DNMT3a epigenetic program regulates the HIF-2alpha oxygen sensing machinery

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In loving memory of Marthe V. Boily and Claire Carrière.

ABSTRACT

Epigenetic regulation of gene expression by DNA methylation plays a central role in the maintenance of cellular homeostasis. Here we present evidence implicating the DNA methylation program in the regulation of hypoxia-inducible factor (HIF) oxygen-sensing machinery. We show that DNA methyltransferase 3a (DNMT3a) methylates and silences the HIF-2α gene (*EPAS1*) in normal cells. Epigenetic silencing of *EPAS1* prevents activation of the HIF-2α gene program associated with hypoxic cell growth, thereby limiting the proliferative capacity of cells under low oxygen tension. Naturally occurring defects in DNMT3a, observed in primary tumours and malignant cells, cause the unscheduled activation of *EPAS1* in early dysplastic foci. This enables incipient cancer cells to exploit the HIF-2α pathway in the hypoxic tumour microenvironment, which is necessary for the formation of cellular masses larger than the oxygen diffusion limit. Reintroduction of DNMT3a in DNMT3a-defective cells restores *EPAS1* epigenetic silencing, prevents hypoxic cell growth, and suppresses tumour growth *in vivo*. In addition, restoring HIF-2α expression in DNMT3a-reintroduced cancer cells restores full tumorigenic potential, including the capacity to traverse the hypoxic barrier. These data support a tumour-suppressive role for DNMT3a as an epigenetic regulator of the HIF-2α oxygen-sensing pathway and the cellular response to hypoxia.

AUTHORIZATIONS

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LIST OF ABBREVIATIONS

36B4 : Ribosomal Protein, Large, P0
5-aza : 5-Azacytidine

aKG : Alpha-Ketoglutarate
AIDS : Acquired Immunodeficiency Syndrome
AML : Acute Myeloid Leukemia
AMS : Acute Mountain Sickness
APC : Adenomatous Polyposis Coli
ARNT : Aryl-Hydrocarbon Receptor Nuclear Translocator
ATCC : American Type Culture Collection

BAP1 : early onset (BRCA1) associated protein-1
BHLHE41 : basic helix-loop-helix family member e41
Bnip3 : autophagy BCL2/adenovirus E1B 19- KDa interacting protein 3

BORIS : CCCTC-binding factor (zinc finger protein)-like
BRCA : Breast cancer 1, early onset
BrdU : Bromodeoxyuridine
Brg1 : Brahma related gene 1

CAIX : Carbonic anhydrase 9
ChIP : Chromatin Immunoprecipitation
CTCF : CCCTC- binding factor
CTCFL: CCCTC-binding factor (zinc finger protein)-like

DCL : Donor Cell Leukemia
DMEM : Dulbecco's modified Eagle's medium
DNMT1 :DNA methyltransferase 1
DNMT3a : DNA methyltransferase 3a
DNMT3b : DNA methyltransferase 3b
DNMT3L : DNA methyltransferase 3L
DNMTase : DNA methyltransferase
DMOG : Dimethyloxaloylglycine

E2FF : E2F transcription factor-like
EGFR : Epidermal Growth Factor Receptor
EGFRVIII : Epidermal Growth Factor Receptor variant 3
EGR : Early Growth Response
eIF4A : Eukaryotic translation initiation factor 4A
eIF4E : Eukaryotic translation initiation factor 4E
eIF4E2 : Eukaryotic translation initiation factor 4E isoform 2
eIF4Ebp1 : Eukaryotic translation initiation factor 4E-binding protein 1
eIF4F : eIF4A, eIF4E, and eIF4G complex
EMT : Epithelial-Mesenchymal Transition
EPAS1 : Endothelial PAS domain-containing protein 1

EPO : Erythropoietin
ETS1 : V-ets avian erythroblastosis virus E26 oncogene homolog 1
ES : Embryonic stem cell
EYA4 : Eye absent homolog 4
EZH2 : Enhancer of zeste 2 polycomb repressive complex 2 subunit

FBS : Fetal Bovine Serum
FH : Fumarate Hydratase
FIH : Factor Inhibiting HIF
FKHD : Fork Head Domain factor

GAPDH : Glyceraldehyde-3-phosphate dehydrogenase
GLUT1 : Glucose transporter 1
GWAS : Genome-Wide Association Studies

HAF : Hypoxia Associated Factor
HDAC1 : Histone deacetylase 1
HIF : Hypoxia-Inducible Factor
HK : Hexokinase
HMBS : Hydroxymethylbilane synthase
HOXF : Homeobox Factor
HRE : Hypoxia Response Element
HSC : Haematopoietic stem cells
HYOU1 : Hypoxia up-regulated 1

ICF : Immunodeficiency, Centromeric region instability, Facial anomalies
IDH : Isocitrate dehydrogenase
IGF1R : Insulin-like Growth Factor 1 Receptor
IgG : Immunoglobulin G
iPSC : induced Pluripotent Stem Cell
ISH : *in situ* hybridization
ITS : Insulin-Transferrin-Selenium

LDHA : Lactate dehydrogenase
LIF: Leukemia Inhibitory Factor
LOH : Loss of heterogenecity

MBD2b : Methyl-Binding Domain 2B
^{m}C : Methylcytidine
MeDIP : Methylated-DNA Immunoprecipitation
MLH1 : mutL homolog 1
MSRPCR : Methylation-Sensitive Restriction digest PCR
Myc : v-myc avian myelocytomatosis viral oncogene homolog

NHA : Normal Human Astrocyte
PAR-CLIP : Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation

PCNA : Proliferative Cell Nuclear Antigen
PDGFR : Platelet-Derived Growth Factor Receptor
PDH : Pyruvate Dehydrogenase
PGC-1α : Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PHD : Plant homeodomain
PHD : Prolyl hydroxylases
PI : Propidium Iodine
PKM2 : Pyruvate dehydrogenase Kinase isoenzyme 1
PTEN : Phosphatase and tensin homolog
PWWP : Proline and tryptophan rich domain

RB1 : Retinoblastoma protein 1
RBM4 : RNA-binding protein 4
Rbx1 : RING-box protein 1
RCC : Renal Cell Carcinoma
REC : Renal Epithelial Cell
rHRE : ribonucleic Hypoxia Response Element
RIP : RNA immunoprecipitation
RTK : Receptor Tyrosine Kinase

SAM : S-Adenosyl Methionine
SDH : Succinate dehydrogenase
SHARP1 : Basic helix-loop-helix family member e41 (BHLHE41)
SMC: Smooth Muscle Cell
SNP : Single Nucleotide Polymorphism
SP1 : Specificity Protein 1
STAT : Signal Transducer and Activator of Transcription
SUV39H1 : Suppressor of variaegation 3-9 homolog 1

Tet : Ten-elevent translocation
TF2B : Transcription Factor IIB
TGF-α : Transforming Growth Factor alpha
TP53 : Tumor Protein p53

TSS : Transcription Start Site

UTR : Untranslated region

VEGF : Vascular Endothelial Growth Factor
VHL : von Hippel-Lindau

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INTRODUCTION

1. Cancer biology

Cancer rates are declining but the disease remains inadequately managed

In occidental countries cancer death rates have declined 20% over the last two decades (American cancer society, cancer statistics 2013). This improvement is thought to be a direct consequence of life style changes such as reduced tobacco smoking, combined with preventive medical screening programs and more targeted radiotherapy and chemotherapeutic treatments. The five-year relative survival rate, an indicator of treatment success, also improved but less significantly (7% over the last 15 years, American cancer society). However, the most aggressive cancers (lung, pancreas and brain) showed little or no change in survival rates. Currently, just 17%, 8% and 15% of patients survive five years after diagnosis of lung, pancreatic and brain cancer compared to 95%, 88% and 50% for prostate, breast and renal cancers, respectively. Unlike AIDS combination therapy or diabetes insulin replacement therapy, cancer patients are not offered a generic treatment to control the progression of the disease upon initial diagnosis. Therefore, without safe anti-cancer agents or a reliable curative treatment, the earliest possible detection of tumour initiating cells yields the best chances of survival. However, aggressive cancers are often detected too late because of a lack of early molecular markers of the disease and an incomplete understanding of the early oncogenic program. Tools to identify early neoplastic masses are progressing with fundamental research. There will likely be an important improvement in cancer treatment once it is possible to target the oncogenic

growth activation in a wide variety of cancers. For this it is imperative that we better understand the biology of cancers.

1.1 The clonal model of oncogenesis

1.1.1 Survival of the fittest

We now have a significant understanding of how a multicellular mass grows from a single rogue cell of the body. Following early breakthrough in cancer research, scientists were optimistic about understanding cancers on the basis of the way tumour cells evolve (national cancer act 1971). Traditionally, the clonal model of tumour formation is the irreversible alteration of a normal cell of the body and the later selection for the most aggressive cancer cell originating from the abnormal ancestral cell (Nowell PC, 1976). The advance of growing cells and the accumulating tumourigenic mass ultimately threatens life by affecting the normal functioning of the organ. Tumour cells can also switch to a metastatic growth and colonise other parts of the body until the host dies.

The definition of the clonal model of oncogenesis implied that all tumour cells that are selected to retain the ability to reform a malignant tumour indefinitely (Iannaccone et al., 1987). This was supported by observations that cancer cells in human neoplastic lesions carried nearly identical karyotypes (Rowley et al., 1980; Yunis et al., 1981), from which stable cultures could be established *in vitro*, and retained their original morphology when induced to form tumours *de novo* in laboratory animals (Fogh et al., 1977). However only a limited number of the tumour cells supported the transition from *in vivo* to *in vitro* growth

(Dobrynin YV, 1963; Turin et al., 2014) and at the time it was already recognised that cancer cells display a large spectrum of phenotypic variability that contrasted with the theory that all tumour cells were genetically identical enough to be regarded and targeted as a monolithic pathology (Makino S, 1956; Levan and Biesele, 1958).

1.1.2 Clonal populations of tumour cells

Now the consequence of heterogeneity in cancers is starting to be re-appreciated using the most recent advances in high-resolution DNA sequencing using freshly isolated tumours. Recent work using deep-sequencing tools supported the clonal model, whereby studies of primary solid tumours showed that, in most cancer cases, a single clonal population accounted for the majority of the tumour mass (Sohrab et al., 2012; Walter et al., 2012; Hugues et al., 2014; Klco, 2014; Lohr et al., 2014; Wang et al., 2014). On the other hand, these studies also confirmed that many different cancer populations coexisted throughout tumour development (Ding et al., 2013) and that tumour growth and metastasis could be occurring in a polyclonal manner (Marusyk et al., 2014; McFadden et al., 2014). It could be argued that only the major clone is relevant for tumourigenesis while other clones might not since they were selected against. However, the emergence of a dominant cancer clone occurs late during tumour development and is generally the point when the tumour mass is detected and extracted for analysis (Shinichi et al., 2010). Therefore, studying relatively late events can mask cells that were small in number but important for the emergence of the most aggressive ones and it does not exclude that other cells could have spread and already colonized other parts of the body (Vanharanta et al., 2013).

1.2 Targeting heterogeneous tumours by understanding their commonalities

In light of these findings, it is now highly debated how to target these human tumour cells that are heterogeneous in many ways and changing over time. Diversity in cancers is a major impediment for understanding cancer and for the design of a systematic therapy. Since it is generally accepted that any given tumour has originated from a common ancestor clone with a finite set of alterations, the shared attributes of tumour cells were most likely acquired at the earliest stages of malignancy. To bypass tumour cell diversity, it is possible to trace back the origin of a common progenitor cell and target these core alterations, before cancer cells coevolved and became varied (Feinberg et al., 2006; Brock et al., 2009). Commonalities also exist between different individual tumours that can be tackled. A set of common cellular traits is conserved and required for cancer development and is generally used to support the view that cancer can be rationalized and understood as a single pathology to some extent.

1.2.1 The common traits of human neoplasia and the cancer hallmarks theory

Transformation of normal cells into growing tumours is a multistep process and involves significant alterations to the cellular compartment and the surrounding tissues (reviewed in Greaves and Maley, 2012). One of the most comprehensive descriptions of tumourigenic cells was coined by Hanahan and Weinberg as “The Hallmarks of Cancer” and describes the common attributes of malignant cells (Hanahan and Weinberg, 2000; 2011). The original theory is cited in over 19,000 scientific publications and states that cancer cells revolve around the common themes of autostimulated and unrestricted growth, evasion of

programmed cell death, limitless replication, self-sustained nutrient supply and the capacity to invade and spread across multiple parts of the host body (**Figure I**). Except for the metastatic process, all of these traits are shared between malignant and benign tumour cells. Substantial evidence suggests that these traits are absolutely required to confer cancer cells their malignant properties (Luo et al., 2008; reviewed in Weinberg RA, 2014).

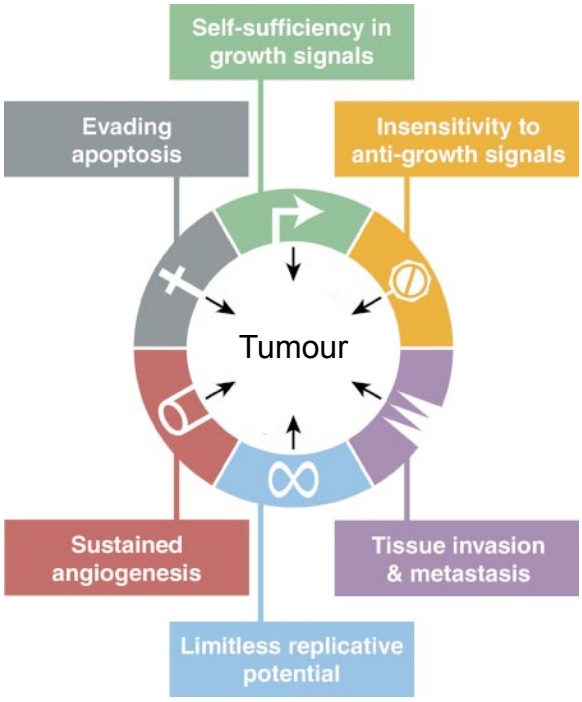


Figure I: Cancer Hallmarks: The Hallmarks of cancer were originally coined by Hanahan and Weinberg to generalise the biology of cancer and consist of the acquisition, by the incipient cancer cells of six cellular traits that are required altogether but not sufficient singularily to cause malignant growth. (Modified from Hanahan and Weinberg. (2000). Cell, *100*, 57-70)

Although every single hallmark is required for malignancy, none alone is sufficient to drive malignant growth. It could be argued that the theory is a reductionist approach but it nevertheless helped researchers ask precise questions to generally obtain definitive answers and predict the outcome of experiments in preclinical settings (Ellenbroek et al., 2014;

Reviewed in Giancotti FG, 2014). Understanding the critical genes and intracellular pathways required for each hallmark and their conservation among different sporadic cancers has been an intense area of research in order to target tumour cells before they irreversibly acquire multiple hallmarks.

1.3. The genetic basis of cancer

1.3.1. Alterations in oncogenes and tumour suppressor genes are the root cause of malignancy

After years of cancer research, it is now generally recognized that at the molecular level cancer is fundamentally a disease of altered gene expression. Technology allows tracking changes in the DNA of cancers to find that a finite number of genes are directly related to cancer formation (The biology of cancer, RA Weinberg 2013). Recurrent DNA mutations are generally found within growth-promoting genes: oncogenes and anti-proliferative genes called tumour suppressors, among others (reviewed in Futreal et al., 2004 and Rahman N, 2014). These genes are directly implicated in the acquisition of the cancer hallmarks (reviewed in Vogelstein and Kinzler 2004; Luo et al., 2010). While some genes are mutated in many different tumours, most are only causal to a subtype of tumour and are, therefore, tissue-specific (reviewed in Croce et al., 2008; Berger et al., 2011). Early findings correlating cancer incidence and age at diagnosis, suggested that genetic mutations accumulated over time and were associated with increased tumour incidence (Nordling et al., 1952; Knudson et al., 1971). The somatic mutation theory of cancer stipulates that a

cancer cell emerges after a normal cell suffers irreversible chromosomal alterations, endowing it with the potential to form a tumour in any environment (Boveri T, 2008).

1.3.1.1 Cancer-related syndromes

Direct support for the somatically-mutated nature of cancer cells includes the data that compounds altering the genetic code were among the first mutagenic substances capable of inducing tumours in treated animals (Muller HJ, 1927; Cook et al., 1933). Cancers have different number of mutations depending on tissue or carcinogen exposure, the most well known mutagen being tobacco smoke (Doll and Hill, 1950; Wynder et al., 1953). Granted by the studies of very rare cancer-causing syndromes, several genes are now known to be specifically mutated in malignant cells and can, therefore, be used to screen for cancer predispositions (Reviewed in Futreal et al., 2004). For example, von Hippel-Lindau (VHL) syndrome is caused by a mutation of the VHL gene, which results in a higher frequency of renal, hemangioma and pheochromocytoma cancers. VHL is a negative regulator of a stress-activated pathway that enhances angiogenesis and survival in the low oxygenated tumour microenvironment (Richard et al., 2013). Details of the oncogenic pathway and the mechanism of VHL-dependent tumour suppression are provided in **section 3.3**. An ever-growing list of cancer-related genes has emerged over the years (Kandoth et al., 2013) and an appreciable number of successful drug therapies have been conducted in late stage cancer patients by targeting these direct drivers of human cancer cells (reviewed in Sawyers C, 2004).

