family of enzymes composes 3 known members to date, TET1, TET2 and TET3, all have been shown to perform DNA demethylation reactions involving TDG DNA repair and successive oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Tahiliani *et al.*, 2009; He *et al.*, 2011; Ito *et al.*, 2011). The consequences of *IDH1/2* mutation were soon to be unraveled, as predicted by Dang in 2009, (Chowdhury *et al.*, 2011) showed how R-2HG inhibited histone lysine demethylases from the jmjC domain containing histone demethylase family (JMJD2A). Koivunen P et.al showed how R-2HG and not S-2HG stimulated PHD1/2 activity in astrocytes, virally transfected with R132H IDH1, successfully diminishing HIF-1α and HIF-2α protein levels (Koivunen *et al.*, 2012). DNA repair enzymes were also shown to be sensitive to R-2HG overproduction (Wang *et al.*, 2015). The authors showed using established glioblastoma cell lines U87-MG and U373-MG stably expressing the *IDH* mutation R132H, increased DNA damage which was attributed to the inhibitory effects of R-2HG on alpha-ketoglutarate-dependent dioxygenase alkB homologs 2/3 (ALKBH2/3). Rendering the IDH1 mutated cancer cells sensitive to alkylating chemotherapy.

Recently, two papers were exploring the hypoxic response to 2HG accumulation in various cell types, (Intlekofer *et al.*, 2015) illustrated how hypoxia incubation of SF188 glioblastoma cell line, accumulated S-2HG in millimolar concentrations. Furthermore, the S-2HG levels were generated in these cells through malate dehydrogenase 1 and 2 (MHD1/2). Through siRNA transient knockdown strategies of MHD1 and 2, total levels of S-2HG could be shown to decrease in hypoxia. Similarly, (Oldham *et al.*, 2015) could show MHD1 and 2 dependence in generating S-2HG during hypoxia, however instead of using cancer cell lines, primary mouse

lung fibroblasts were used. Both of these papers suggested that HIF-1α was not necessary for the induction of S-2HG during hypoxia, although they could see decreases in the overall 2HG pool in siRNA knockdown experiments targeting HIF-1α. Furthermore, these papers incubated the cells in 0.5 % and 0.2 % oxygen tension respectively, these very low almost anoxic conditions could have effects on the redox balance in these cells. These studies beautifully explored the probability of a HIF-1α driven 2HG effect, however in their model systems HIFα was not necessary. Tyrakis P et.al (Tyrakis *et al.*, 2016) highlighted the necessity of hypoxic HIF-1α driven S-2HG and R-2HG production in mouse primary $CD8^+$ T-cells. Using the power of knockout mice models, Tyrakis P et.al could show that *VHL*$^{-/-}$ negative $CD8^+$ T-cells produced the highest amount of 2HG and the dominant enantiomer was S-2HG. The accumulated hypoxic S-2HG was lost in the HIF1a$^{-/-}$ $CD8^+$ T-cells, however the HIF-2a$^{-/-}$ $CD8^+$ T-cells had no effect on the 2HG pool. S-2HG was shown to accumulate in $CD8^+$ T-cells during activation which peaked between days 2 and 4, suggesting that S-2HG had some endogenous effects. Exploring the possibility of S-2HG affecting T-cells, the authors characterized both the phenotypic markers of activation and differentiation, showing when treating T-cells exogenously with a cell permeable S-2HG dose, effector cytokines as well as proliferation was reduced, suggesting that S-2HG is skewing T-cells towards a more memory like phenotype. In adoptive transfer experiments, the authors could show robust recall effects of S-2HG treated $CD8^+$ T-cells. In tumor xenograft models, pre-treated OT-1 $CD8^+$ controlled tumor growth *in vivo* more successfully compared to vehicle treated. Thus, it appears that hypoxia stabilized HIF-1α drives S-2HG accumulation in T-lymphocytes, furthermore, promotes a higher proportion of memory T-cells *in vivo*, potentially being of clinical value.

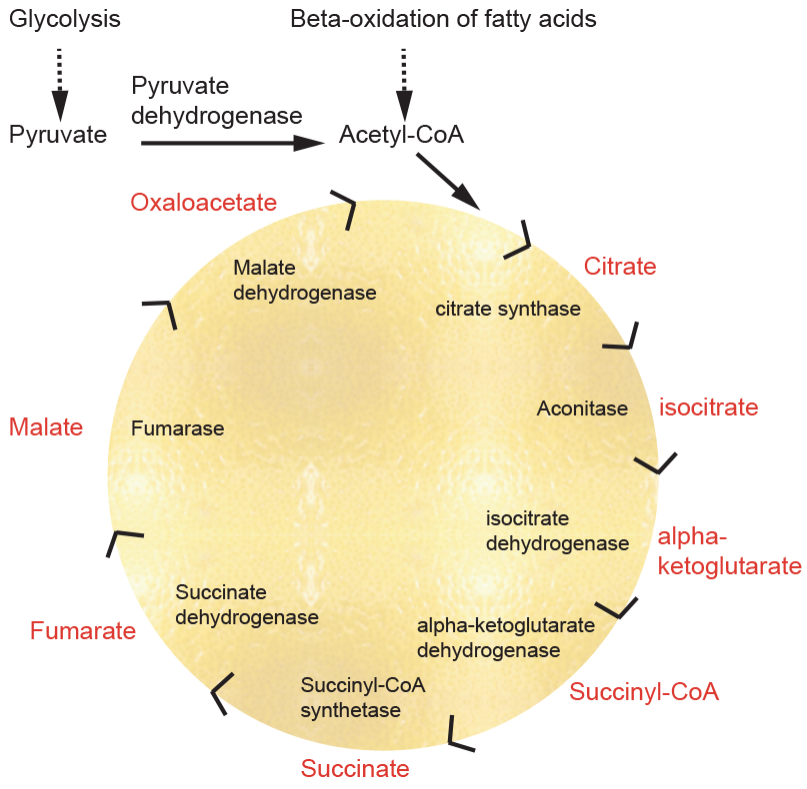


Fig 3. TCA cycle schematic. Krebs cycle, or TCA cycle schematic, illustrated here in red, are the products in each step of the cycle. In black, the corresponding enzymes responsible for the said product. 2-hydroxyglutaric acid (2HG) products, are produced in the steps between isocitrate dehydrogenase and alpha-ketoglutarate dehydrogenase.