1.3.2 Mitigated success for targeting the cancer-driving genes

As mentioned before, tumours do not necessarily all carry the same spectrum of mutations and, therefore, marginal clones can repopulate after targeted cells are destroyed. In-depth analysis of relapsing tumour cell genomes revealed both highly divergent and surprisingly similar recurrent cancers emerging with few additional mutations compared to the primary tumour cells (Turner et al., 1999; Klein et al., 2002; Zhang et al., 2013; Almendro et al., 2014; Hedberg et al., 2014; Johnson et al., 2014), reflecting at least two modes of acquired resistance.

Acquired resistance and clonal diversity

Sequencing the genome of the mutant cells after initial therapy allows for the anticipation of compensatory pathways so that combinatory drugs may be applied in a more systematic manner (Wu et al., 2012; Bettegowda et al., 2014; Diaz and Bardelli, 2014). This is the conventional approach of multidrug therapy aimed at circumventing the acquired resistance to anti-cancer agents (Kim et al., 2013). The inherent drawback to this approach is that non-mutational changes have a significant impact on the function of mutated oncogenes and tumour suppressor genes and drives alternative phenotypes after anti-cancer therapy (Cancer Genome Atlas, 2012; Mao et al., 2012; Kottke et al., 2013). Clearly, the gene sequencing technology overlooks the changes occurring in cancer cells independent of the DNA code. This would explain why, in some cancers, the presence of a mutated Braf, for example is not a reliable predictor of cancer resistance to Braf-targeted therapy (Canadas Garre et al., 2011; Kim et al., cancer 2012; Gouveia et al., 2013; Walczyk et al., 2014).

Recent studies suggest that the epigenetic changes that accompany the relapse of new resistant tumour cells could be a reliable marker of recurrence for these types of Braf-mutated cancers (Mancikova et al., 2014).

A research team used a panel of five different epigenetically-regulated genes in order to test changes in their DNA methylation status over time, before and after an anti-cancer regiment. This set of differentially methylated genes was found to be the best predictor of recurrence in various cancer types (Dauksa et al., 2012). Hyper and hypomethylated genes can be tested with urine samples and are good predictors of tumour recurrence (Su et al., 2014). In addition, studies suggest that a stem cell-like epigenetic signature, characterized by changes in methylated DNA could be implicated in early tumour development (Brock et al., 2008; Bartholdy et al., 2014). Since these cancer-related epigenetic alterations are readily reversible, a fundamental question remains that needs to be addressed: Are epigenetic alterations, including DNA methylation, direct drivers of tumour cells?

2. Cancer epigenetic

Epigenetic alteration of cancer cells involves non-mutational modifications of DNA, change in gene expression and confer reversible phenotypic alterations.

The field of epigenetics focuses on gene regulatory mechanisms that are independent of changes in the genetic code. Epigenetic modifications of DNA and chromatin are reversible and heritable. Non-coding RNAs, chromatin remodelling, post-translational modification of histones, microRNAs and DNA methylation are examples of non-mutational modifications

of genes that serve to regulate cellular gene expression (reviewed in Jaenisch and Bird, 2003). First support for the involvement of epigenetic control of gene expression came from animal cloning experiments. Researchers rationalized that if the genome of cancer cells was irreversibly altered when transferred into an enucleated normal cell (oocyte), the cancer cell's nucleus would have the capacity to transform the normal cell and, therefore, no viable animal would be created. Transfer of a cancer cell's nucleus into an enucleated oocyte generates normal animals, albeit with increased tumour incidence, supporting the notion of irreversibly altered genetic information (Gurdon et al., 1958; Blelloch et al., 2004). The nucleus of melanoma, leukemia, lymphoma and breast cancer cells has been shown to be reprogrammable into a pluripotent state of differentiation and contribute to the normal development of the embryo. Altogether this suggests that many different cancer types can have their gene expression program at least partially reversed to a normal cell type (Hochedlinger et al., 2004; Jaenisch et al., 2004).

The following sections will focus on gene regulation by DNA methylation and alteration of methylated DNA in cancer (epimutations), although other epigenetic regulators are critically involved in cancer and often overlap with other DNA epimutation to genetically regulate oncogenic pathways (reviewed in Baylin and Jones, 2011).

2.1 DNA methylation

DNA methylation is implicated in regulating X-chromosome inactivation and the expression of imprinted genes, tumour suppressors and oncogenes (Jaenisch and Bird, 2003). In addition, DNA methylation triggers the formation of heterochromatin and

represses the expression of endogenous retroviruses, recombination of transposable DNA fragments, and ensures telomere and centromere stability (Ehrlich M, 2005). In general, methylated genes tend to be transcriptionally silent. DNA methylation suppresses transcription by altering the binding of transactivating factors such as transcription factors and also by promoting heterochromatin formation that is associated with a repressive state of transcription (Jones PA, 2012). Significant interest in DNA methylation occurred when it was discovered that classical tumour suppressor genes such as mutL homolog 1 (MLH1), Adenomatous polyposis coli (APC), Phosphatase and tensin homolog (PTEN), Retinoblastoma protein (RB1), Breast cancer type1 susceptibility protein (BRCA1) and VHL, among others, were inactivated via DNA hypermethylation in cancer cells (Kalari and Pfeifer, 2010). In some instances, such as pancreatic and renal cancers, an estimated rate of 20% of the total VHL tumour suppressor gene inactivation events are occurring in sporadic cancers via promoter hypermethylation (Herman et al., 1994; Banks et al., 2006; Schmitt et al., 2009). Reversing the tumour suppressor gene's hypermethylation with generic allosteric inhibitors of the enzymes catalyzing the formation of methylated DNA, the DNA methylransferases (DNMTases), efficiently reactivates tumour suppressor gene expression (Momparler RL, 2005). Thus, targeting the epigenetic modifications of tumour cells could be a viable approach to target the abnormal gene expression of cancer cells.

2.1.1 DNA methylation-based drug therapies

Despite the fact that both mutation and epimutation are hereditary by definition, epigenetic-mediated modification of DNA can also be modulated by changes in the environment (Jaenisch and Bird, 2003). This triggered the therapeutic approach aimed at reverting

cancer-associated epimutations back to normal. One of the first epigenetic drugs tested in clinical settings, 5-azacytidine (Vidaza) or its more stable derivative 5-azadeoxycytidine (Dacogen), are now used in leukemia late-stage therapy and further testing is ongoing for their use on solid tumours (Ahuja et al., 2014). Vidaza or Dacogen are approved for use on patients with myelodysplastic syndrome that are not eligible for bone marrow transplant (Gracia-Manero et al., 2014). Leukemia patients treated with Vidaza or Dacogen have a significantly increased overall survival supporting the rationale that loss of DNA methylation would reactivate epigenetically silenced tumour suppressor genes and kill cancer cells (Estey EH, 2013; Xie et al., 2014). However, several independent studies report poor correlation between changes in DNA methylation and drug response (Voso et al., 2014), suggesting alternative tumour suppression effects for those drugs. Vidaza and Dacogen also increase recurrent mutations in relapsing cancers and DNMTase-mutated tumours display inconsistent responses to epigenetic drug therapy, supporting the existence of several bystander effects of the systematic DNA methylation blocking strategy (Metzeler et al., 2012; Tobiasson et al., 2014).

Compared to cancer-targeted therapies, epigenetic-targeting drugs have widespread effects that are only starting to be understood. We particularly lack insight into the effect of inhibiting DNA methylation on normal cells. A major obstacle to our understanding of the epigenetic contribution to cancer is the lack of experimental findings directly linking altered DNA methylation activity to the intracellular pathways causing the disease.

2.2 Cancer cells display frequent epigenetic alterations

One of the most consistent epigenetic changes in cancer cells is the overall loss of methylcytosine accompanied with focal hypermethylation of tumour genomes (Ehrlich M, 2002; Timp et al., 2014). DNA methylation alterations can be observed in preneoplastic lesions and are thought to support the abnormal gene expression of cancer cells (Goelz et al., 1985; Feinberg et al., 1988; Salem et al., 2000; Compare et al., 2011; Sonnet et al., 2014). The first causal evidence linking the rate of methylated DNA and the pathology of cancer came from non-selective inhibition of DNMTase activity by competitive incorporation of methylation-resistant cytosine analogs. Exposure of normal rats to 5-azacytidine was shown to be carcinogenic and induce formation of multiple primary tumours including leukemia, cancer of the testis, skin and bronchus (Carr et al., 1984; Carr et al., 1988). Other experiments with 5-azadeoxycytidine showed that the compound directly caused or facilitated cellular transformation (Olsson and Forchhammer, 1984; Frost et al., 1987; Rainier et al., 1988; Rimoldi et al., 1991), depending on pre-existing genetic alterations in the cells. It was, therefore, suspected that acute changes in DNA methylation could directly cause cancer.

2.2.1 The Cellular DNA methylation machinery

DNA methylation is typically a modification of cytosine within CpG rich regions of the genome (CpG islands). Non-CpG methylation occurs predominantly in embryonic stem cells and its relevance to cancer is currently unknown (Patil et al., 2014). Generally, the CpG islands are clustered in the vicinity of gene regulatory regions such as gene promoters

(Jones PA, 2012). Mammals express four different DNMTases: DNMT1, DNMT3a, DNMT3b and DNMT3L. All DNMTases, except DNMT3L, catalyze the formation of methylcytosine (^{m}C) utilizing S-adenosyl methionine (SAM) as a cofactor (Jeltsch A, 2006). DNMT3L does not have a complete catalytic domain and acts by enhancing the activity of *bona fide* DNMTases DNMT3a and DMT3b (Chedin et al., 2002; Gowher et al., 2005). The DNA methylation pattern of somatic cells is established *de novo* during differentiation and conserved by the action of maintenance DNMTase activity (**Figure II**). Support for the existence of a *de novo* DNMTase stems from the observations that DNMT3a and DNMT3b single- and double-knockout animals are incapable of generating new DNA methylation post-implantation and embryos are not able to proceed to late stages of development because of failure to establish DNA methylation patterns of imprinted and tissue-specific genes, for instance (Okano et al., 1999; Chen et al., 2003). Methylation patterns are established *de novo* during embryogenesis and cell lineage commitment by DNMT3a, DNMT3b and the participation of DNMT3L (Okano et al., 1999; Suetake et al., 2004). DNMT3L knockout animals phenocopy DNMT3a ablation (Hata et al., 2002; Kaneda et al., 2004). After the establishment of the epigenetic marks on the DNA, cytosine methylation is preserved through cell divisions by the maintenance DNMTase DNMT1. DNMT1 enzyme copies the template strand's ^{m}C pattern onto the newly synthetized DNA, thus maintaining the epigenetic state of the chromatin after replication and the overall level of DNA methylation in the genome. *De novo* DNMTases have been shown to exhibit DNA methylation activity during replication (Gonzalo et al., 2006), which would explain why DNMT3a and DNMT3b are expressed in terminally differentiated tissues (Watanabe, et al., 2006; Wu, et al., 2010; Colquitt et al., 2014). DNMT1 has been shown to have higher affinity for hemi-methylated DNA than unmethylated DNA, lending support for its role as

a maintenance DNMTase (Bashtrykov et al., 2012). Notably, acute deletion of DNMT1 causes progressive loss of DNA methylation synchronized with cellular replication (Li et al., 1992; Howell et al., 2001; Egger et al., 2006; Chen et al., 2007). Also, DNMT1 is the DNMTase with the most processivity in mammalian cells and finally, DNMT1 directly binds proliferative cell nuclear antigen (PCNA) on newly synthesized DNA at the replication fork (Schermelleh et al., 2007).

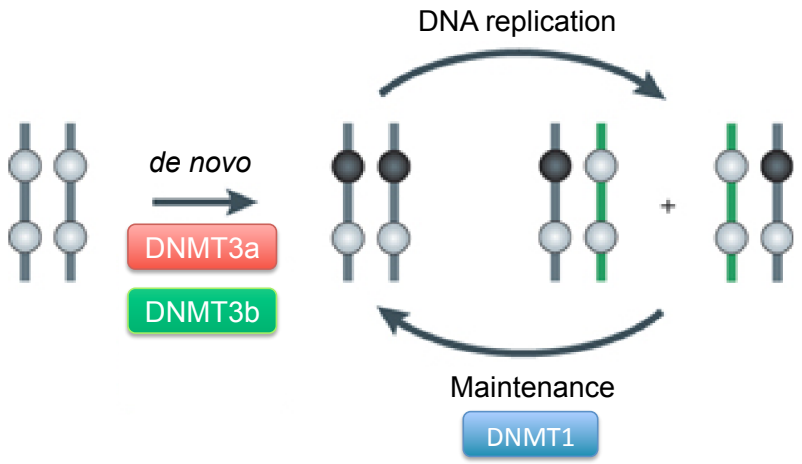


Figure II: Model of *de novo* and maintenance DNA methylation. The unmethylated CpGs (gray spheres) are targeted by DNMT3a and DNMT3b for the addition of a methyl group (black sphere). During DNA replication, the new DNA (green line) is not methylated and this hemimethylated status is corrected by the action of DNMT1 that replicates the DNA pattern established by DNMT3a and DNMT3b (Modified from Jones PA., and Liang G.. (2009). Nature review genetics,*10*, 805-811).

2.3 Support for causal role of DNA methylation in cancer

2.3.1 DNA hypomethylation and cancer

Several lines of evidence argue for a role of DNA methylation loss in cancer cells. A low S-adenosyl methionine (SAM) diet systematically depletes ^{m}C and can spontaneously induce

tumour formation in animals, indirectly supporting a causal role for loss of DNA methylation in cancer cells (Pogribny et al., 1995; Cooney et al., 2002). Additionally, DNMT1 knockout was shown to favour cellular transformation in mice (Holm et al., 2005; Yamada et al., 2005; Howard et al., 2008). Since DNMT1 is mainly required to replicate the DNA methylation pattern from the mother strand onto newly synthesized DNA during mitosis, these data suggest that maintenance of DNA methylation activity could be implicated in cellular transformation. However levels and activity of DNMT1 in normal tissue is not lower in the vast majority of cancers and several discrepancies confound its direct role in tumour formation (Etoh et al., 2004; Agoston et al., 2005; reviewed in Kar et al., 2012; Yu et al., 2014). For example, the genetic deletion of DNMT1 decreases formation of polyps in the APC model of colon cancer (Laird et al., 1995) and decreases tumourigeneity of leukemic cells (Trowbridge et al., 2012), suggesting that some cancer cells require maintenance of DNA methylation. Instead, several investigators proposed that hypomethylation plays an indirect role in cancer by causing DNA instability and stimulating chromosomal recombination (Chen et al., 1998). In support of this, several genome-wide DNA methylation mapping experiments performed on early human tumours or animal models of cancer suggest that significant loss of DNA methylation occurs in structurally important regions such as centromeres and telomeres as well as regulatory regions such as CpG islands (Eden et al., 2003). Indeed colorectal cancer cells in which DNMT1 was inactivated via homologous recombination displayed genome-wide hypomethylation and loss of viability related to chromosomal instability (Egger et al., 2006; Chen et al., 2007). Therefore it is currently debated whether loss of methylcytosine is a direct causal factor for cancer or a passive consequence of highly replicating cells outpacing endogenous DNMT1 activity.

2.3.2 DNA hypermethylation and cancer

On the other hand, CpG hypermethylation is frequently observed in cancer cells but in various patterns and to different extents. Overexpression of a constitutively active DNMT3b was shown to promote tumour formation (Linhart et al., 2007). However there is no report to date of DNMT3b mutations naturally occurring in cancers. Nevertheless, DNA hypermethylation is perceived to be more relevant to cancer biology because it targets a large number of cancer-related genes, including a wide array of tumour suppressor genes (Issa JP, 2004). In contrast, many hypermethylated and silent genes in cancer are often not expressed in normal tissue (Keshet et al., 2006; Weber et al., 2007; Simmer et al., 2012) making the relevance of DNA hypermethylation and subsequent loss of expression events in tumours unclear. Several lines of evidence suggest that both loss and gain of DNA methylation in cancer cells could be explained by a compensatory mechanism. Experimental data suggest that DNA hypo and hypermethylation occur in the same tumour sample but at different loci (Ehrlich et al., 2006). Also, DNA hypomethylation precedes genomic damage (Suzuki et al., 2006) and DNA damage subsequently induces hypermethylation of the DNA by recruiting DNMT1 to the repair site (Mortusewicz et al., 2005).

Therefore, this suggests there is a widespread reprogramming of the neoplastic epigenome, which would be supported by the establishment of alternate DNA methylation patterns in cancerous cells (Corces-Zimmerman et al., 2014). In normal cells, DNA methylation patterns are implemented during embryogenesis by the action of DNMT3a and DNMT3b *de novo* DNA methyltransferase enzymes. *De novo* DNA methylation is known to be

specifically required for maintenance of the terminally differentiated states of some somatic cells (Wu et al., 2010) but is also involved in editing the epigenome in response to external cues (Laplant et al., 2010; Guo et al., 2011; Hon et al., 2013; Azzi et al., 2014). Tumours induced with methyl-low diets could not reinstall DNA methylation patterns after normalization of methyl intake suggesting that *de novo* activity is deregulated in cancer cells (Pogribny et al., 2006). In support of this, ablation of *de novo* DNMTase genes DNMT3a, DNMT3b alone or together in colorectal cancer cells had no or little impact on overall DNA methylation, respectively, suggesting that cancer cells could spare *de novo* DNA methylation (Jair et al., 2006). For a long time, no mutations directly affecting DNMTase activity could be linked to cancer (Baylin and Bestor, 2002), greatly hampering our understanding of cancer's epigenome. Recently, a cancer-causing mutation in human DNMT3a gene was discovered, overcoming this hurdle (Yamashita et al., 2010).

2.4 DNMT3a

The recent discovery of recurrent somatic mutations in the DNMT3a gene further implicated *de novo* DNA methyltransferase activity in epigenetic reprogramming of cancer cells. Whole-genome exome sequencing of DNA from acute myeloid leukemic (AML) cells revealed C to T transitions in the catalytic domain of the DNMT3a gene and a R882H/A hot spot mutation causing a missense substitution of arginine to histidine or alanine (Ley et al., 2010; Yamashita et al., 2010; Yan et al., 2011). A significant amount of independent analyses confirmed the presence of a DNMT3a mutation in about 20% of all AML cases enlisting them among the five most common genetic alterations in this cancer type (Ley et al., 2010; Lin et al., 2011; Thol et al., 2011; Walter et al., 2011; Yan et al.,

2011; Marcucci et al., 2012; Huang et al., 2013; Gaidzik et al., 2013; Liang et al., 2013; Shivarov et al., 2013; Im et al., 2014; Kihara et al., 2014). DNMT3a R882 heterozygote mutations were found in cells of the major leukemic clone in several independent individuals, suggesting that DNMT3a defects were conferring a selective advantage for AML cells in the tumour niche (Bisling et al., 2013; Yasuda et al., 2014). Furthermore, several investigators suggested that the DNMT3a-mutated AML patients had a significantly worsened survival outcome compared to wild-type DNMT3a patients (Ostronoff et al., 2013; Bejar et al., 2014). DNMT3a mutations have also been found in solid colorectal and lung malignancies (Kim et al., 2013) and reduced expression of its mRNA occurs in some cancers (Kim et al., 2013; Li et al., 2014). DNMT3a mutations are associated with particularly poor prognosis in a wide array of solid cancers similar to Tumour protein p53 (TP53) and Breast cancer 1, early onset (BRCA1) associated protein-1 (BAP1) mutations (Kandoth et al., 2013; Skipper M, 2013).

2.4.1 Dominant negative mutation of DNMT3a

Recent experiments clearly established that the DNMT3a R882 mutant causes dominant loss of DNA methylation when expressed in somatic cells and transgenic animals (Russler-Germain et al., 2014). Heterozygous expression of the DNMT3a R882 variant competes with endogenous DNMT3a activity. DNMT3a R882 blocks the formation of DNMT3a-tetramers, later recognized as its functional tertiary structure, and thereby blocks DNMT3a activity (Holz-Schietinger et al., 2012). The R882 dominant negative mutation occurs in the C-terminal region of DNMT3a polypeptide. Functional domains of DNMT3a include N-terminal plant homeodomain (PHD), PWWP domain and the C-terminal catalytic domain.

The PHD sequence is responsible for interaction of DNMT3a with other proteins including transcription factors (v-myc avian myelocytomatosis viral oncogene homolog (Myc)), histone modifying enzymes (Histone deacetylase 1 (HDAC1), Enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), suppressor of variaegation 3-9 homolog 1 (SUV39H1)) and chromatin remodelling factor Brahma related gene 1 (Brg1) (Aapola et al., 2002; Fuks et al., 2003; Viré, et al., 2006; Hervouet et al., 2009; Rush et al., 2009). The PWWP domain is required to target and bind DNMT3 enzymes to pericentromeric heterochromatin (Chen et al., 2004). It remains unclear why the dominant negative mutation of DNMT3a occurs principally at R882 since no experiments have been conducted to test the interactions of DNMT3a mutant with its cofactors. There is some indication that the DNMT3a mutation could promote its degradation through a post-translational citrullination mechanism involving arginine modification (Deplus et al., 2014).

2.4.2 DNMT3a is implicated in early tumourigenesis

Recurrent mutations in *DNMT3a*, described in primary tumours, were stable throughout progression of the disease (Ding et al., 2012; Hou et al., 2012) and were shown to persist during and after cancer therapy, suggesting that these mutations are enriched in a clinically important AML cellular population (Ding et al., 2012; Welch et al., 2012). The presence of circulating tumour cells found during and after an anti-cancer treatment often causes tumour relapse and is referred as the minimal residual disease (Hourigan and Karp, 2013). DNMT3a mutant cells are found in minimal residual disease (Wakita et al., 2013) and relapsing tumour cells, suggesting that DNMT3a loss is directly implicated in cancer

resistance to conventional therapies and possibly a causal event in AML recurrence as well (Kapp-Schwoerer et al., 2013). Lineage tracing of DNMT3a mutations based on purified cell fractions from whole blood of AML patients identified the R882 mutants to be present in haematopoietic stem cells (HSC), preneoplastic progenitor cells and AML blasts (Shlush et al., 2014). This finding is best explained by the discovery of DNMT3a mutations in normal T cells albeit without the additional mutations occurring in DNMT3a-mutated AML cells, confirming the common origin of DNMT3a mutation at the earliest stages of AML disease in the haematopoietic progenitor cells (Shlush et al., 2014).

Several experiments suggest that DNMT3a could be involved in translating change in the environment into a stable inheritable epigenetic modification of the genome (Feng et al., 2010; LaPlant et al., 2010; Rugo et al., 2011; Day et al., 2013; Morris et al., 2014). In cancer, some data suggests that the cellular environment could have an impact on the tumour formation capacity of DNMT3a-mutated cells. For example, an unlikely case of donor cell leukemia (DCL) occurred in an AML patient under remission after receiving allogeneic transplantation of peripheral stem cells from his brother. Whole-exome sequencing found a rare DNMT3a mutation present in 8% of the donor stem cells to be largely enriched in the AML patient (61% of DCL cells) after transplantation but completely absent from all the previous stages of the disease's progression. Surprisingly, the donor with the DNMT3a mutation remained disease-free 10 years after his stem cell donation (Yasuda et al., 2014). These findings suggest that an anterior cancer environment from the patient under remission could enrich and enable DNMT3a-mutated cells with a tumour growth phenotype that is otherwise absent from the original cellular context where DNMT3a was not promoting tumour formation.

To further improve our understanding of cancer epigenetics, it is critical to determine the pathways altered in DNMT3a-defective cancer cells. The fact that a previous cancer conditions could impact the penetrance of DNMT3a mutation in cancer cells suggests that DNMT3a-mediated epigenetic program is possibly sensing or collaborating with the cancer microenvironment.

3. Cancer microenvironment

The tumour microenvironment is a ubiquitous and evolving trait of cancer cells

Abnormal proliferation is a core determinant of tumour cell biology and paradoxically also a flaw that prevents unlimited tumour growth. As a result of the excessive tissue proliferation and disruption of the normal vasculature, the obstructed or insufficient blood flow to cancer cells causes a shortage of essential nutrients such as glucose, amino acids and oxygen (**Figure III**, Jones and Thompson, 2009). The adverse tumour microenvironment, comprised of abundant stromal cells, extracelullar matrix and recruited immune cells, pressures the cancer cells to adapt cellular characteristics that circumvent these limitations in order to maintain growth and viability (Quail and Joyce, 2013). Examples of enabling cancer hallmarks include: blunting the immune system, reprogramming the cellular morphology (EMT) to grow and invade the tissue, and metabolic adaptation using alternative energy sources and catabolic reactions (Hanahan and Weinberg, 2011). A significant body of evidence suggests that the tumour microenvironment is common to the vast majority of solid cancers (Hanahan and Coussens,

2012; Doig et al., 2013) but it is debated whether the tumour microenvironment is a constant or transient feature of human malignancies (Swartz et al., 2012; O'Toole et al., 2014).

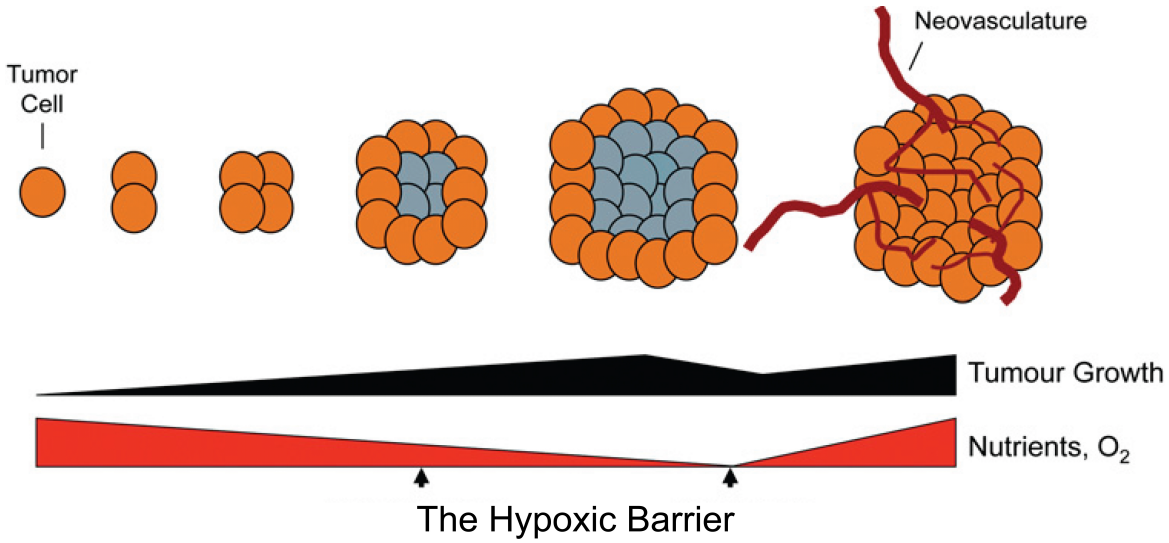


Figure III: The hypoxic barrier. The unrestricted growth of cancers results in depletion of the nutrients available from the environment including reduction of oxygen levels. This hypoxic microenvironment tiggers tumour cells to adapt and establish alternative strategies, such as neovascularization to pursue anarchical growth. (Modified from Jones, RG, Thompson, CB. (2009). Genes and development *23*, 537-548, 2009).

It is not well understood how the tumour microenvironment fluctuates during development of the tumour or what stages of tumour development are most sensitive to changes in the surrounding environment. For example, the hypoxic fraction of a tumour mass is dynamic, with a low oxygenated region that can be acute (stably hypoxic) or chronic (alternating between hypoxic and normoxic state) (Bennewith and Durant, 2004; Matsumoto et al., 2010). Nevertheless several findings support the fact that abnormal growth in the toxic microenvironment is particularly associated with increased tumour aggressiveness and worsened patient prognosis (Barcellos-Hoff et al., 2013). For example hypoxic cancer cells are significantly more resistant to radiotherapy (Brizel et al., 1994; Brown JM, 1999;

Shannon et al., 2003; Subarsky and Hill, 2003; Semenza GL, 2004; Sullivan and Graham, 2009; Strese et al., 2013; Ng et al., 2014), supporting the idea that the tumour microenvironment fundamentally alters the biology of cancer cells. More precisely, findings suggest that the altered tumour microenvironment is interconnected at the molecular level with cancer driving genes (Jones and Thompson, 2009; Wang et al., 2010; Patel et al., 2014).

3.1 Cancer causing genes and the tumour microenvironment

An ever-increasing body of knowledge supports that oncogenes and tumour suppressor genes are involved in regulating metabolism, blood supply, extracellular matrix and immunity in cancer (Faubert et al., 2014). Epidermal growth factor receptor (EGFR), one of the most frequently upregulated oncogenes, colocalizes with markers of the hypoxic tumour microenvironment and correlates with aggressive cancers, arguing for anti-cancer therapy targeting the hypoxic fraction of the tumours (Hoogsteen et al., 2012). As a proof of concept, several combination therapies used metabolic drugs such as Dichloroacetate with classical anti-cancer regiments and improved the efficacy of the treatment in preliminary experiments (Weiss et al., 2011; Dai et al., 2014; Xuan et al., 2014). We are starting to understand how cancer cells stimulate intracellular pathways to circumvent the adverse tumour microenvironment and progress further into the disease. By investigating these means of evasion, strategic targets for therapy are being developed to overcome the bottleneck in current treatment approaches.

3.2 Tumour hypoxia

For a large proportion of solid cancers, the lack of oxygen or hypoxia is one of the most consistent attributes of the tumour's microenvironment (Bertout et al., 2008). Humans breathe air containing 21% O_2, inside the human body normal cells live with 5 to 15% of O_2, while cancer cells are exposed to O_2 levels ranging from 0 to 5% in tumours (Carreau et al., 2011). Pathological intratumoural hypoxia has been demonstrated directly by use of oxygen electrode measurements (Cruickshank et al., 1994; Mortensen et al., 2011) and indirectly, based on hypoxia-activated fluorescent compounds, radioisotope tracers, gene markers and the proliferation of endogenous and exogenous anaerobe bacteria in primary tumours (Cronin et al., 2012; Yu et al., 2012; Warren et al., 2013). Cancer-related hypoxia is a consequence of tumour cells outgrowing normal tissue structure, disrupting blood vessel homeostasis and oxygen supply (Jain RK, 2005; Koch et al., 2012). Cells at the periphery of the tumour receive the most oxygen with decreasing delivery towards the core of the tumour mass (Olive et al., 1992; Rijken et al., 2000; Lambrechts et al., 2013). Cells in the core region are largely deprived of oxygen (anoxic) and display features of necrotic cell death (Tomlinson and Gray, 1955).

3.2.1 The hypoxic barrier

The extent of oxygen deprivation varies depending on the rate of oxygen diffusion from existing blood vessels and the metabolic rate of oxygen consumption by the tumour cells (Goel et al., 2011). Consequently, intracellular hypoxia can occur in well-vascularized tissues as a consequence of metabolically hyperactive cancer cells depleting their

intracellular oxygen levels (Prior et al., 2014). Interestingly, the oxygenation of some tumours can be inferred from the thickness of the viable ring of cells compared to the radius of the necrotic layer. Seminal histological analysis of 160 primary lung tumour cross sections suggested that at about 100-200 μm deep within tumours, the limited blood supply causes oxygen shortage and limits cell viability (Thomlinson and Gray, 1955). *In situ* oxygen electrode measurements and immunostaining using hypoxic chemical tracers such as pimonidazole (hypoxyprobe) and its etanidazole deriviative EF5 corroborated that the approximated diffusion limit of oxygen in tumourigenic vascularized tissue is about 100 μm (Reviewed in Kunz and Ibrahim, 2003). Interestingly, normal and tumourigenic tissues have roughly the same oxygen diffusion radius (Powis and Kirkpatrick, 2004). Although oxygen limitation can be toxic to both normal and cancer cells (Yamamoto et al., 2004; Azad, et al., 2008; Ginouves et al., 2008; Zheng et al., 2012), some malignant cells persist and escape hypoxia to sustain proliferation (Harris AL, 2002).

Several studies in genetically diverse cancers including ^{3}H thymidine labelling showed that proliferation of cancer cells declined with increasing distance from blood vessels (Tannock IF, 1970). Cell division is limited in low oxygen due to reduced ATP availability (Heerlein et al., 2005; Parks et al., 2013) and consequently, cancer cells have been shown to reduce the rate of ATP-consuming processes such as protein translation and use alternative catabolic processes in order to survive in the low oxygen environment (Wouters and Koritzinsky, 2008; Leprivier et al., 2013). Overall, several reports suggest that oxygen diffusion from blood vessels could limit growth in early stages and constrain the

multiplying cancer cells to a maximal size (**Figure III**) (Folkman et al., 1989; Hanahan et al., 1996; Lehman et al., 2009; Plodinec et al., 2012).

Unrestricted tumour growth is thought to occur when tumour cells self-stimulate the development of new blood vessels, prolong oxygen supply or evade the limitations of the microenvironment to sustain growth and metastasis (Gimbrone et al., 1972; Hanahan and Folkman, 1996). In support of this, the growth rate of cells in subcutaneous chambers or avascular corneas is initially slow and linear but rapid and nearly exponential after neovascularization (Algire et al., 1945; Gimbrone et al., 1972). In addition, *in vivo* tumour growth and expansion from micro (1-2 mm^3) to macroscopic tumours (1-2 cm^3) correlates with the growth of blood vessels and neovascularization of tumour cells (Folkman et al., 1966). A large spectrum of cancer phenotypes is affected by the hypoxic tumour microenvironment including withdrawal from growth arrest/senescence (Leontieva et al., 2012), telomere elongation (Seimiya et al., 1999), inhibition of apoptosis (Dong et al., 2001; Zhang and Hill, 2004), autophagy, anchorage independent growth, invasion and metastasis (Gatenby et al., 2007; Liao et al., 2007), and resistance to anti-cancer treatment (reviewed in Koutcher et al., 1992; Ruan et al., 2009; Zhou et al., 2014). Tumours with hypoxia-driven genetic signatures were shown to be associated with more aggressive, more resistant and increasingly recurrent tumours after various anti-cancer regiments (Vaupel et al., 2001; Milosevic et al., 2012; Zannella et al., 2013; Govaert et al., 2014; Helbig et al., 2014). For some cancers, identification of hypoxia gene signatures can predict beneficial hypoxia-targeting therapy (Eustace et al., 2013), supporting that insight into how tumour cells, in general, withdraw from oxygen restriction could normalize anti-cancer therapy and possibly restrict tumour growth to a microscopic stage. For many of those hypoxia-driven

phenotypes, improved understanding of the molecular pathways triggering adaptation to low oxygen is needed.