2 METHODS

2.1 CHROMATIN IMMUNOPRECIPITATION (CHIP)

Chromatin immunoprecipitation (ChIP) was first described (Boyd and Farnham, 1999), many advances have been made to both antibody quality as well as the bead quality, which ultimately generate better binding maps. Further advances have been made coupling the power of ChIP to other platforms such as microarray and DNA-sequencing. The first study to couple ChIP to microarray was performed by Lieb.D et.al, (Lieb *et al.*, 2001), they studied the Rap1 telomere binding protein, and had a striking resolution of 2 kb binding maps. The second revolution was achieved when ChIP was coupled to DNA whole genome sequencing, (Barski *et al.*, 2007). These studies paved the way for current advances in molecular biology and beyond.
The current protocol used in Papers I-III are described in detail in the methods section, the conceptual methodology is as follows. Living cells are fixed with formaldehyde, generating protein-protein as well as DNA-protein cross-links. The chromatin is sheared through either sonication or enzyme digestion or both, to produce chromatin at variable sizes. Following chromatin shearing, immunoprecipitation with specific antibody targeting the fraction of DNA-protein crosslinked molecules is performed. Subsequently, protein A or protein A-G beads are added, which bind to the DNA-protein-antibody molecules. De-crosslinking of formaldehyde linkages frees up the DNA-protein-antibody-beads molecules, which are then eluted from this complex to generate the small fragments of DNA which should be specific towards the primary antibody used. The DNA is purified and compatible for further analysis, such as qPCR, microarray or sequencing.

2.2 SODIUM DODECYL SULPHATE – POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE or more commonly referred to as Western blot, was in its current form first described by U.K Laemmli in 1970. Western blot technique allows for separation of protein size of the lysate, through acrylamide gel electrophoresis. The SDS in the solution will linearize and together with a strong reducing agent such as β-mercaptoethanol, keep the proteins in the primary structure. SDS is also binding the linearized protein which produces an overall negative charge of the molecule. This together with the acrylamide gel and applied current, separates proteins by size only, ensuring that larger proteins are migrating slower and smaller proteins faster. After separation has completed, transfer of the proteins to a membrane (either PDFV or nitrocellulose) is performed. Subsequently the membrane is blocked with milk or commercially available blocking solutions, after blocking, the membrane is incubated with primary antibody solution targeting the protein of interest. Another wash step is performed and finally secondary antibody targeting the primary IgG isotype. Lastly, membrane development and data interpretation.

2.3 QUANTITATIVE REVERSE-TRANSCRIPTASE PCR

Polymerase chain reaction (PCR) is a very powerful technique which is widely used in many fields, from forensic science to determination of illnesses. The principle of PCR is based upon DNA polymerase needing to have a primer sequence to start its reaction, therefore, by designing primer sequences for the target genetic region of interest, one can determine if the gene is expressed, how much of the gene product is present or simply if the gene is present. Primer design is consequently very important, there are multiple tools available online for free as well as paid versions, that calculate the GC content, specificity and self-alignment characteristics of the primer sequences. GC content is an important factor because GC rich regions are notoriously harder to amplify and self-alignment of the primer pairs is also important which generates primer dimers which in turn lowers the specificity.

Quantitative reverse transcriptase PCR is performed by extracting the mRNA of the target cell or organ, performing complementary DNA (cDNA) synthesis and measuring the amount of mRNA present in the elute with a PCR reaction. The determination of mRNA is put into relation to a housekeeping gene which is known not to change its expression in the actual experiment, and the data is represented as fold change over housekeeping gene expression.

2.4 MICROCOCCAL NUCLEASE (MNASE) PROTECTION ASSAY

Micrococcal nuclease (MNase) enzyme digests non-histone bound DNA. MNase has been widely used to map enhancer regions, promoter regions and other active sites in the genome. The MNase protection assay is used to map HIFα responsive gene promoter auxiliary factor remodeling. Nuclear extracts from normoxia and hypoxia treated cells are either undigested or subjected to 15 minutes of MNase digestion in 37° C and stopped by adding SDS and EDTA solution for 5 minutes. The DNA is purified and RT-qPCR is performed. The data is then analyzed and represented as % protection of digestion, using the qPCR data from the undigested values and calculating using the undigested values as threshold.

3 AIMS

The aim of the research presented in this thesis, was to acquire a more in depth understanding of HIFα dependent processes, such as hypoxic epigenetic effects as well as, cell intrinsic effects of loss of HIFα.

- The role of hypoxia in cell epigenetic effects, in cancer cell models
- The loss of HIFα during $CD8^+$ cell proliferation and function
- HIF-1α downstream effects, such as accumulation of S-2HG, and its instrinsic effect on $CD8^+$ T-lymphocytes

4 RESULTS

4.1 PAPER I

It is has been widely observed that tumor hypoxia is an important hallmark of cancer progression (Nobre *et al.*, 2018). Although the mechanism(s) vary depending on cell of origin, hypoxia exerts its function nonetheless. Most solid tumors will outgrow their oxygen diffusion limits, hence slowly and gradually become more hypoxic, which are characterized usually with a malformed vasculature. Hypoxia is also not uniformly occurring throughout the tumor (Jiang *et al.*, 2012), giving rise to areas which are well vascularized, as well as, areas which are highly hypoxic. Overall, a hypoxic signature is correlated to lower disease free survival (Vaupel and Mayer, 2007).
In this present study, we focused our attention to the epigenetic effects of hypoxia, and the role HIF-1α during hypoxic insults. Established cancer cell line models, SK-N-Be(2)c and HepG2, neuroblastoma and hepatoma cancer cells respectively, two model cell lines widely used in the field. We incubated the cells at various time points of hypoxia and reoxygenation. Cell nuclei were harvested and micrococcal nuclease digestion was performed. DNA was purified and qPCR was performed, with primers targeting hypoxia regulated gene promoters.