3.2.2 The hypoxia-driven gene response and the cellular adaptation to low oxygen

Oxygen restriction triggers distinct layers of cellular responses primarily regulated by acute changes in gene expression (reviewed in Yang et al., 2013). A great body of knowledge on hypoxia biology was granted by understanding the canonical pathways regulating the transcription of hypoxia-regulated genes (Tsai and Wu, 2012), although emerging evidence supports the existence of a non-transcriptional cellular response to hypoxia and is discussed in **section 5.1**. A cis-regulatory 5'-RCGTG-3' DNA sequence called Hypoxia Response Element (HRE) is commonly found in transcription regulatory regions, such as gene promoters, of oxygen-enriched mRNA transcripts (Semenza et al., 1991). The HRE tethers a set of three transcription factors (Hypoxia-inducible factors alpha (HIFα)s) called HIF-1α, HIF-2α and HIF-3α, which are activated in low oxygen and are involved in orchestrating coordinated cellular responses to oxygen scarcity by modulating levels of mRNA transcripts, rate of protein synthesis and anaerobic metabolism, to name a few (Semenza GL, 2013; Tsai et al., 2013).

3.3 HIF transcription factor complex

HIF-mediated transcription requires the formation of a heterodimer between the hypoxia-inducible HIF alpha subunit and the constitutive HIF beta subunit called aryl-hydrocarbon

receptor nuclear translocator (ARNT) that includes three different genes; ARNT1 (HIF-1β), ARNT2 (HIF-2β), and ARNTL (HIF-3β). RNA polymerase recruitment and transactivation of hypoxia-related genes also requires several HIF-interacting proteins such as E1A binding protein p300 (P300) transcriptional co-factor (Arany et al., 1996). Other HIF cofactors specifically drive the expression of a subset of hypoxia-related genes. For example, binding of HIF to V-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS1) drives expression of growth-promoting genes (Aprelikova et al., 2006), Myc binding promotes cellular proliferation in hypoxia (Gordan et al., 2007-b), binding of HIF to Pyruvate kinase muscle specific isoform (PKM2) regulates expression of mitochondria-related gene (Luo et al., 2011) and binding to Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) drives expression of muscle-specific transcripts associated with the slow twitch skeletal muscle type (Rasbach et al., 2010). For instance, between 350 and 550 gene loci have an HRE that has been experimentally tested for HIFα binding by chromatin immunoprecipitation (ChIP) in hypoxic MCF-7 breast cancer cells (Mole et al., 2009; Schödel et al., 2011). Whole genome RNA sequencing and microarray analysis of hypoxic cells identified thousands of unique gene transcripts upregulated 3 fold or greater after exposure to low oxygen among a microarray of more than 20,000 unique elements representative of the human transcriptome. More transcripts were upregulated in hypoxia, including non-coding RNAs using direct sequencing (Wang et al., 2005; Chi et al., 2006; Mole et al., 2009; Choudhry et al., 2014). Gene products of HIF-mediated transcription include glycolysis-regulating enzymes; lactate dehydrogenase (LDHA), pyruvate dehydrogenase (PDH) kinase isoenzyme 1 (Pdk1), PKM2, Hexokinase (HK), vascular regulators; vascular endothelial growth factor (VEGF), growth promoting factors;

Transforming growth factor alpha (TGF-α), cyclin D1, autophagy BCL2/adenovirus E1B 19-KDa interacting protein 3 (Bnip3) and protein synthesis machinery (reviewed in Semenza GL, 2013) to exemplify a few. With the exception of few growth-promoting genes, hypoxia-regulated genes are part of a normal regulatory response of both normal and cancer cells to cope with oxygen shortage (Verma A, 2006; McKeown SR, 2014). This is supported by microarray analysis of 4712 hypoxia-regulated transcripts, from which 168 are epithelial cell type specific and 123 are often found upregulated in epithelial cancers (Chi et al., 2006). Overall, several lines of evidence suggest that the mechanistic response to low oxygen, at the cellular level, might be shared between distinct normal and cancerous cell types.

3.3.1 Mechanism of oxygen sensing and the HIF cellular response

Oxygen sensing and HIFα activity is primarily regulated at the posttranscriptional level and by tissue-specific expression of the HIFα genes (Fandrey et al., 2006). HIF-1α is ubiquitous (Semenza GL, 1998; Stroka et al., 2001), HIF-2α is expressed primarily in endothelial cells (Tian et al., 1997) and HIF-3α is far less studied and is thought to be a mixed-regulator of the hypoxic gene response (reviewed in Giaccia et al., 2004; Zhang et al., 2014). In normoxic conditions, Prolyl hydroxylases (PHD), a class of enzymes utilising oxygen and α-ketoglutarate (α-KG), catalyze the hydroxylation of proline residues 402 (405) and 564 (531) within the HIF-1α (HIF-2α) transcriptional coactivation domain and marks HIFα factors for systematic proteasomal degradation (reviewed in Schofield and Ratcliffe, 2004). Hydroxylated HIFα are recognised by a Von Hippel-Lindau (VHL) E3-

ubiquitin ligase complex composed of RING-box protein 1 (Rbx1), Cullin 2, Elongin C and Elongin D protein cofactors that ubiquitinates and targets HIFα for proteasomal degradation (**Figure IV)** (Ohh et al., 2000; Jaakkola et al., 2001; Kaelin and Ratcliffe, 2008). VHL functions as a critical negative regulator of all three HIFαs and is a tumour suppressor gene (Wykoff et al., 2000). Other post-translational regulators of HIF include Factor Inhibiting HIF (FIH), a negative regulator, and a HIF-1α asparagine 803 hydroxylase (Mahon et al., 2001; Hewitson et al., 2002). Hypoxia Associated Factor (HAF) protein is also implicated in switching HIF-1α to HIF-2α-dependent transcription (Koh et al., 2008; Koh et al., 2011).

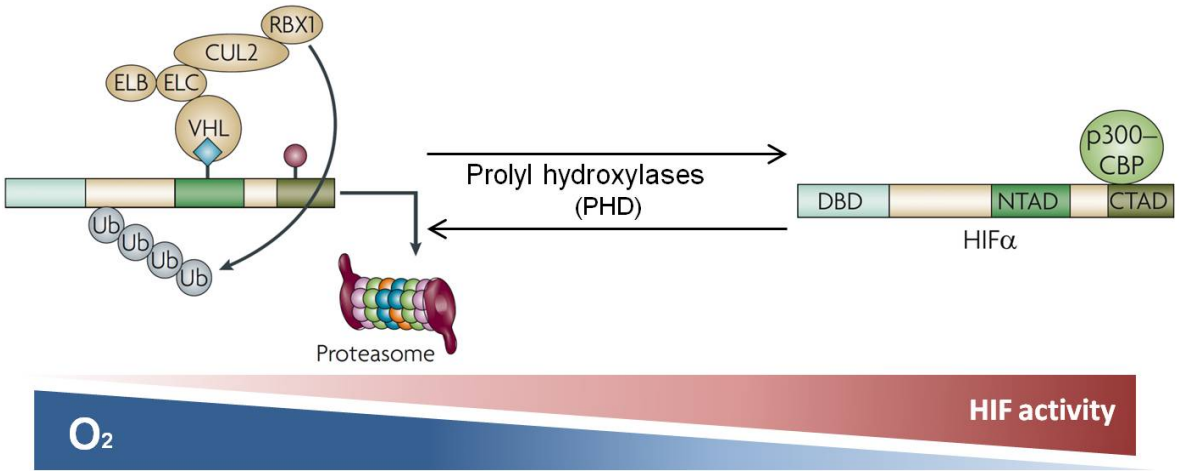


Figure IV: Post-translational control of HIFα by oxygen-dependent hydroxylation. The HIFα transcription factors (HIF-1α and HIF-2α) are targeted by Prolylhydroxylases at normal steady-state conditions. The hydroxylproline residue shown in blue is then recognized by VHL tumour suppressor gene and triggers the recruitment of the VHL ubiquitin ligase complex containing elongin C (ELC), elongin B (ELB), cullin 2 (CUL2) and RING-box protein 1 (RBX1). Ubiquitinated HIFα molecules (Ub) are then degradated by the proteasome. When oxygen is limited hydroxylation is lost and HIFα factors are stabilized. Factor inhibiting HIF (FIH) can repress HIF activity by hydroxylating proline residues on the HIFα C-terminus transactivation domain (CTAD). DNA binding domain (DBD). (Modified from Kaelin WG. (2008). Nature reviews cancer, *8*, 865-873).

3.3.2 Post-translational control of HIF-mediated gene expression

Altogether, hypoxia acts chiefly by disrupting PHD activity causing stabilization of the intracellular pool of HIFα, reprogramming gene expression and inducing a coordinated cellular response to low oxygen (reviewed in Mole and Ratcliffe, 2008). Oxygen diffusion is similar between normal and tumourigenic tissues (Hockel and Vaupel, 2001; Janssen et al., 2002) and thus there are only a handful of transcription factors, mostly encompassing the canonical HIF-dependant gene expression system, that are directly implicated in oxygen sensing and cancer development. Finding new HIF-related or HIF-independent, differentially regulated systems will elucidate new anti-cancer targets. In addition, several genes upregulated by cancer cells are expressed in normal cells under low oxygen conditions (Chi et al., 2006), suggesting that cancer cells hijack mechanisms of the normal hypoxic response to progress further into the malignant process. To obtain insight into biologically relevant hypoxia regulators, large-scale genetic screening technology is being used to understand hypoxia-related traits and associated genes in conditions of naturally occurring hypoxia such as adaptation to high altitude environments.

3.3.3 HIF-2α and the adaptation to low oxygen

Several experimental findings support a prevalent role for HIF-2α gene (*EPAS1*) in the adaptive response to low oxygen. A number of independent investigations reported a genetic link between *EPAS1* and adaptation to life at high altitude in several different mammalian species including pigs (Dong et al., 2013), horses (Hendrickson et al., 2013),

wolves (Zhang et al., 2014), dogs (Gou et al., 2014; Li et al., 2014; Wang et al., 2014) and humans (Beall et al., 2010; Simonson et al., 2010). Other hypoxia-related genes such as *EGLN1* (PHD1), Basic helix-loop-helix family member e41 (BHLHE41 or SHARP1), Hypoxia up-regulated 1 (HYOU1) and Hydroxymethylbilane synthase (HMBS) are involved in the hypoxic response with functions in HIFα stability, protein folding and haemoglobin synthesis respectively, and were also linked to high altitude populations in some studies (Huerta-Sanchez et al., 2013). In humans, *EPAS1* was consistently enriched in populations adapted to high altitude. Noticeably, a single nucleotide polymorphism (SNP) variant of *EPAS1* was represented in 87% of the Tibetan group (high altitude subgroup) compared to only 9% for low lander Han Chinese group (Yi et al., 2010) and similar results were found in an Ethiopian population living at high altitudes (Scheinfeldt et al., 2012), Sherpas (Hanaoka et al., 2012), and Mongolians (Xing et al., 2013). However, while genetic evidence suggests that HIF-2α is particularly important for low oxygen adaptation, parallel investigations did not find HIF-1α to be genetically connected to low oxygen adaptation in humans (Van Patot and Gassmann, 2011).

3.3.3.1 Regulation of blood delivery

The normal biological functions of HIF-2α are particularly relevant for low oxygenated environment fitness. First, HIF-2α is specifically expressed in endothelial cells that play a fundamental role in regulating oxygen delivery in the body and HIF-2α expression is particularly important during placental development (Tian et al, 1997; Rossant and Cross, 2001). HIF-1α and HIF-2α are required for trophoblastic differentiation and normal

placenta development (Cowden Dahl et al., 2005). Whole body deletion of HIF-2α affects the normal development of the yolk sac in early mouse embryo and is embryonic or perinatal lethal, depending on the transgenic strain (Ema et al., 1997; Tian et al., 1998; Peng et al., 2000). Notably, endothelial-specific re-introduction of HIF-2α is sufficient to rescue vascular development defects of early embryogenesis in HIF-2α-/- animals (Duan et al., 2005). Studies have reproducibly shown that newborn baby weight inversely correlates with elevation, suggesting that decreased oxygenation at high altitude directly affects pregnancy (Howard et al., 1957; Haas et al., 1977; Jensen and Moore, 1997). Indeed, the risk of preeclampsia, a pregnancy disorder characterized by abnormal placenta development, is aggravated at high altitudes, possibly a consequence of reduced placental oxygenation (Krampl E., 2002; Zamudio S., 2007; Julian et al., 2008). Interestingly, overexpression of HIF-2α is also associated with increased incidence of preeclampsia (Rajakumar et al., 2001).

Secondly, HIF-2α is responsible for the transcriptional regulation of erythropoietin (EPO), a hormone controlling red blood cell production (Rankin et al., 2007). Whole body ablation of *EPAS1*, but not HIF-1α, in adult mice causes anemia (Gruber et al., 2007). Exon 12 mutations of HIF-2α are associated with erythrocytosis and drugs targeting HIFα for anemia have been tested clinically (Hsieh et al., 2007; Percy et al., 2008; Bernhardt et al., 2010; Percy et al., 2012). Studies revealed that HIF-2α was required for red blood cell maintenance, by directly regulating the expression of EPO and indirectly ensuring the maintenance of haemoglobin levels and red blood cells (Scortegagna et al., 2003; Scortegagna et al., 2005). Genome-wide association studies (GWAS) showed a genetic link

between *EPAS1* and haemoglobin levels in normal humans and, interestingly, Tibetans with *EPAS1* variant genes have the lowest concentration of haemoglobin in the blood compared to lowlander populations (Beall et al., 2010). Finally, during low oxygen adaptation, the human body consistently loses weight, resulting mostly from loss of muscle mass (Boyer and Blume, 1984). This phenomenon is thought to help transition the body's metabolism towards ketone body production to sustain brain function and prevent acute mountain sickness (AMS), the deadly maladaptive response to high altitude (Veech RL, 2004; Murray and Montgomery, 2014).

3.3.3.2 Regulation of metabolism

HIF activity is a critical regulator of the metabolic switch from slow to fast twitch muscle and the adaptive response of muscle tissues to hypoxia (Slot et al., 2014). Mice with muscle-specific deletion of HIF-2α have increased expression of molecular markers associated with the fast-twitch muscle fiber-type and an increase in oxidative glycolytic metabolism in muscle tissues, mimicking the acute adaptation of the muscle apparatus to low oxygen (Hoppeler and Vogt, 2001; Rasbach et al., 2010). Additional studies also reported a link between *EPAS1* SNP and endurance athlete performance, further supporting a central function for *EPAS1* in the biological response to low oxygen (Hendersen et al., 2005; Voisin et al., 2014).

4. HIF-regulated mechanisms in cancer

Hypoxia and HIF-regulated mechanisms are core components of human neoplasia

Although it seems that adaptation to naturally occurring hypoxia enriches variants of hypoxia-regulated genes, there is support for the idea that the selected traits could be linked to loss of function in specific HIFα–regulated pathways (Lorenzo et al., 2014; Petousi et al., 2014). In the case of the pathological hypoxia found in tumours, several lines of evidence suggest that cancer cells utilise HIFα's oncogenic activity. First, loss of negative regulators triggers constitutive HIFα expression and activity in cancer cells. About 80% of sporadic renal cell carcinoma, accounting for 90% of all renal cancers, have loss of VHL function and thereby the capacity to target HIFα for proteasomal degradation in normoxia (Moore et al., 2011). These cancer-specific alterations are creating a state of pseudo hypoxia, whereby HIFα expression and activity is stimulated independently of oxygen concentration (Favier and Gimenez-Roqueplo, 2010; Pietras et al., 2010).

4.1 Direct and indirect evidence supporting HIF implication in cancer

4.1.1 Post-translational regulation of HIFα in cancer

Interestingly, abrogating HIFα activity either by reintroducing VHL, directly silencing HIFα via RNA interference or blocking the expression of selected HIF-target genes abrogates the ability of kidney cancer cells to form tumours *in vivo* (Abdulrahman et al.,

2007; Zhang et al., 2013), supporting the requirement of hypoxia-activated pathways in renal tumourigenesis. Similarly to VHL in renal cells, basic helix-loop-helix family member e41 (BHLHE41) or Sharp1 depletes HIFα levels in normoxic breast tissues (Piccolo et al., 2013). Breast-cancer specific deletion of Sharp1 is found in human breast cancer specimens and genetic deletion of Sharp1 in mice causes spontaneous malignant breast cancer (Montagner et al., 2012). Of importance, human breast cancer cells also rely on HIF-directed activity to cause cancer (Montagner et al., 2012; Liao et al., 2014). In VHL-dependant cancers, mutations in metabolic regulators can also indirectly cause HIFα expression by blocking endogenous PHD activity (Bratslavsky et al., 2007; Sudarshan et al., 2007). In support of this, fumarate hydratase (FH) and succinate dehydrogenase (SDH), both enzymes of the citric acid cycle pathway are required for HIFα-dependent tumour suppressor activity in paraganglioma, renal and breast tissues (Ricketts et al., 2012; Linehan and Rouault, 2013). Abrogating FH or SDH function in the cell causes abnormal build-up of metabolites such as succinate and citrate, which are competitive inhibitors of PHD activity in the cell (Reviewed in Esteban and Maxwell, 2005; Mackenzie et al., 2007; Sudarshan et al., 2009; Menendez et al., 2014).

4.1.2 Isoform-specific roles of HIFα in cancer

Cancer-causing mutations were found in the HIF-2α isoform of HIFα transcription factors (Comino-mendez et al., 2013). Mutations of HIF-2α, P531T and P531S, occur on proline residues, which are hydroxylated by PHD enzymes and cause constitutive HIF-2α expression when mutated (Maranchie et al., 2002). These and other gain-of-function

mutations of HIF-2α are specifically found in highly vascularized tumours of the autonomic nervous system (pheochromocytomas and paragangliomas) (Zhuang et al., 2012; Comino-mendez et al., 2013). Overexpression of HIF-2α P531T mutant causes a state of pseudo hypoxia in somatic cells via altered gene expression (Kondo et al., 2002). In these tumours, constitutively expressed HIF-2α mutant promote tumour proliferation and shorter latency *in vivo* (Toledo et al., 2013).

Gene silencing experiments suggested that some cancers, irrespectively of the genetic mutational landscape, require expression of both HIF-1α and HIF-2α or HIF-2α alone to form tumours *in vivo* (Burkitt et al., 2009; Franovic et al., 2009; Li et al., 2009; Menrad et al., 2010; Wang and Schneider, 2010; Bordoli et al., 2010; Chiavarina et al., 2012; Xue et al., 2012; Xue et al., 2014). While human cancer cell systems display rather consistent support for the requirement of HIFα oncogenic activity for cancer cells, animal models have displayed several discrepancies in HIF-2α cancer phenotypes (reviewed in Richard et al., 2013). Normal adaptation to reduced levels of oxygen requires switching metabolism away from oxidative phosphorylation towards glycolysis, downregulating oxygen and ATP consumption (reviewed in Wouters and Koritzinsky, 2008). We do not know if cancer cells partially or fully utilize HIFα–activated pathways to survive and thrive in low oxygen. HIF stabilization can occur independently of oxygen levels, as in the so-called pseudo hypoxic state of some cancer cells (Pietras et al., 2010; Piruat and Millan-Ucles, 2014), suggesting that cancer cells utilise HIF-regulated pathways differently than normal cells (reviewed in Bertout et al., 2008). Understanding how the HIF-2α oncogenic axis is directly implicated in conferring hallmarks to incipient cancer cells is an important aspect of tumour biology.

5. HIF-2α oncogenic axis

HIF-2α oncogenic axis involves the acquisition of autonomous growth and the activation of the EGFR pathway to incipient cancer cells.

Tumourigenic cells experience the limitation of a hypoxic microenvironment differently than normal cells. In the adverse conditions where oxygen supply is limited, malignant cancer cells have to resolve oxygen shortage while simultaneously maintaining growth-activating pathways (Bertout et al., 2008). On the contrary, somatic cells have a very low rate of cellular division and are not pressured to sustain energy-consuming processes (Reviewed in Giaccia et al., 2004). Therefore, hypoxia-activated pathways are expected to be sustaining the growth autonomy of tumour cells. In fact, several studies of primary tumours and cell lines revealed that core growth promoting genes and cancer-related phenotypes were driven by HIFα transcription factors (reviewed in Qing and Simon, 2009; Pietras et al., 2010). For example, HIF-2α was shown to interact with c-Myc and increase its transcriptional activity leading to a glycolytic switch and increased proliferation of hypoxic renal and colorectal cancer cells (Gordan et al., 2007-a). Myc amplification occurs in breast, colorectal, pancreas, uterus and ovarian cancers (Chen et al., 2014). HIF-2α was shown to synergise with c-Myc transformation of normal hypoxic fibroblastic cells (Gordan et al., 2007-a). Interestingly, HIF-1α had the opposite effect in this setting in that it antagonised the c-Myc proliferative effect in hypoxic cancer cells (Gordan et al., 2007-a). These differential effects of HIFα transcription factors can be observed in naturally

occurring renal cancers. A subgroup of RCC express solely HIF-2α and have increased c-Myc target gene expression, a higher proliferation index and a reduced DNA damage phenotype (Reviewed in Dang et al., 2008; Gordan et al., 2008). Other important growth-promoting genes activated by HIF-2α include the mitogen polypeptide transforming growth factor-alpha (TGFα), an oncogenic ligand, and its cognate receptor, epidermal growth factor receptor (EGFR), in brain, renal and pancreatic cancers (de Paulsen et al., 2001; Smith et al., 2005; Zhao et al., 2014). HIF-2α interacts with ETS-1 cofactor to specifically drive the expression of TGFα and promote the capacity to grow autonomously, a hallmark of cancer (Holterman et al., 2010).

5.1 HIF-2α mediated post-trancriptional regulation of growth signalling

Targeting mitogens such as TGFα in cancers has proven to be unsuccessful partly because growth factor receptors are expressed at such a high level in cancer cells that the random stochastic interactions of membrane-bound receptors are sufficient to activate growth signalling in a ligand-independent manner (Boerner et al., 2003). Hypoxia was shown to directly and indirectly increase receptor tyrosine kinase (RTK) activity and the autonomous growth of cancers in various ways. First, hypoxia increases the Rab5-dependent recycling of ligand-bound EGFR resulting in a faster rate of growth-promoting EGFR kinase activity (Wang et al., 2009). In addition, HIF-2α specifically increases EGFR association with polysomes resulting in a higher rate of translation and overexpression of EGFR, providing a non-mutational explanation for its overexpression in brain, lung, colorectal and renal cancers (Franovic et al., 2007; Franovic et al., 2009). Interestingly, HIF-2α also drives

expression of additional RTK, platelet-derived growth factor receptor (PDGFR) and insulin-like growth factor 1 receptor (IGF1R), suggesting a novel oncogenic translational regulatory response of HIF-2α that differs from its canonical transcriptional activity (Franovic et al., 2009).

RTKs are commonly upregulated and required for the growth of many diverse cancers (reviewed in Blume-Jensen and Hunter, 2001). EGFR mutations in epithelial cancers are concentrated on the auto-phosphorylated tyrosine residues that control the downstream signalling cascade, including growth promoting transcription factors (Jutten and Rouschop, 2014). EGFRVIII truncated mutant (deletion of exons 2-7) is enriched in glioblastoma multiforme brain cancers and both of these mutations cause constitutive activation of receptor tyrosine kinases (Gan et al., 2013). The activation of EGFR in low oxygen has pleotropic effects beyond autonomous growth promotion, including the genetic reprogramming of cellular metabolism via miRNA maturation (Shen, et al., 2013) and increased resistance to apoptosis and anti-cancer therapy (Peng et al., 2006; Jutten and Rouschop, 2014). Overall the hypoxic microenvironment could select for cancer cells with aggressive growth phenotypes. In support of this, EGFR and hypoxia markers colocalise in aggressive and therapy-resistant squamous cell carcinomas (Hoogsteen et al., 2012) and hypoxic tumours or tumours expressing high levels of HIFα transcription factors and RTK are more resistant to anti-growth chemotherapies (Hung et al., 2009; El Guerrab et al., 2011).

Taken together, many findings describe a conserved cellular response of cancer cells exposed to low oxygen: cancer cells activate a number of growth-promoting pathways, many of which are at least partially regulated by the HIF-2α isoform of the hypoxia-inducible transcription factor system. It is now important to understand how these mechanisms help to shape the early oncogenic activation of developing cancers, and for this purpose, the VHL loss syndrome is an ideal system because it portrays the step-wise acquisition of autonomous growth in HIF-2α-activated cells (Mandriota et al., 2002).

5.2 Inherited VHL disease and the biallelic inactivation of VHL tumour suppressor gene is a tool to study early oncogenic growth activation of HIF-2α.

The VHL syndrome is a classical case of a tumour suppressor gene causing autosomal dominant syndrome of renal cell carcinoma. Patients afflicted with VHL disease carry a mutated, non-functional, version of the VHL gene. For these individuals, the incidence of renal cancer increases substantially with time, a characteristic of tumour suppressor gene alteration. Eventually, additional mutations acquired in the remaining wild type VHL allele causes the complete loss of VHL function and subsequent tumour initiation. Another particularity of VHL syndrome is that only a limited number of tissues develop tumours (tumour incidence is restricted to specific organs). Transformed cells arise in the central nervous system (hemangioblastoma), adrenal glands (pheochromocytoma), neuroendocrine cells of the pancreas and epithelial cell of the kidney (Renal Cell Carcinoma (RCC)). Studies have shown that the mortality rate of VHL patients is attributable to the development of RCC tumours and haemangioblastomas (Richard et al., 2013). Studies of

VHL loss and oncogenic HIF activation is also relevant for both hereditary and sporadic kidney cancers since VHL is inactivated in about 80% of all naturally occurring RCC (Moore et al., 2011).

Renal tumours of VHL patients tend to lose HIF-1α expression and gain HIF-2α expression during the malignant progression of cancer cells (Shen et al., 2011). In this constitutive model of HIFα expression, HIF-2α was the oncogene responsible for tumourigeneity of VHL-/- cancers and HIF-1α was downregulated and implicated as a putative tumour suppressor (Raval, et al., 2005; Shen et al., 2011). In support of this, stable shRNA silencing of HIF-2α was shown to suppress VHL-/- directed tumour growth *in vivo* (Kondo et al., 2003). In addition, silencing HIF-1α alone is sufficient to bestow tumour formation capability to human HIF-2α/HIF-1α expressing RCC cell lines that were formerly non-malignant and not tumourigenic *in vivo* (Shen et al., 2011). A fundamental understanding of RCC is particularly relevant to cancer biology because VHL loss is one of the earliest steps of cellular transformation, bridging the transition between normal and precancerous human cells (Kaelin WG, 2004). Although it is difficult and often controversial to establish the origin of many tumours, RCCs originate from either the proximal or distal epithelial cell compartments of the nephron (Wallace and Nairn, 1972; Motzer et al., 1996; Mandiota et al., 2002; Raval et al., 2005; Schietke et al., 2012).

5.3 HIF-2α expression is an early oncogenic step coinciding with growth activation in VHL null multicellular lesions and likely occurs in a two-step mechanism in human renal cancers.

Genetic disruption of VHL causes HIFα misregulation but not tumourigenesis in VHL null mice, suggesting that the loss of VHL function is not tumourigenic *per se* (Mack et al., 2003; Rankin et al., 2006; Schietke et al., 2012; Pritchett et al., 2014). In human cells, silencing of VHL stops proliferation and provokes an oxygen-dependant senescent phenotype (Kim and Sharpless, 2008; Young et al. 2008; Welford et al., 2010), altogether confirming that the initiation of abnormal proliferation in VHL disease occurs as a multi-step mechanism.

Interestingly, normal epithelial cells of the kidney express HIF-1α but do not express HIF-2α in hypoxia, a condition where normally all expressed HIFα subunits are stabilized because of PHD inhibition (Rosenberger et al., 2002; Wiesener et al., 2003). In comparison, HIF-2α is expressed in interstitial cells of the kidney that are responsible for EPO expression (Paliege et al., 2010). The vast majority of renal cancers express HIF-2α necessitating that renal cancer cells experience a mutation that enables HIF-2α expression at some point in their development.

Studies of VHL cancers uniequivocally showed that HIF-2α expression was a limiting factor for cancer progression (Kondo et al., 2002; Mandriota et al., 2002; Maranchie et al., 2002; Kondo et al., 2003) Normal renal epithelial cells preferentially express HIF-1α

instead of HIF-2α and most overt RCC expressed HIF-2α, suggesting additional control of HIFα isoform expression in early transformation of RCC (Mandriota et al., 2002). Therefore studying HIF-2α expression in histological kidney sections of VHL disease patients is the ideal system to understand oncogenic activation in naturally occurring RCC. Using this system, it was shown that early VHL loss lesions retained normal morphology and were only expressing HIF-1α and not HIF-2α (Mandriota et al., 2002). Staining for a HIF target gene, CAIX confirmed the lesions have constitutive HIFα activation, supporting the idea of an additional mechanism controlling the repression of the HIF-2α isoform (Mandriota et al., 2002; Schietke et al., 2012). HIF-2α expression was specifically induced in renal epithelial cells displaying abnormal structure and correlated with the expression of cyclin D1, a hypoxia-target gene implicated in tumourigenic growth (Raval et al., 2005). Of note, HIF-2α expression occurred in a subsequent step following the initial loss of VHL function and was correlated with the presence of abnormal multicellular foci (Mandriota et al., 2002; Raval et al., 2005; Schietke et al., 2012). GWAS showed the HIF2α gene, *EPAS1*, to be linked to RCC suggesting a putative gene-related mechanism of HIF-2α expression in RCC (Purdue et al., 2011; Han et al., 2012).

6. Description of the rationale and hypothesis

The HIF-2α gene promoter is located in a portion of DNA with high CpG content that meets the criteria of a putative epigenetic regulation site via CpG island DNA methylation (**Figure 2A**). In support for a gene-related expression mechanism, studies suggested that the mRNA of HIF-2α was enriched in RCC tissues compared to the matching normal

adjacent renal tissue (Xia et al., 2001; Turner et al., 2002; Sandlund et al., 2009). Overall, several lines of evidence suggest that after the initial loss of VHL tumour suppressor gene function, normoxic HIF-1α expression occurs first and HIF-2α expression follows after the loss of a second putative silencing mechanism leading to the acquisition of several cancer hallmarks including autonomous growth. We thus hypothesized that an epigenetic silencing mechanism prevents HIF-2α gene expression in normal cells and this mechanism is lost in early tumourigenesis.

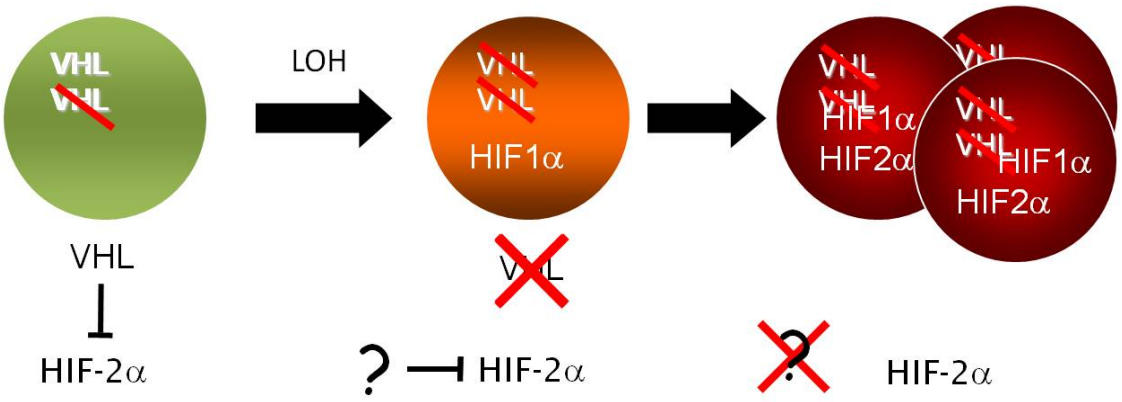


Figure V: The multistep initiation of early abnormal growth in VHL cancer. (Left) Somatic cells with VHL predisposing mutation (hatched red) still maintain normal degradation of HIF-2α by retaining a normal copy of VHL. (Middle) VHL loss of heterozygocity (LOH) causes stabilization of HIF-1α but not HIF-2α suggesting that a second repressing mechanism is silencing HIF-2α (question mark). (Right) A second mutational event in parallel to VHL loss likely causes HIF-2α expression that correlates with multicellular foci formation and early tumourigenesis in VHL disease.

MATERIALS AND METHODS

Cell culture and reagents

Human renal epithelial primary cells (REC) were kindly provided by Dr. Chistopher Kennedy (Kidney Center, University of Ottawa) and maintained in epithelial cell medium (ScienCell). Normal human astrocytes were a kind gift of Dr. Alexandre Prat (Université de Montréal, Montréal) and grown in astrocyte growth media. The human renal carcinoma cell lines; 786-O, KTCL and RCC4 and the human colorectal carcinoma, HCT116 were obtained from the American Type Culture Collection (ATCC). Astrocytomas U118mg and U87mg and lung carcinoma A549 cell lines were generously provided by Dr. Ian Lorimer (Ottawa Regional Cancer Center, Ottawa). All non-primary cell lines were maintained at 37^{o}C in a 5% CO_2 environment with high glucose DMEM (HyClone) media supplemented with 5% FBS (Wisent Inc.), 100U/ml penicillin, 100µg/ml streptomycin. Hypoxia was induced by incubating cells at 37^{o}C in a 1% O_2, 5% CO_2 and N_2-balanced atmosphere. Serum-free media consisted of DMEM supplemented with 1% insulin-transferrin-selenium (Invitrogen). 5-azacytidine (Sigma) was added to the cell media fresh every day and incubated for 72 hours at a final concentration of 10µM. ActinomycinD (EMD Bioscience) was used at a final concentration of 10µg/ml for the indicated times. Hypoxyprobe^{TM} was used at a final concentration of 100µM and incubated for 1 hour prior to analysis.