We observed that HIF-1α and HIF-2α proteins were expressed and detectable under our hypoxia timepoints. We could see HIF-1α protein accumulation in SK-N-Be(2)c cells as early as 4 hours of hypoxia incubation at 1% oxygen, however, HIF-1α protein levels were less at later hypoxia time points, 24 and 48 hours respectively, with complete degradation of HIF-1α at our reoxygenation timepoints 4 and 24 hours. Conversely, HIF-2α protein levels were increased gradually from 4 hours of hypoxia and maintained stability until 48 hours of hypoxia. In HepG2 cells, HIF-1α protein was stably visible from the early 4 hour timepoint of hypoxia, with strong HIF-1α protein band even after 4 hours of reoxygenation. mRNA expression of target genes, CA9, PGK1, PFKFB4, GYS1, P4HA2, KDM3A, PDK2, TMEM45A, and BNIP3, showed hypoxia dependent increases in mRNA relative to housekeeping gene β-actin.

We continued the analysis, by performing ChIP-qPCR following hypoxia incubations, using HIF-1α and HIF-2α specific antibodies. We could detect HIF-1α and HIF-2α protein binding to HRE sequences in CA9, PGK1, PFKFB4, and PDK2 in both cell lines. The majority of HIFα protein was bound during our hypoxia incubations, with sharp decline in association with the HRE´s at our reoxygenation timepoint of 1 hour at 21% oxygen.

We used DNase 1 hypersensitivity and ChIP-qPCR, using antibodies for histone 3 protein and, histone 3 lysine 9 acetylation. As our DNase-seq illustrates, the peaks were concentrated on the transcription start sites (TSS) of CA9, PGK1, PFKFB4, GYS1, P4HA2, KDM3A, PDK2, TMEM45A, and BNIP3, with GYS1 and KDM3A, having peaks over their respective HRE sequences. Our histone 3 lysine 9 acetylation ChIP-qPCR shows hypoxia dependent increases in hypoxia timepoints 4 and 24 hours at the CA9 HRE/TSS, and PGK1 TSS locus respectively. With decreasing amounts of histone 3 lysine 9 acetylation being detected at reoxygenation timepoints 4 and 24 hours.

We observed chromatin organization differences in target gene promoters of hypoxia regulated genes, our micrococcal nuclease coupled to qPCR (MNase) assay allowed us to carefully distinguish between two types of chromatin conformations. Nucleosome organization differences were divided into inducible NFRs (iNFRs) and constitutive NFRs (cNFRs). iNFRs were observed for target genes such as CA9, whereas, cNFRs were observed for PGK1, PDK2 and PFKFB4. Our reoxygenation time points, 4 and 24 hours post 24 hours of hypoxia, enabled us to observe a pattern of reversibility. Through siRNA technology, targeting SIN3A, an adaptor protein present in several chromatin remodeling complexes, we could observe a necessity for SIN3A in the reestablishment of chromatin structure.

4.2 PAPER II

Cytotoxic T-lymphocytes or CTLs, are a major part of the adaptive immune response. They infiltrate tumors, clear pathogens, and mediate the cytolytic effects of the T-cell lineage. The HIFα transcription factors are upregulated in CTLs during low oxygen tensions, as well as, thought to aid CTLs when encountering hypoxic niches. To explore the differences between the main two HIFα isoforms role in T-lymphocyte biology and function, we employed a conditional knockout technique, using the distal lymphocyte protein tyrosine kinase gene (Lck) promoter (dLck) LoxP CRE system. Deleting exon2 from *Hif1a* and *Epas* alleles, generating ablated HIF-1α and HIF-2α proteins in $CD8^+$ T-cells.

Our characterization of the two main HIFα isoforms was initiated in wild type, splenic extracted $CD8^+$ T-cells, showing activation and time dependent mRNA expression of HIF-1α and HIF-2α relative mRNA compared to unstimulated T-cells. With peak levels at 72 hours post *ex vivo* αCD3ε/αCD28 stimulus. We detect HIF-1α, and HIF-2α protein levels during our activation time points, with HIF-1α protein stability being highest at 24 hours post activation, while, HIF-2α maintains a stable protein level throughout our activation time.

Deletion efficiency was almost 100% in the HIF-$1\alpha^{fl/fl}$ $dLck^{CRE}$ and HIF-$2\alpha^{fl/fl}$ $dLck^{CRE}$ T-cells, measured through genomic DNA PCR, and protein quantification of nuclear extracts. Proliferation was not affected by either HIFα isoform deletion. However, HIF-1α target gene expression of glucose genes, Hk2, Pdk1, Mct4, and Pgk were significantly reduced in 1% oxygen, in HIF-$1\alpha^{fl/fl}$ $dLck^{CRE}$ T-cells. Furthermore, extracellular flux, lactate, and glucose uptake were impaired in HIF-$1\alpha^{fl/fl}$ $dLck^{CRE}$ cells, which were not affected in HIF-$2\alpha^{fl/fl}$ $dLck^{CRE}$ T-cells.

Next, we determined how hypoxia, HIF-1α and HIF-2α impact T-cell effector differentiation. Properly activated and differentiated effector $CD8^+$ T-cells downregulate CD62L, a chemokine receptor targeting T-cells to secondary lymphoid tissues. HIF-1α deficient, not HIF-2α deficient, effector $CD8^+$ T-cells failed to downregulate CD62L. Additionally, T-cells lacking HIF-1α, decreased the production of effector cytokine interferon gamma (IFN_γ), and tumor necrosis factor alpha (TNFα). However, hypoxia treatment increased the production of granzyme B (GzmB) and activation related costimulatory molecules CD137, OX40, and GITR. Hypoxia also increased the production of checkpoint receptors PD-1, TIM3, and LAG3. The production of these molecules was HIF-1α dependent, and not HIF-2α, with HIF-2α deficient T-cells, displaying almost identical levels to wild type littermates. Lastly, we observed activation dependent and hypoxia dependent VEGF-A production, which was also, HIF-1α dependent. Thus, hypoxia, and HIF-1α, increase the production of activation related molecules, and hypoxia target genes, such as VEGF-A.

In line with the aforementioned, improper activation phenotype observed in the HIF-1α deficient T-cells, we sought to identify *in vivo* specific effects of these observations. To this end, we subcutaneously injected Lewis lung carcinoma (LLC), and B16-F10, melanoma cells into mice lacking specifically, HIF-1α or HIF-2α in T-lymphocytes (HIF-$1\alpha^{fl/fl}$ $dLck^{CRE}$ and HIF-$2\alpha^{fl/fl}$ $dLck^{CRE}$). We compared tumor growth to wild type mice, and the corresponding loxP allelic mice (HIF-$1\alpha^{fl/fl}$ and HIF-$2\alpha^{fl/fl}$). HIF-1α deficient T-cells, showed increased tumor volume, and weight, compared to HIF-$1\alpha^{fl/fl}$ animals. HIF-2α deficient T-cells, showed similar tumor volume and weight,

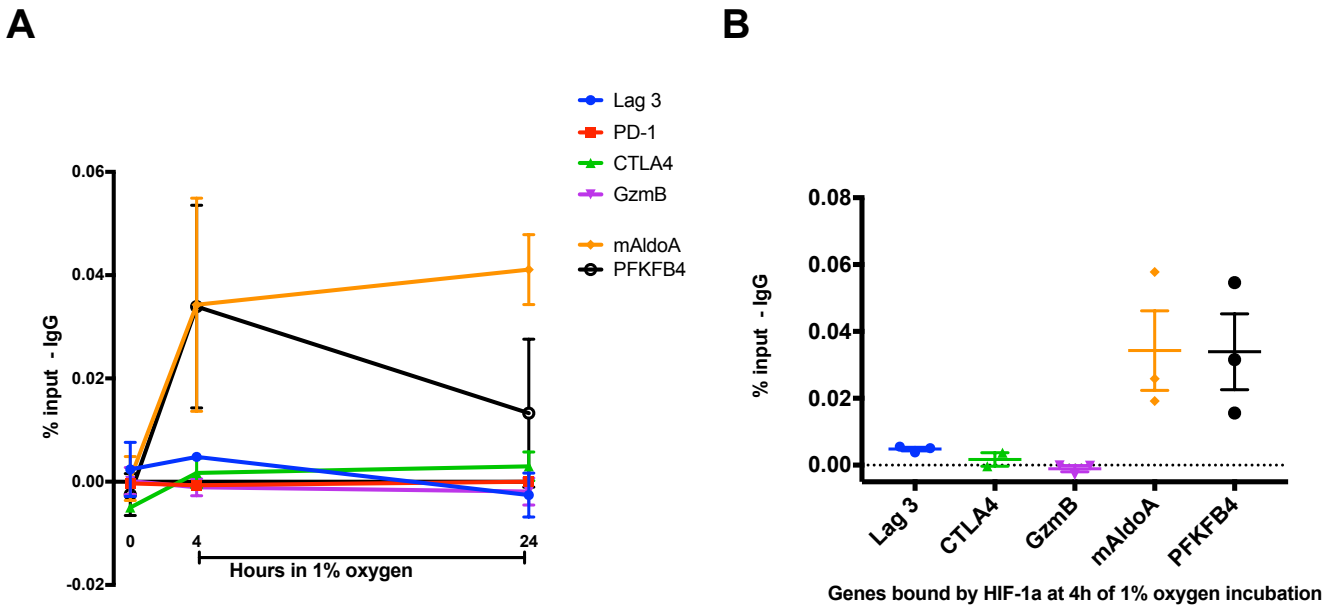
compared to HIF-2α$^{fl/fl}$ animals. When analyzing the proportion of tumor-infiltrated CD8 T-cells to CD4 T-cells, we could observe a skewing in the HIF-1α deficient T-cells, showing less CD8$^+$ infiltration in the tumors. Correspondingly, lower levels of co-stimulatory markers CD137 and GITR, expression was also observed in the tumor-infiltrated T-cells, with lower expression of checkpoint receptors PD-1, LAG3, and TIM3. The endothelial nature of the subcutaneous tumors, and the notion that HIF-1α deficient T-cells have lower tumor-infiltration capacity, we employed an *in vitro* co-culture experiment, with Boyden chamber plates. We cultured early passage of pulmonary endothelial cells, with either, HIF-1α deficient T-cells or wild type control T-cells, in hypoxic conditions. We observed, approximately 50% less migration in HIF-1α deficient T-cells, in hypoxia 1% oxygen, after 24 hours, compared to wild type control cells. Lastly, we injected mice with fluorescence-conjugated tomato lectin, in order to assess tumor vascularization, in mice bearing LLC tumor. Mutant T-cell, tumor bearing mice, showed vascular segment length differences, and altered vessel straightness compared to wild type mice. The *in vivo* results point therefore, to a pivotal role of intact HIF-1α in CD8$^+$ T-cells, to properly control tumor size, weight, and tumor-infiltration.

Immunotherapy, is beginning to emerge as a viable option for certain tumors, in clinical settings. We therefore, explored if HIF-1α was necessary for the acquisition of effector function. By using the established ovalbumin recognizing TCR receptor expressing transgenic mouse, OT-1 mouse. We generated HIF-1α conditional knockout OT-1 CD8$^+$ T-cells, with and without dLck^{CRE} expression. CD8$^+$ T-cells were then activated by addition of SINFEKL, the cognate TCR ligand in the OT-1 system. As with previous CD8$^+$ T-cells lacking HIF-1α, the OT-1 HIF-1α knockout T-cells, were able to proliferate and survive during antigen stimulation, although, CD62L downregulation was defective with similar phenotype to non-OT-1 CD8$^+$ T-cells lacking HIF-1α. OT-1 mutant T-cells, showed altered expression of TNFα, GzmB, and IFN$_\gamma$ after re-stimulation. We then went on to perform adoptive transfer experiments, to assess how HIF-1α deficient OT-1 cells, migrate *in vivo*. By co-transferring control CD45.1$^+$ and mutant CD45.1$^+$ TdTomato$^+$ CD8$^+$ T-cells in a 1:1 ratio, into CD45.2$^+$ host mice, challenged with B16-OVA tumors. Subsequently, determining the relative amount of each genotype, migrating to the lymph node, spleens, and tumors 48 hours after adoptive transfer. We could observe, that HIF-1α deficient OT-1 cells, migrated more to the lymph node, with less migration to the B16-OVA tumors. OT-1 HIF-1α$^{fl/fl}$ T-cells managed to control tumor growth, compared to OT-1 HIF-1α dLck^{CRE} mice, which also manifested as decreased survival of the mice. Lastly, we used a colon cancer cell line, MC38, which we injected into HIF-1 dLck^{CRE} and HIF-1α$^{fl/f}$ mice and subjected them to combinatorial antibody-based immunotherapy by administering αCTLA4 and αPD-1 antibodies. HIF-1 dLck^{CRE} mice tumors grew larger, compared to HIF-1α$^{fl/f}$ mice. The combinatorial therapy controlled the tumor volume in HIF-1α$^{fl/f}$ animals.