Human tissues and sample preparation

Frozen primary tumours and normal adjacent kidney biopsies were obtained from The Ontario Tumour Bank (Ontario Institute for Cancer Research, Toronto, ON). Samples were

chosen arbitrary from the repository and patients under 18 years old were excluded (Ontario cancer research ethics board TEC#018-10). To extract DNA/RNA, samples were crushed in liquid nitrogen and the homogenate powder underwent nucleic acid purification using the TriPure reagent (Roche) and following manufacturer's instructions.

Multicellular spheroid assays

50 or $1x10^5$ cells were plated in multi-well plates pre-coated with 1% SeaPlaque agarose (Cambrex) to prevent anchorage-dependant growth. To promote cell-cell adhesion and induce formation of a multicellular sphere, plates were rotated for 1 hour and grown under standard conditions for the indicated number of days. Images were taken daily with Zeiss Axiovert S100 TV microscope to measure cell growth. For hypoxia analysis, spheroids were, incubated with hypoxyprobe for 1h, washed with PBS, flash frozen in OCT compound, sliced and fixed in paraformaldehyde prior to immunohistochemical analysis of pimonidazol adducts.

Xenograft tumours

10^7 exponentially growing cells (786-0, U87MG, A549 and HCT116) were resuspended in 200μl PBS and injected subcutaneously into the flanks of CD-1 nude female mice (Charles River). Tumour growth was recorded by weekly measurement and animals were sacrificed when the end-point was reached or 8-9 weeks post-injection as per animal care protocol (Canadian Council on Animal Care).

Plasmids and transfection

The full-length coding sequence of wild-type HIF-2α was PCR-amplified and cloned in pcDNA3.1(+). pEGFP-C1-DNMT3a and pEGFP-C1-DNMT3b1 were kindly provided by Dr. Keith Robertson (Georgia health sciences university cancer center, Augusta, GA). FLAG-DNMT3a and HA-HIF-2α lentiviruses were purchased from GeneCopoeia. For the transient experiments, cells were transfected with the different expression vectors mentioned above using Effectene transfection reagent (Qiagen).

MSRPCR, MeDIP and Bisulfite sequencing

The MSRPCR assay amplified genomic DNA (1μg) that was previously restriction endonuclease digested for 48 hours with the isoschizomers MspI or HpaII. Genomic DNA was extracted by lysing cultured cells or tissue in 1% SDS followed by Proteinase K digestion, ethanol precipitation and phenol-chloroform purification. MeDIP was performed with 1μg of purified genomic DNA precipitated using the methylcytidine monoclonal antibody (Eurogenetec) as previously described (Mohn et al., 2009). For methylation specific PCR and bisulfite sequencing, genomic DNA was bisulfite deaminated using EpiTect Bisulfite Kit (QIAGEN**),** which converted unmethylated cytosine to uracil prior to PCR amplification using primer sets specific to the native or deaminated DNA. For bisulfite sequencing, DNA fragments amplified by methylation insensitive primers were cloned into pGEM-T Easy vector (Promega**)** and sent for DNA sequencing.

In situ hybridization

Riboprobes were prepared by *in vitro* transcribing and digoxigenin (DIG)-labeling PCR-amplified fragments of HIF-2α 5'UTR and *GAPDH* cloned into pGEM-T-easy (Promega) using the DIG RNA Labeling Kit (Roche). Prior to riboprobe hybridization, cells were paraformaldehyde-fixed, Triton-X-100 permeabilized and pre-hybridized in DIG Easy Hybridization Buffer (Roche). Probes were hybridized in 50% formamide/DIG Easy Hybridization Buffer overnight at 37°C. Riboprobe signal was detected using alkaline phosphatase conjugated anti-DIG antibody and NBT/BCIP staining solution (Roche).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation was carried out using the EZ ChIP kit (Millipore) according to manufacturer's instructions. DNA was precipitated through its association with RNA polymerase II (Millipore), DNMT3a (Santa Cruz), DNMT3b (Novus), GFP-Trap (Chromotek) and rabbit IgG was employed as a negative control.

RNA isolation and real-time PCR

Total RNA was extracted using TriPure Reagent (Roche) according to the supplier instructions. Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) using random hexamer primers. Samples were subjected to Real-Time quantitative PCR with IQ SYBR Green Supermix (Bio-Rad) and data was recorded and analyzed using a MX3000P thermocycler (Stratagene). The relative abundance of target mRNA or DNA was calculated using the delta-delta CT method and otherwise stated all samples were normalized the housekeeping gene *36B4* mRNA.

Primers used in the study

Primers	Sequences (5'->3')	
qPCR		
HIF2α	GTCTGCAAAGGGTTTTGGGG	TGTGAGGTGCTGCCACCAG
HIF1α	CACAGCCTGGATATGAAATTTTCTTATT	AGAATTCTTGGTGTTATATATGACAGTTGC
DNMT1	GCCCCTGAGCCCTACCGAATTG	AGCTCGCTGGAGTGGACTTGTGG
DNMT3a	CGGACATGTGGGTGGAACCTG	GCACTTCTGCCGCACCTCGT
DNMT3b	GACGGGGAAGATGGGGATGG	CGGGTGGAACGTGGGGAAG
MBD2b	TGGGCTAAGTGCTGGCAAGAGC	TGATTGAGAGGATCGTTTCGCAGTC
EYA4	AACTGAGGCAGCCACTCTGT	TCCCCACAGCTGTATCCTTC
36B4	CGACCTGGAAGTCCAACTAC	ATCTGCTGCATCTGCTTG
Ch-IP		
EPAS1	TGACAGCTGACAAGGAGAAGAA	GCAGAAGTCTCCACTGCAAA
H19	GCGAGCCGTAAGCACAGC	GCCGATTCCCATCCAGTTGAC
EYA4	TGGTTGTGGCATACACACG	GTATAGGCATCCGTCCCAGA
MSRPCR		
GAPDH	CCCGTCCTTGACTCCCTAGTG	TCTTGAGGCCTGAGCTACGTG
H19	GCGAGCCGTAAGCACAGC	GCCGATTCCCATCCAGTTGAC
EPAS1-1	CTCCCAGACGGTCAAGTCAG	CGTCGGTGTCCTCGTACC
EPAS1-2	GTCCTGCCCTCGTTCCAA	CGCACTTCGAGGACTTGTTT
EPAS1-3	TACAATCCTCGGCAGTGTCC	GCTGTCAGACCCGAAAAGAG
EPAS1-4	TGACAGCTGACAAGGAGAAGAA	GCAGAAGTCTCCACTGCAAA
MeDIP		
GAPDH	CCCGTCCTTGACTCCCTAGTG	TCTTGAGGCCTGAGCTACGTG
H19	GCGAGCCGTAAGCACAGC	GCCGATTCCCATCCAGTTGAC
EPAS1-1	CTCCCAGACGGTCAAGTCAG	CGTCGGTGTCCTCGTACC
EPAS1-2	GTCCTGCCCTCGTTCCAA	CGCACTTCGAGGACTTGTTT
EPAS1-3	TACAATCCTCGGCAGTGTCC	GCTGTCAGACCCGAAAAGAG
EPAS1-4	TGACAGCTGACAAGGAGAAGAA	GCAGAAGTCTCCACTGCAAA
Bisulfite sequencing		
EPAS1	ATAAGGAGAAGAAAAGGTAAGT	RAAAACATACACCCACAAACATT
DNMT3a exon sequencing		
Pair 1	CATCTGCTGTGGGGGCCGTG	TGTGTGACGCTGCGGACGTC
Pair 2	AGCCCATCCGGGTGCTGTCT	CCCCAGAAGTAGCGGGCCCT
Pair 3	ATGATGCGCGGCCCAAGGAG	CAGCGGAGCGAAGAGGTGGC

Preparation of cell lysates and immunoblotting

For total protein extracts, cells were washed with PBS prior to harvesting in 4% SDS and denaturation at 95 °C for 5 min. For immunoblotting, equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 h in 5% skim milk in TBST before overnight incubation at 4 °C with polyclonal antibodies against HIF-2a (Novus), HIF-1a (Novus), DNMT3a (Santa Cruz Biotech), DNMT3a2 (Abcam), DNMT3b (Novus), DNMT1 (NEB), EGFR (LabVision), pEGFR (Cell signaling), eIF4E (Santa Cruz Biotech), eIF4E2 (Genetex), RBM4 (ProteinTech), Hsp90 (Cell signaling), GAPDH (Genetex) and β-actin (Sigma). Horseradish peroxidase-conjugated anti-mouse (Amersham Biosciences) or anti-rabbit (Jackson ImmunoResearch) secondary antibodies were used to detect bands by enhanced chemiluminescence (Pierce) and Kodak film.

Cell proliferation and viability assay

Cell proliferation assays were performed as previously described (Franovic et al., 2010). Briefly, cells were incubated for 48h in serum-free media and then labeled with bromodeoxyuridine (BrdU) (Roche) for 2h. Cells were ethanol fixed and immunostained using the BrdU Labeling and Detection Kit I (Roche). The ratio of BrdU-labeled to Hoechst-stained cells was determined by counting a representative field acquired by fluorescence microscopy. A minimum of 200 cells per field were counted, three times per experiment. Cell death was measured by tryspinizing monolayer of cells in culture and counting the number of trypan blue positive cells versus non-stained cells on a hemocytometer.

Lentivirus infection

Lentiviral vectors expressing human DNMT3a shRNAs were purchased from Open Biosystems, while HIF-2α and DNMT3a cDNA expression vectors were purchased from GeneCopoeia. Scramble and empty vector were obtained from Addgene Inc and GeneCopoeia, respectively. Viruses were produced by quadruple transfection of HEK293T cells with: effector plasmids, pLP1, pLP2 and pLP/VSV-G. 72h after Lipofectamine (Invitrogen) transfection, viral particles were collected by sucrose gradient centrifugation. Cells were infected 24h after plating with supernatant from transfected HEK293T and 4ng/ml Polybrene (Sigma). Medium was replaced 24h after infection and every 48h afterwards.

RNA immunoprecipitation

Formaldehyde (1%) was added to cells for 30 min at room temperature. Glycine (200 mM) was added for 5 min to stop the reaction, followed by two washes with cold PBS. Cells were lysed in 1 ml of modified RIPA. RNase inhibitor (Ambion) was added to modified RIPA just before use. Samples were sonicated at 50% amplitude for two cycles of 30 s (2 s on, 2 s off) with a 1-min pause between cycles followed by a DNase treatment 30 min. RNase A solution (Fermentas) was added for 30 min for RNase-treated control samples. Samples were pre-cleared by using 10 ml of Dynabeads (Invitrogen) and Immunoprecipitation was performed with 2 mg primary antibody overnight at 4 °C. Samples were centrifuged for 15 min at 12,000g and 4 °C and the supernatant was incubated with Dynabeads equilibrated for 1 h in 2% BSA at 4 °C. Beads were recovered and washed five times with modified RIPA and eluted with 20 ml of 0.1 M glycine (pH

3.0). Bound proteins were removed by adding 200 mM NaCl and 20 mg of Proteinase K to the supernatant and incubating for 1 h at 42 °C. Crosslinking was reversed by incubation overnight at 65 °C. RNA extraction and RT–PCR analysis were performed to identify interacting RNA segments. Inputs were 2% of the sample.

Protein synthesis by [^{35}S]Met incorporation

Cells were grown for 48 h in 10-cm plates. Serum-free conditions supplemented with 1% insulin-transferrin-selenium (Invitrogen) were used when cells were incubated under hypoxia to detect stronger *de novo* protein accumulation. At 1 h before the end point, the medium was changed to glutamine-free, methionine-free and cysteine-free DMEM and labelled 30 min later with [^{35}S]Met (33 mCi/ml) for 30 min. Cells were lysed for 30 min in 1 ml of modified RIPA (50 mM Tris-HCl pH 7.4, 1% Igepal, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na3VO4, 1 mM NaF, and 1 mg/ml aprotinin, leupeptin and pepstatin) at 4 °C. Samples were run on a 6% SDS–PAGE gel, dried for 90 min at 80 °C and exposed overnight to X-ray film at -80 °C. For total cellular protein synthesis rates, samples were loaded on a per-cell basis (500,000 cells).

RESULTS

1. DNMT3a-mediated epigenetic silencing of *EPAS1* in renal cancers

1.1 *EPAS1* mRNA is preferentially expressed in human renal cell carcinomas (RCC) compared to normal epithelial cells of the kidney.

EPAS1 expression is tightly regulated in humans resulting in only a subset of cells in organs having detectable levels of the transcript (Favier et al., 2001; Wiesener et al., 2003). For example, HIF-2α mRNA is rarely observed in renal tubular epithelial cells, the cell type thought to give rise to RCC (Paliege et al., 2010; Wiesener et al., 2003). Interestingly, immunohistochemistry studies have demonstrated that expression of *EPAS1* is associated with the earliest multicellular foci following VHL loss and constitutive HIF activation (Mandriota et al., 2002; Schietke et al., 2012). This implies that *EPAS1* is silent in normal renal epithelial cells but activated in early RCC and may play an important role in oncogenesis. Indeed several reports show strong expression of the *EPAS1* gene in RCC (Sandlund et al., 2009; Turner et al., 2002; Xia et al., 2002; Onita et al., 2002).

To investigate the molecular pathway restricting HIF-2α expression in renal epithelial cells we first confirmed that HIF-2α was upregulated at the mRNA level in a panel of normal adjacent kidney and matching RCC tumour samples obtained from the Ontario tumour bank. Expectedly, HIF-2α mRNA was consistently lower in normal kidney compared to corresponding RCC tumour samples from stage I and stage III tumours (**Figure 1A**).

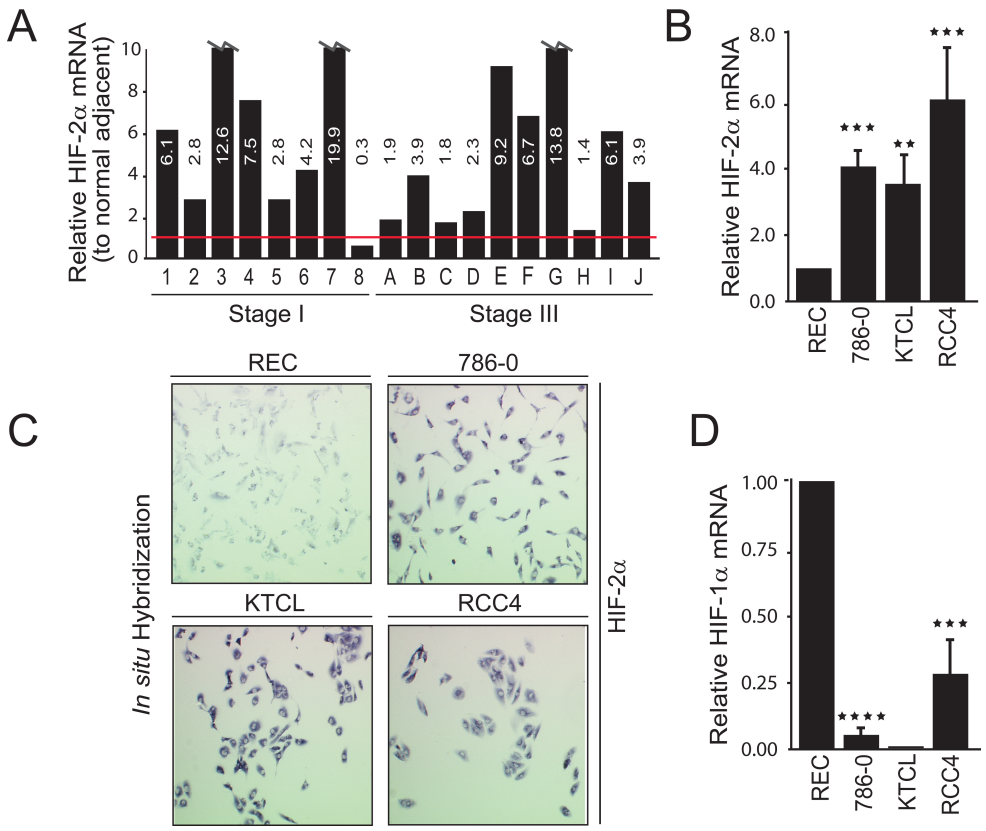


Figure 1

Figure 1. HIF-2α mRNA is upregulated in RCC: (A) Real-time PCR quantification (qPCR) of HIF-2α mRNA in a panel of stage I and III renal cell carcinomas (RCC). RNA was extracted from homogenate tissue of primary tumours and normalized to the corresponding normal adjacent kidney. (B) qPCR of HIF-2α transcripts in total RNA samples extracted from human primary renal epithelial cells (REC) and from three different RCC cell lines (786-0, KTCL, RCC4). (C) Cultures of RECs and RCC cell lines were fixed and tested for HIF-2α mRNA expression by *in situ* hybridization (ISH) using HIF-2α specific DIG-labelled riboprobe. See **Appendix 1** for validation of the ISH riboprobe. (D) qPCR of HIF-1α in total RNA samples extracted from RECs and three different RCC cell lines. Error bars represent SEM. Significance was measured by Student t test; **P < 0.01, ***P < 0.001.