Our findings showed altered tumor vascularization in HIF-1α dLck^{CRE} mice bearing LLC tumors, which, had lower levels of VEGF-A expression. We sought to gain further insight into the role VEGF-A plays in tumorigenesis, expressed from the T-lymphocyte lineage. We generated VEGF$^{fl/fl}$ dLck^{CRE} CD8$^+$ T-cells, as well as OT-1 VEGF-A deficient T-cells. The VEGF$^{fl/fl}$ dLck^{CRE} CD8$^+$ T-cells were cultured in 21% oxygen and 1% oxygen respectively, to assess if VEGF-A affects T-cells more generally. The mutant T-cells were responding in similar fashion in regards to, survival, glycolytic metabolism, and expression of cytolytic and costimulatory/checkpoint receptors relative to wild type counterpart cells. OT-1

VEGF-A deficient T-cells, expressed similar levels of CD62L, and CD44, as their wild type counterpart. However, with a small reduction in expression of costimulatory molecules, which did not impact the OT-1 VEGF-A deficient T-cells in an *in vitro* tumor killing assay, in which tumor cells expressed ovalbumin, their cognate TCR recognition motif. We adoptively transferred OT-1 $VEGF^{fl/fl}$ $dLck^{CRE}$ or wildtype OT-1 T-cells, to mice harboring B16-OVA tumors, the resulting tumor volume was not significantly changed between wildtype and $VEGF^{fl/fl}$ $dLck^{CRE}$ OT-1 cells, consequently, the survival of the mice was overlapping. We injected LLC subcutaneously into $VEGF^{fl/fl}$ $dLck^{CRE}$ mice, $VEGF^{fl/fl}$ mice, and wildtype C57/BL6. The growth of the tumors, were similar for $VEGF^{fl/fl}$ and wildtype animals, however, $VEGF^{fl/fl}$ $dLck^{CRE}$ tumor bearing mice, had an increase tumor volume and weight. Analysis of the tumor-infiltrated lymphocytes, illustrated less $CD8^+$ T-cell migration into the LLC tumors, in $VEGF^{fl/fl}$ $dLck^{CRE}$ mice. Similar results were obtained with HIF-1α deficient T-cells, in regards to infiltration capabilities, however, PD-1 expression was not affected in the VEGF-A deficient T-cells, as was in the HIF-1α deficient T-cells. The $VEGF^{fl/fl}$ $dLck^{CRE}$ mice LLC tumors, showed some signs of vessel normalization, which included, increased perfusion, segment length, and decreased vessel stiffness. Interestingly, we observed less hypoxia in these tumors, by Piminidazole staining, coupled with less *Vegfa* mRNA from tumor lysates. With the signs of normalization of vasculature, we observed in the $VEGF^{fl/fl}$ $dLck^{CRE}$ mice harboring tumors, we administered 3 doses of cyclophosphamide and vehicle control to $VEGF^{fl/fl}$ $dLck^{CRE}$ mice and $VEGF^{fl/fl}$ mice harboring LLC tumors. Vehicle control tumors displayed similar pattern as $VEGF^{fl/fl}$ $dLck^{CRE}$ mice having larger tumors, compared to $VEGF^{fl/fl}$ mice, however, the cyclophosphamide group, showed smaller tumors in the $VEGF^{fl/fl}$ $dLck^{CRE}$ mice. Lastly, to test the role of VEGF-A in spontaneous models of breast cancer, we used the MMTV-PyMT model of spontaneous adenocarcinomas, backcrossed to our $VEGF^{fl/fl}$ and $VEGF^{fl/fl}$ $dLck^{CRE}$ mice. We did not observe any difference in tumor latency, however, at 17 weeks past, tumor weight is significantly increased in $VEGF^{fl/fl}$ $dLck^{CRE}$ mice compared to $VEGF^{fl/fl}$ mice. With a more advanced tumor form acquired in the $VEGF^{fl/fl}$ $dLck^{CRE}$ mice, scored histologically.

Finally, considering the striking observations in $CD8^+$ T-cell effector differentiation loss, and costimulatory/checkpoint dysregulation, in HIF-1α deficient T-cells, we sought to understand if HIF-1α was directly binding to $CD8^+$ genes which are important for these functions. To this end, we performed ChIP-qPCR analysis on wildtype mice, with HIF-1α specific antibody, on $CD8^+$ T-cells cultured at 4 and 24 hours of 1% oxygen, with corresponding 21% oxygen control. As can be seen in Paper II Figure 1, HIF-1α was able to bind mAldoA and PFKB4 canonical target genes during hypoxia 1% oxygen, however Lag3, PD-1, CTLA4, and GzmB were not bound by HIF-1α at the putative HREs.



Paper II Figure 1. ChIP-qPCR analysis in CD8$^+$ T-lymphocytes. (A) ChIP-qPCR analysis of putative target gene promoters containing at least one HRE sequence. Cells were incubated at (0) normoxia, (4) 4h of hypoxia and (24) 24 hours of hypoxia. mAldoA and PFKFB4 were used as positive control. (B) ChIP-qPCR analysis at the 4-hour, 1% oxygen timepoint. n=3 mice.

4.3 PAPER III (MANUSCRIPT)

When treating $CD8^+$ T-cells, exogenously with S-2HG (300μM) for 3 days, numerous phenotypical observations can be made. Cell size, as well as cell number are affected by this treatment, compared to vehicle (H_20) treated cells. The cell proliferation effects of S-2HG, during 3-day activation, are seemingly striking, S-2HG seems to inhibit cell proliferation with a total amount of cells, after 3-day activation being almost 50% less then vehicle treated cells. We know from previous findings that, 2-Hydroxyglutaric acids, both R and S enantiomers, will competitively bind to 2-oxoglutarate dependent enzymes. We therefore, focused our search for epigenetic differences, between vehicle, and S-2HG treated $CD8^+$ T-cells. After 3-days of activation, T-cells were harvested for histone proteins, naïve cells were harvested day 0. Through SDS-PAGE analysis, of histone extracted $CD8^+$ T-cell lysates, we could observe a striking acetylation decrease in the S-2HG treated cells. The relative amount of H3K9Ac, observed between naïve cells, vehicle treated activated cells, and S-2HG, activated cells, was highest in the vehicle activated group. This lead us to test if, H3K27Ac, was equally affected by S-2HG. To this end, we performed SDS-PAGE analysis on the three aforementioned groups, with H3K27Ac antibody. Although the vehicle activated group was highest in the signal of H3K27Ac, these experiments were not as consistent as with the H3K9Ac antibody. We used several approaches, in order to recapitulate the observed effects of S-2HG on H3K9/27Ac. With readily available histone acetyltransferase inhibitors, we treated $CD8^+$ T-cells with p300 inhibitor Bix as well as Gcn5 inhibitor CPTH2. Bix treatment proved to be toxic for T-cells, irrespective of concentration used, however, CPTH2 was well tolerated, with a final concentration of 20μM administered during 3-day activation. Our SDS-PAGE analysis, of CPTH2, S-2HG and vehicle treated cells, with H3K9Ac antibody, showed that S-2HG treatment, yet again, had lowest amount of H3K9Ac, CPTH2 HAT inhibitor, could not recapitulate the observed effects of S-2HG alone and had no effects on H3K27Ac levels.

Histone acetylation is enzymatically driven, as well, the removal of lysine-acetylations, with HDAC enzymes. We treated our cells with TSA (3nM), an HDAC inhibitor, with and without S-2HG, as well as vehicle treated group. The subsequent SDS-PAGE analysis, demonstrated potent H3K9Ac level increases in the TSA treated group, however, TSA in combination with S-2HG, showed similar H3K9Ac as vehicle treated group. Possibly linking deacetylation as a potential mechanism for how S-2HG lowers H3K9Ac in T-cells.

We continued our focus on H3K9Ac, by performing ChIP-qPCR and ChIP-seq analysis. The ChIP-qPCR analysis, demonstrated how several genes, showed differential H3K9Ac levels during 3-day activation in the presence of S-2HG, compared to vehicle and naïve cells. Genes such as, CD44, PD-1 and CD127 showed differential H3K9Ac levels, in their proximal promoters, having highest enrichment in vehicle treated cells. Naïve and S-2HG treated cells, had comparable amounts of H3K9Ac in their promoters, albeit, with naïve cells showing slightly more enrichment of H3K9Ac. Other genes tested, such as Eomes, TCF7 and IL-2, showed no difference in promoter H3K9Ac between the three groups, in our ChIP-qPCR analysis. Our ChIP-seq analysis, was performed by Bioinformatics and Expression Analysis (BEA) core facility, at KI campus Huddinge. Our ChIP-seq analysis, demonstrates how vehicle activated cells, compared to S-2HG treated cells, have globally higher levels of H3K9Ac, across the genome. The peak density plots, show

how the distribution of peaks are similar between the two groups, with increases in H3K9Ac roughly 2Kb upstream of the transcription start sites (TSS), with the highest observed peaks being in the gene bodies.

5 DISCUSSION

5.1 PAPER I

Our study highlights some important features of stress inducible gene transcription. Severe hypoxia (1% O_2), is one of the many ways cells can experience stress. During hypoxia, HIFα protein stabilization occurs, with concomitant gene up-regulation. However, our focus was on promoter regions of HIFα target genes. Many transcription factors require the nucleosomes to be removed or displaced upon *cis*-regulation, HIFα proteins are necessary for the remodeling to occur at iNRFs in hypoxia regulated gene promoters. Thus, in our study hypoxia acts as a stress inducible model system, and the corresponding epigenetic effects observed herein, are likely to be applicable to similar models.

The important observation of reversibility, mediated through SIN3A protein, adds elegance to the model presented forth. HIF-1α binds to target gene promoters, remodels the chromatin, and induces gene transcription. Through recruitment of SIN3A and its corresponding complex, either HDACs or NuRD, (McDonel, Costello and Hendrich, 2009; Clark *et al.*, 2015), manages to mediate the reestablishment. This elegant mechanism of requiring HIF-1α for the initiation of remodeling and transcription, with the necessity of SIN3A and corresponding complexes for restoration of the chromatin landscape, enables versatility, and co-dependence of multiple pathways for proper chromatin management.

Lastly, cell differentiation is linked to the inherent epigenetic state of each cell. One way of rationalizing this phenomenon, is to assume that the epigenetic states are “fixed” in differentiated cells. However, through the pioneering work of Yamanaka and colleagues, we now know, that epigenetic states can be reversed, albeit, through overexpression of specific “Yamanaka” factors. The reversibility of cell types in normal physiology, is likely to be low, notwithstanding, stem cell niches in the gut and the hematopoietic system. Cell type specific epigenetic effects can be observed, for instance, nucleosome positioning in an enhancer element of the *CSF2* gene, which is expressed in mast cells and T-lymphocytes, was shown to recruit specific transcription factors in different cell types (Bert *et al.*, 2007). In undifferentiated T-cells, the enhancer element was induced by NFAT/AP-1 transcription factor complexes, that displaced nucleosomes downstream of the TSS. In myeloid cells, the enhancer was remodeled by the GATA-1 transcription factor, however, GATA-1 remodeled nucleosomes which were located upstream of the TSS of CSF2 gene (Bert *et al.*, 2007). This illustrates how, conserved genetic elements such as enhancers, can be utilized between different cell types for their specific needs.