This trend was also recapitulated in a pure culture of primary human renal epithelial cells (REC) when compared to three human RCC cell lines and HIF-2α mRNA levels were consistently higher in these cancer cells (**Figure 1B**). Because the qPCR averages the mRNA levels of HIF-2α from the samples, we used *in situ* hybridization to verify in parallel experiments that the expression of HIF-2α mRNA was systematically low in all primary renal epithelial cells compared to RCC cell lines (**Figure 1C**). Prior to the *in situ* detection experiments, the DIG-labelled riboprobe was validated by northern blotting total RNA samples of cells transfected with HIF-1α and HIF-2α cDNA constructs or cells expressing shRNA targeting HIF-2α (**Appendix 1**). To further support that HIF-2α was differentially expressed in normal renal cells, we compared the levels of HIF-1α and HIF-2α mRNA in RECs and RCC cancer cell lines. As expected from previous observations reporting the preferential expression of the HIF-1α isoform in normal kidney and precancerous lesions (Mandriota et al., 2002; Kondo et al., 2002; Raval et al., 2005), our analysis verified that normal kidney cells preferentially expressed HIF-1α mRNA and not HIF-2α (**Figure 1D**). Overall this data recapitulated the scenario where primary renal cells repressed HIF-2α mRNA expression and established that the renal epithelial cells and corresponding cancer cell lines was a suitable system to study HIF-2α oncogenic expression.

1.2. *EPAS1* is methylated in normal human kidney and renal epithelial cells

HIF-2α mRNA expression is mitigated in tissue under normal cellular growth conditions. The mechanism regulating this silencing is unknown, however one of the salient features of

the *EPAS1* promoter is the presence of a large CpG island (**Figure 2A**) that is often associated with DNA methylation and epigenetic gene silencing (Bird A, 2002). Therefore, we investigated the DNA methylation status of the *EPAS1* promoter using the methylation-sensitive restriction digest PCR (MSRPCR) assay. This technique harnesses the property of DNA methylation-sensitive recombinant restriction enzymes to measure differentially methylated genes throughout the genome (Frühwald and Plass, 2002). For example HpaII and MspI restriction endonucleases both recognise the palindromic DNA sequence 5'-CCGG-3', but HpaII is incapable of cutting the DNA if any cytosine is methylated (Frühwald and Plass, 2002). In contrast MspI is not inhibited by cytosine methylation and can cut DNA irrespective of the DNA methylation status of the genes (Lei et al., 1996; Okano et al., 1999) therefore providing an internal control ensuring the proper digestion of the samples.

The MSRPCR assay is highly sensitive but has the caveat of restricting the DNA methylation analysis to genes containing the 5'-CCGG-3' sequence. *EPAS1* CpG island contained multiple HpaII/MspI recognition sites and sometimes up to 4 adjacent 5'-CCGG-3' sequences over the 200bp DNA fragment of the *EPAS1* promoter (**Figure 2A**). In order to test for DNA methylation in human kidney samples and cultured renal epithelial cell genomic DNA, we used MSRPCR and focused our analysis on sections of the CpG island surrounding Exon1 and the *EPAS1* promoter since these were located within the CpG Island and enriched in HpaII/MspI sites (**Figure 2A**). A total of four different sites were tested and the expected PCR amplicons are depicted in **Figure 2A**. Normal human kidney and primary cells showed minimal cleavage by the methylation-sensitive restriction enzyme HpaII (**Figure 2B**) but consistent digestion by MspI throughout, indicating the presence of

DNA methylation within these regions, as each amplicon possessed at least two HpaII cleavage sites. As expected, the imprinted gene *H19* was also methylated, while the constitutively expressed *GAPDH* was digested efficiently indicating a lack of methylation at this site (**Figure 2B**).

The presence of methyl-cytosine within the *EPAS1* promoter was further validated in REC using methylated-DNA immunoprecipitation (MeDIP) (**Figure 2C**) and bisulfide deamination (**Figure 2D**) assays. The MeDIP assay enables detection of methylated DNA in a sequence-independent manner and was used to complement our MSRPCR analysis of the methylated cytosine (^{m}C) within the *EPAS1* CpG Island. For this approach we used a commercially available monoclonal antibody raised against methylcytidine to specifically enrich for methylated DNA in pre-sonicated genomic DNA collected from RECs. We used a mouse IgG preparation to control for non-specific interactions and compared by PCR amplification the enrichment of ^{m}C to 5% of the input DNA used for immunoprecipitation as described before (Mohn et al., 2009). These experiments confirmed the fragment of *EPAS1* CpG Island previously found to be resistant to HpaII to be specifically enriched in ^{m}C, therefore providing additional proof of DNA methylation within *EPAS1* in normal kidney epithelia (**Figure 2C**).

Finally to consolidate our DNA methylation analysis and to identify the precise nucleotides targeted for DNA methylation on *EPAS1* we mapped the DNA sequence corresponding to *EPAS1* first exon and part of the first intron using bisulfite sequencing. This region was not specifically selected *a priori* but because regions upstream of *EPAS1* transcription start site (TSS) generated PCR amplicons with inconsistent quality. Despite this, we were able to

detect three nucleotides methylated in the primary cells genomic DNA and interestingly two of those ^{m}C corresponded to HpaII recognition sites that were also found to be associated with DNA methylation with our MSRPCR and MeDIP analyses (**Figure 2D**). Taken together, significant evidence correlated the restricted expression of HIF-2α mRNA in normal kidney cells with the presence of DNA methylation on this gene.

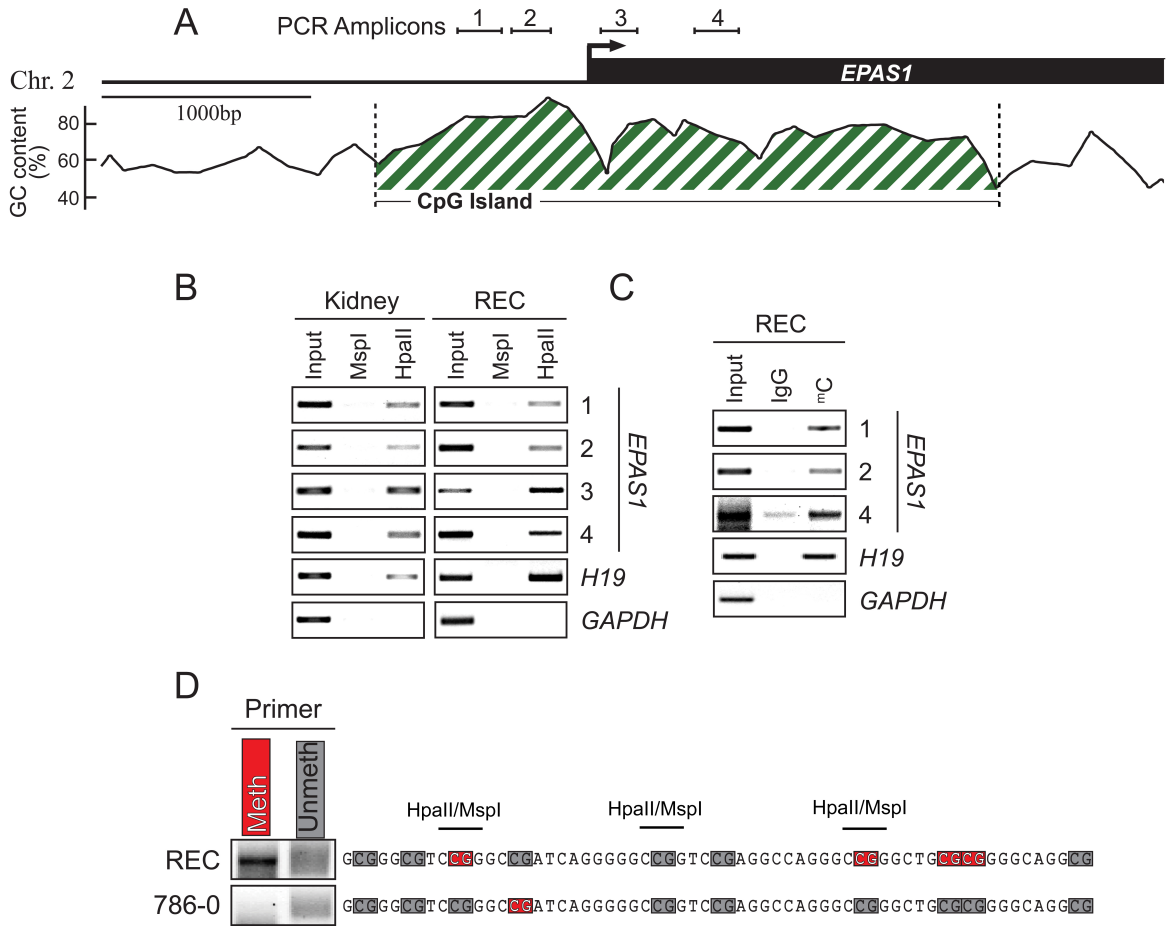


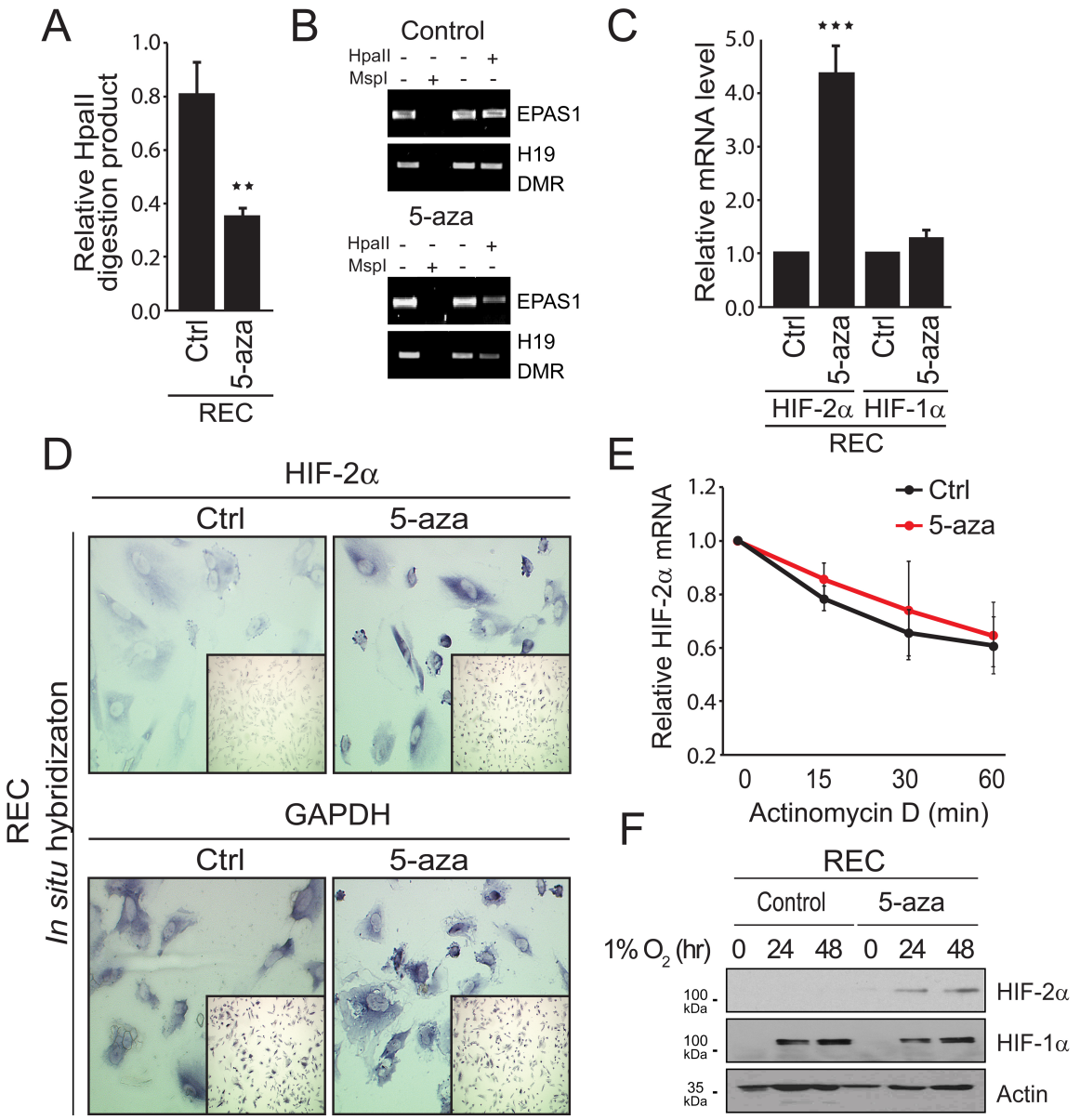
Figure 2

Figure 2. *EPAS1* is methylated in normal renal cells: (A) Primers used in this investigation are shown with the *EPAS1* locus and the CpG island defined as a region of DNA with at least 50% of GC content over a minimum of 200bp (dashed green) and in this case surrounding the transcription start site (arrow). (B) Methylation-Sensitive Restriction digest PCR (MSRPCR) assay performed first by digesting genomic DNA isolated from normal human kidney and primary renal epithelial cells (REC) with isoschizomers HpaII and MspI endonucleases. Various primers covering *EPAS1* CpG Island were used in the analysis as depicted in A). Input was total DNA in the reaction, MspI digests controlled for methylation-insensitive cutting and HpaII digests were enriched in methylated DNA. *H19* and *GAPDH* locus were used as positive and negative controls of DNA methylation, respectively. (C) Methylated DNA immunoprecipitation (MeDIP) of the *EPAS1*, *H19* and *GAPDH* loci from primary RECs. 5-methylcytosine specific antibody (^{m}C) was used to pull down methylated DNA. PCR was used to detect DNA methylation with the same primers as in B). Inputs represent 1% of the lysates, and IgG was used as a negative control. (D) Genomic DNA extracted from RECs and 786-0 cells was deaminated by bisulfite conversion and primers complementary to unmethylated (U) or methylated (M) *EPAS1* DNA were used to measure the DNA methylation state. Using primers designed to amplify both unmethylated and methylated DNA, the bisulfite-treated genomic DNA was sent for sequencing and the precise nucleotides that underwent CpG methylation are identified in red. HpaII and MspI recognition sites are highlighted above the *EPAS1* genomic sequence that was tested for bisulfite sequencing and correspond to part of the region amplified by primer set 4 in B) and C).

1.3. DNA methylation regulates the expression of HIF-2α in human renal epithelial cells

To determine whether these DNA methylation events were responsible for inhibiting *EPAS1* expression in normal renal epithelia, we treated REC with the general DNA methylation inhibitor 5-azacytidine (5-aza). 5-aza is a synthetic mimetic of cytidine that lacks the site for the addition of the methyl group and represses whole cell DNA methyltransferase (DNMTase) activity (Chiak A, 1974). 5-aza is prominently incorporated in replicating cells and causes passive demethylation of their genome by covalently trapping DNMTases (Christman JK, 2002). Upon exposure of renal primary cells to 5-aza, the *EPAS1* promoter became sensitive to HpaII digestion (**Figure 3A-B**), demonstrating a significant loss in DNA methylation at this locus. An independent DNA methylated locus, H19 also showed loss of HpaII resistance (**Figure 3B**). Using qPCR, we correlated this effect with an increase in HIF-2α expression (**Figure 3C**) that was not seen for HIF-1α mRNA (**Figure 3C**). Further analysis using *in situ* hybridization demonstrated that the induction of HIF-2α mRNA by 5-aza was not limited to a few cells but instead produced a uniform and global upregulation (**Figure 3D**) that was not caused by alterations in HIF-2α stability (**Figure 3E**). At steady state in normoxia, HIFα factors are post-translationaly targeted by PHDs for VHL-dependent ubiquitination leading to their proteasomal degradation but these reactions are inhibited in low oxygen. Thus to confirm that the general loss of DNA methylation was affecting the differential expression of HIFα transcription factors, we tested the protein levels of HIF-1α and HIF-2α in hypoxia with or without 5-aza using western immunoblotting.

As expected the combined effect of 5-aza treatment and hypoxia caused the misexpression of HIF-2α protein but did not change the status of HIF-1α in primary renal epithelial cells (**Figure 3F**). Interestingly, the mRNA levels of HIF-2α in RCC cells were similar to those observed in 5-aza treated REC cells (**Figure 4A**). In addition, 5-aza treatments had no significant effect on HIF-2α expression in the VHL mutated cell line 786-0 (**Figure 4A, B**). To further validate the contribution of DNA methylation towards the oncogenic expression of HIF-2α protein we compared the effect of 5-aza treatment between tumourigenic 786-0 cells that constitutively express HIF-2α and hypoxia-treated RECs with or without 5-aza, to find that they were similar (**Figure 4B**). Overall these results agreed for a role of DNA methylation in the epigenetic silencing of *EPAS1* in normal kidney cells and suggested that loss of DNA methylation could be accounting for *EPAS1* oncogenic expression in renal cancer cells.