5.2 PAPER II

To better understand and combat, tumor progression, we need to characterize the interactions that occur between the different cell types within a given tumor. This will ultimately lead to better cancer therapies, and better patient overall survival. Although many cell types have been shown to express HIF-1α, the role of HIFα in T-lymphocyte differentiation and activation, was lacking. We therefore tried to examine the role of each HIF isoform in T-lymphocyte biology. We show that $CD8^+$ T-cells express both of the HIFα transcripts and proteins after αCD3ε/αCD28 *ex vivo* TCR activation. Furthermore, we observe HIFα target gene up-regulation, such as VEGF-A, which had the capability to modulate tumorigenesis. Our previous findings (Doedens *et al.*, 2013) examined the consequences of loss of VHL in T-cells. With increased HIF-1α expression driving effector differentiation phenotype accompanied with enhanced viral clearance, with an exacerbated immune response (Doedens *et al.*, 2013). Other components in the HIFα pathway have been deleted in T-lymphocytes, such as the PHD enzymes, responsible for hydroxylation and degradation of the HIFs (Clever *et al.*, 2016). The authors used $CD4^{CRE}$ to ablate the three PHD proteins in CD4, CD8, and NKT T cells, generating triple KO T-lymphocytes. They observed increased IFN_γ expression from the $CD8^+$ T-cells, accompanying increased frequency of effector $CD8^+$ T-cell differentiation (Clever *et al.*, 2016), which reflects our findings, in which HIF-1α is important for effector differentiation of $CD8^+$ T-cells.

The effector differentiation state of $CD8^+$ T-cells is accompanied by a glycolytic switch, generating ATP from oxidative and fatty acid metabolism, to glycolysis being the predominant form of energy production (Pearce *et al.*, 2009). Our HIF-1α deficient T-cells, are impaired in extracellular flux, lactate, and glucose uptake in hypoxia conditions, with enzymes such as Hk2 and PDK1 being significantly reduced at hypoxia 1% oxygen. Glucose metabolism has been shown to be an important mediator and signaling mechanism for $CD8^+$ T-cells (Chang *et al.*, 2013), were the authors showed how GAPDH, generated in part through glycolysis, is necessary for IFN_γ production in T-cells. Our HIF-1α deficient T-cells showed reduced expression of IFN_γ during 1% hypoxia. Furthermore, we show loss of HIF-1α, but not HIF-2α, results in reduced expression of several cytolytic, and costimulatory molecules during hypoxia 1% oxygen. In our xenograft experiments, HIF-1α deficient T-cells have increased tumors, probably due to, our defective cytokine and costimulatory effector profile being impaired. Thus, HIF-1α is necessary for proper $CD8^+$ T-cell effector differentiation, with *in vivo* tumor killing capabilities being lessened, proving how cell type specific changes, modulate tumor progression.
The effector molecules classically produced by CTLs such as TNFα, IFN_γ and GmzB, were reduced in HIF-1α deficient T-cells, costimulatory and checkpoint molecules CD137, OX40, and GITR, and TIM3, CTLA-4, PD-1, and LAG-3, respectively, are also dependent upon HIF-1α in T-cells. Our previous findings could show PD-1 downregulation was VHL regulated, but not ablated in the VHL/HIF-1α/ HIF-2α triple knockout cells (Doedens *et al.*, 2013), however, in our HIF-1α deficient, tumor-infiltrated cells, PD-1 levels were lower, compared to control, this could reflect model specific differences, such as inflammatory milieu between chronic viral load, and tumor engraftment. PD-1 regulation could also be hypoxia dependent, but not HIF-1α dependent, as our ChIP-qPCR of wildtype mice could not detect any HIF-1α binding to the putative HRE of the PD-1 promoter.

There is a correlation between robust lymphocyte infiltration, and patient survival in a multitude of cancers, documented in melanoma, ovarian, head and neck cancer, breast, and colorectal (Galon *et al.*, 2013). As we could observe, in our HIF-1α deficient T-cells, the tumor infiltration of $CD8^+$ T-cells, was less compared to wildtype cells, accompanying lower PD-1 expression as well. Importantly, PD-1 levels in tumor-infiltrating T-cells, are associated with tumor reactive T-cell subsets (Gros *et al.*, 2014). Furthermore, our HIF-1α deficient T-cells were impaired in their migration over endothelial barriers, which could possibly explain the low infiltration observed inside tumors.

Immunotherapies are an emerging and exciting new field of cancer treatment, with the first PD-1L1 monoclonal antibody used in cancer therapeutics, as recently as 2012 (Brahmer *et al.*, 2012), with CTLA-4 monoclonal antibody therapy preceding only two years earlier (Hodi *et al.*, 2010). We employed immunotherapy techniques to observe if HIF-1α was necessary for effector function. HIF-1α deficient T-cells seemed unaffected by the combinatorial immunotherapy, conversely, the wildtype cells responded to αCTLA-4/αPD-1 antibodies having smaller tumors. Thus, HIF-1α seems able to modulate immunotherapy-based modalities, which might be of clinical importance.

As HIF-1α target genes are plentiful, one important and hallmark gene is VEGF-A. Our observations that VEGF-A is highly expressed by $CD8^+$ T-cells during activation and was HIF-1α dependent, made us interested in the contribution of T-lymphocyte expressing VEGF-A during tumor progression. Our VEGF-A deficient T-cell mice, grew larger tumors, compared to control. The VEGF-A deficient T-cells activated properly, so we rule out cell-intrinsic effects in regards to overall T-cell biology. Furthermore, we also observed that VEGF-A deficient T-cells has less tumor infiltration, one important finding in regards to VEGF-A role in regulating tumor/endothelial cell barriers, and plays a role in the interactions between immune and vascular cells, stimulating recruitment (Melder *et al.*, 1996; Detmar *et al.*, 1998). The many effects of VEGF-A in these complex environments illustrates how important a factor it is, in regards to cell intrinsic loss, as well as, secretion from surrounding tumor microenvironment. VEGF-A deficient T-cells also seemed to normalize tumor vasculature, by increasing perfusion and oxygenation, with pericyte coverage also being increased on the vessels. Although, tumor vasculature is a hallmark of solid tumors, normalization of the vasculature is not always correlated to increases in tumor size (Carmeliet and Jain, 2011; Claesson-Welsh and Welsh, 2013). Tumor vasculature is known to play important roles in regards to drug delivery and chemotherapy, as well as the oxygen status of the cells and surrounding tissues (Goel, Wong and Jain, 2012). In our VEGF-A deficient T-cells, chemotherapy administration, and probably delivery to the tumor, is more successful, in comparison to wildtype. The normalization of tumor vasculature, with increased perfusion and oxygen, likely contributes to these effects. In this complex tumor microenvironment, consisting of several other immunological components, macrophages have been shown to secrete VEGF-A, and with VEGF-A deletion in macrophages, susceptibility to chemotherapeutic cytotoxicity was also observed (Stockmann *et al.*, 2008). Collectively, our findings demonstrate the importance of HIF-1α in the regulation of T-cell effector responses in the tumor microenvironment. Moreover, HIF-1α regulation of VEGF-A has functional consequences for effector $CD8^+$ T-cells in infiltration, vascularization, and progression.

5.3 PAPER III (MANUSCRIPT)

Today, we still lack an understanding of how 2-hydroxyglutaric acids (2-HG) affects the epigenome. 2-hydroxyglutaric acids have two enantiomers, R and S, and thus far, almost all studies have been focusing on the R-form of 2-HG, showing how R-2HG is upregulated upon $IDH1^{132}$ mutations (Dang *et al.*, 2009). Further studies have implicated R and S-2HG in being histone lysine demethylase inhibitors (Chowdhury *et al.*, 2011), JMJD2A and JMJD2C, albeit, at different potencies. Furthermore, 2-oxoglutarate dependent enzymes, are affected by increased levels of R/S-2HG (Xu *et al.*, 2011), by competitively binding to the 2-OG pocket and inhibiting the enzyme(s).

The unsuspected finding, that S-2HG, decreases the overall acetylation level of histone N-terminal tails, is, previously, not known. Acetylation of histone proteins, neutralizes the overall positive charge of histone proteins, and by doing so, it loosens the interaction between DNA and the NCP (Verdone, Caserta and Mauro, 2005). This is believed to increase the access of transcription factors, and other chromatin binding proteins, to DNA (Castillo, López-Rodas and Franco, 2017). Hence, rendering an unattainable *cis* regulatory sequence, suddenly more accessible. Thus, these data imply that the HIF-1α/S-2HG axis induces changes in gene expression by lowering acetylation levels. It has previously been shown that differential H3K9Ac levels in $CD8^+$ T-cells control the Eomes locus transcriptional output (Araki *et al.*, 2008). In the future, it would be interesting to combine our ChIP-seq analysis with gene-expression analysis, such as RNA-sequencing analysis, to identify acetylation-regulated genes important for T-cell effector regulation.

Previously it has been shown that S-2HG restrains cell expansion after 3 days of activation (Tyrakis *et al.*, 2016) in accordance to our findings. The IL-7 receptor (CD127) has been shown to promote proliferation in virus specific $CD8^+$ T-cells (Cellerai *et al.*, 2010). We find that this gene had less acetylation during our S-2HG treatment compared to our vehicle control treated cells. This implies that at least some part of the suppression of proliferation may be driven through promoter acetylation of CD127.

T-cell proliferation is controlled through multiple mechanisms and one important regulator of this is the PD-1 receptor and its cognate ligand PD-1L. S-2HG treated $CD8^+$ T-cells express less PD-1 after 7 days of 500μM of S-2HG treatment (Tyrakis *et al.*, 2016). We observe less promoter acetylation at the PD-1 promoter in 300μM S-2HG treated cells after 3 days of activation. This might indicate that acetylation is one mechanism of control over PD-1 expression in $CD8^+$ T-cells.

Acetylation and deacetylation of histone proteins are mediated through enzymatic reactions. We treated our $CD8^+$ T-cell with TSA, which is a potent inhibitor of histone deacetylation, leading to an overall increase in H3K9Ac. Also under these conditions, S-2HG robustly decreased H3K9Ac.

Several markers are associated with long term memory formation of T-cells, of which CD44 and CD62L seem to predict memory phenotypes, either T_{EM} ($CD62L^{low}$ $CD127^{hi}$ $KLRG1^{hi}$ $CCR7^{low}$) or T_{CM} ($CD62L^{hi}$ $CD44^{hi}$ $CD127^{hi}$ $CCR7^{hi}$ $KLRG1_{low}$) (Lazarevic, Glimcher and Lord, 2013). In our previous study, we could observe how S-2HG treatment increases the persistence and proportion of $CD44^{hi}$ T_{cm} memory cells after adoptive transfer experiments (Tyrakis *et al.*, 2016). Our results however

show that the CD44 promoter after 3 days of activation in the presence of S-2HG is less acetylated, indicating that CD44 expression has decreased. The reasons for this potential discrepancy could be plentiful. Although acetylation is a good predictor of transcription, it might not be the sole determinant and thus CD44 expression might not be controlled through H3K9Ac. Moreover, the acetylation analysis of CD44 is performed at 3 days post activation whereas the adoptive transfer experiments in (Tyrakis *et al.*, 2016) persisted for 30 days. Our *ex vivo* analysis is therefore difficult to compare with the *in vivo* experimental results. Careful mRNA analysis of the *CD44* gene during the course of T-cell activation with and without S-2HG treatment is therefore warranted.

Collectively, our results show how acetylation is affected through S-2HG treatment of $CD8^+$ T-cells, and thus provide a novel insight and potential modularity of effector differentiation through the addition of S-2HG during these processes.

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