A
Relative HpaII digestion product
1.0
0.8
0.6
0.4
0.2
0.0
**
Ctrl
5-aza
REC
B
Control
HpaII - - - +
MspI - + - -
EPAS1
H19 DMR
5-aza
HpaII - - - +
MspI - + - -
EPAS1
H19 DMR
C
Relative mRNA level
5.0
4.0
3.0
2.0
1.0
0.0
***
Ctrl
5-aza
Ctrl
5-aza
HIF-2α
HIF-1α
REC
D
HIF-2α
Ctrl
5-aza
REC
In situ hybridizaton
GAPDH
Ctrl
5-aza
E
Relative HIF-2α mRNA
1.2
1.0
0.8
0.6
0.4
0.2
Ctrl
5-aza
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15
30
60
Actinomycin D (min)
F
REC
Control
5-aza
1% O2 (hr)
0 24 48 0 24 48
100 kDa
HIF-2α
100 kDa
HIF-1α
35 kDa
Actin

Figure 3

Figure 3. DNA methylation regulates *EPAS1* expression: (A,B) Quantification of DNA methylation loss after 5-azacytidine (5-aza) treatment of primary renal epithelial cells (REC). MSRPCR analysis was done by qPCR using primer set 4 and genomic DNA of RECs left untreated (Ctrl) or exposed to 10μM 5-azacytidine (5-aza) for 72h. Values were calculated by assessing the percentage of DNA uncut following HpaII digestion relative to input and a representative experiment is shown in B). (C) Expression of HIF-2α and HIF-1α mRNA was quantified by qPCR and compared between control and 5-aza treated RECs. (D) *In situ* hybridization (ISH) assays performed on low passage RECs treated or not with 10μM 5-aza for 72h using DIG-labeled antisense riboprobes specific to HIF-2α and GAPDH mRNAs. Insets are lower magnification. (E) RNA polymerase inhibition treatment using actinomycin D and comparison of HIF-2α mRNA levels by real-time PCR as a function of time in control (PBS) or 5-aza pre-treated cells. (F) Western blot analysis of HIF-2α and HIF-1α hypoxic protein levels in control or 5-aza treated RECs incubated in a low oxygenated environment for 24h and 48h. Error bars represent SEM. Significance was measured by Student t test; $^{**}P < 0.01$, $^{***}P < 0.001$.

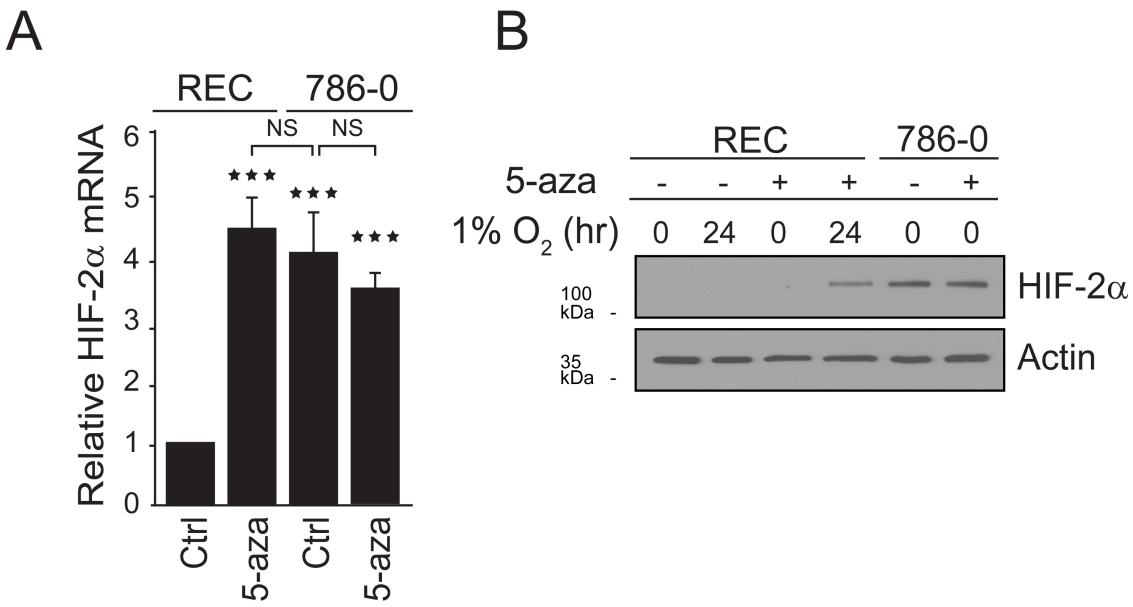


Figure 4

Figure 4. DNA methylation regulates HIF-2α oncogenic expression: (A) qPCR analysis of HIF-2α mRNA levels in total RNA samples extracted from RECs and 786-0 cells treated in parallel with control and 5-azacytidine (5-aza). (B) Western blot experiment to measure the hypoxic protein levels of the samples corresponding to A). Error bars represent SEM. Significance was measured by Student t test; ***P < 0.001.

1.4. *EPAS1* is demethylated in human renal cancers and cell lines

Numerous groups (Mandriota et al., 2002; Raval et al., 2005; Schietke et al., 2012) have demonstrated that HIF-2α expression is induced in the earliest multicellular foci of VHL disease, though the mechanism regulating this expression has not been elucidated. To test if loss of DNA methylation accounted for HIF-2α expression in clinical kidney cancer cases, we compared the DNA methylation pattern observed at the *EPAS1* locus in pairs of renal tumours and matched normal human kidneys (**Figure 5A**). MSRPCR assays revealed that the normal methylation of the *EPAS1* promoter was lost in multiple tumours tested, while DNA methylation levels at the control *H19* and *GAPDH* loci were unaffected (**Figure 5A**). On a total of 8 tumour samples tested, 5 showed loss of *EPAS1* DNA methylation, 3 had no change and none had higher DNA methylation on the fragment of DNA tested. Next we mapped *EPAS1* DNA methylation in three established RCC cells lines (786-0, KTCL and RCC4). Compared to the primary culture of REC cells, all three RCC cell lines displayed marked hypomethylation in the *EPAS1* promoter (**Figure 5B**). This loss of DNA methylation was corroborated by bisulfite analysis as well (**Figure 2D**), altogether supporting the implication of DNA methylation in the events causing *EPAS1* expression in RCC. Overall these results rallied with the general reduction in epigenetic silencing of *EPAS1* in renal cancers and suggested that parts of the normal DNA methylation machinery could be altered in these cancer cells.

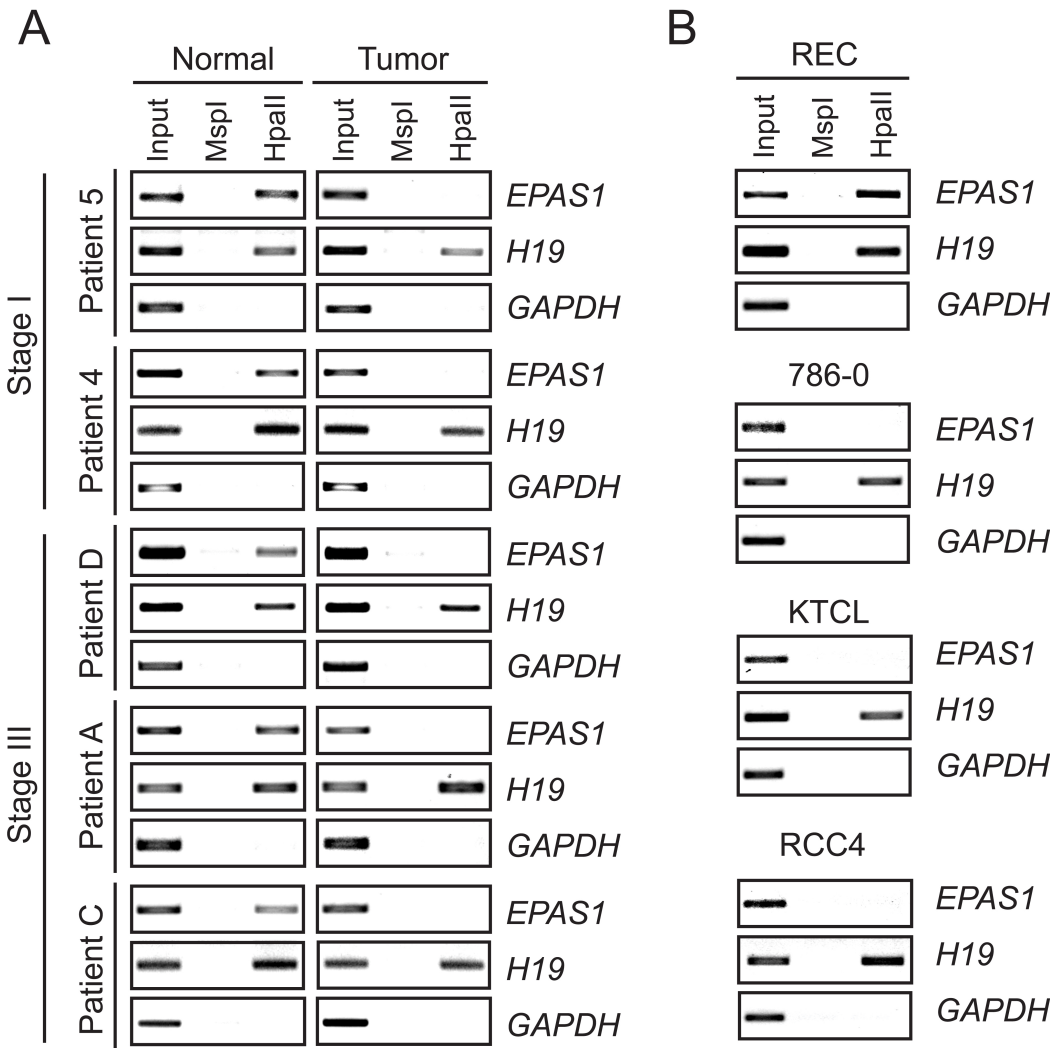


Figure 5

Figure 5. *EPAS1* DNA methylation is lost in RCC: (A) *EPAS1* CpG methylation was tested on genomic DNA extracted from two stage I and three stage III primary renal cell carcinoma (RCC) tumours and compared to matching normal human kidney tissues using MSRPCR with primer set 4 (see **Figure 2A**). (B) Three cancer cell lines 786-0, KTCL and RCC4 were compared with primary renal epithelial cells (REC) for *EPAS1* DNA methylation using MSRPCR technique and the same primers as in A). *H19* and *GAPDH* were controlling for methylated and unmethylated genes, respectively.

2. DNMT3a expression is lost in human renal cancers and cell lines

Our investigation concerning the loss of epigenetic regulation of *EPAS1* in renal cancers lead us to presuppose the loss of activity of one or many DNMTase enzymes. Cytosine methylation is catalyzed by a family of DNMTases including: DNMT1, DNMT3a and DNMT3b (Bird A, 2002). DNMT1 acts to maintain the methylation pattern from the template strand to the newly synthesized strand during DNA replication (Turek-Plewa and Jagodzinski, 2005), while DNMT3a and 3b are *de novo* methyltransferases that establish post-replicative methylation patterns to regulate gene expression (Bird A, 2002). Therefore, we sought to determine which of these enzymes was responsible for the DNA methylation observed on the *EPAS1* CpG Island. We first interrogated the protein atlas databank for renal normal and cancerous tissues stained with different DNMTase antibodies hoping to find a correlation between expression of these enzymes and RCC development. In many instances we found DNMT3a to be unusually downregulated or mislocalised to the cytoplasm of tumour cells (**Figure 6A**).

To further confirm this observation we tested independently the expression of DNMT1, DNMT3a, DNMT3b and control MBD2b mRNAs in our RCC samples and matched normal adjacent tissues from stage I and stage III primary renal tumours. Because of the low amount of tissue, especially for stage I RCC, we extracted total RNA and performed qPCR analysis to test gene expression change between tumour and matching normal kidney samples.

As shown before, loss of *EPAS1* DNA methylation during early tumourigenesis was correlated with an increase in HIF-2α mRNA levels (**Figure 1A; Figure 5A**) and we further discovered a systemic reduction of DNMT3a expression (**Figure 6B**) in these samples, while DNMT3b, DNMT1 and a methyl-binding domain cofactor MBD2b had highly variable expression profiles, when comparing tumour samples to adjacent kidney tissue of stage I primary RCC (**Figure 6C**). Three selected tumour and matching normal tissue samples were tested for DNMT3a and HIF-2α protein levels in parallel western blots and thus confirmed the correlation between DNMT3a loss and activation of HIF-2α expression (**Figure 6D**).

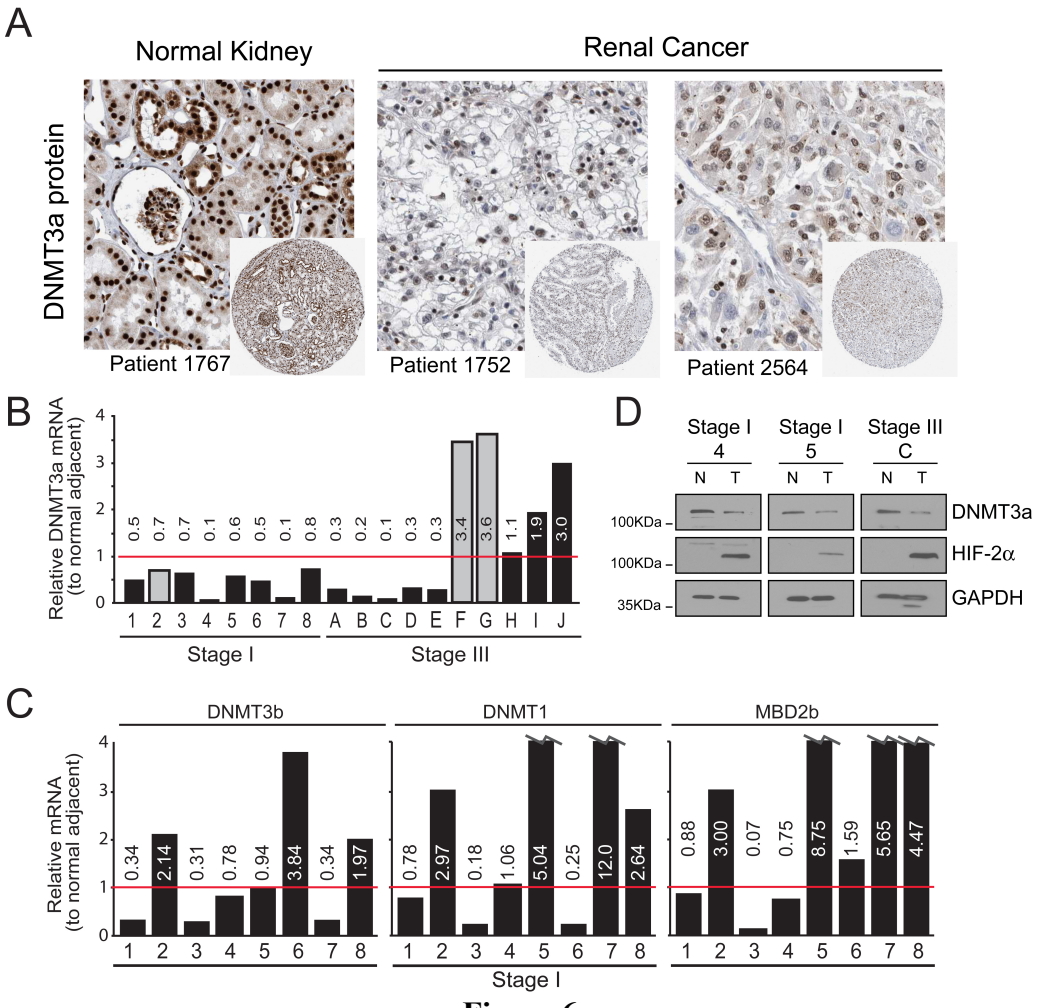


Figure 6

Figure 6. DNMT3a is lost in RCC: (A) The protein atlas databank was interrogated for the expression of DNMT3a in normal kidney and renal tumour samples stained with DNMT3a antibody and representative samples are shown. (B) Relative expression of DNMT3a in a panel of primary renal cell carcinoma (RCC) tumours samples as measured by qPCR. Tumour samples were normalized to their normal adjacent tissue counterparts. Samples in grey represent DNMT3a truncated mutant mRNA (see **Figure 7**). (C) Relative mRNA expression of several components involved in DNA methylation was measured by qPCR in stage I RCC and relative expression was normalized to adjacent normal kidney. Genes tested were DNA methyltransferase 3b (DNMT3b), DNA methyltransferase 1 (DNMT1), and Methyl-Binding Domain 2B (MBD2B). (D) DNMT3a and HIF-2α protein levels were assessed in three primary tumours and matching normal kidney samples from B) and GAPDH was used as loading control.

Since DNMT3a loss-of-function mutations have been described in a number of different human cancers, we also tested for the presence of a coding sequence variant of DNMT3a using simple RT-PCR amplification and direct DNA sequencing. We focused our analysis on the catalytic domain of the enzyme and found three deletion mutants expressed specifically in tumour samples (**Figure 7,** see also **Figure 6B** grey bars). Interestingly, HIF-2α mRNA was upregulated in these RCC cases alleging loss of DNMT3a catalytical activity (**Figure 1A; Figure 7**).

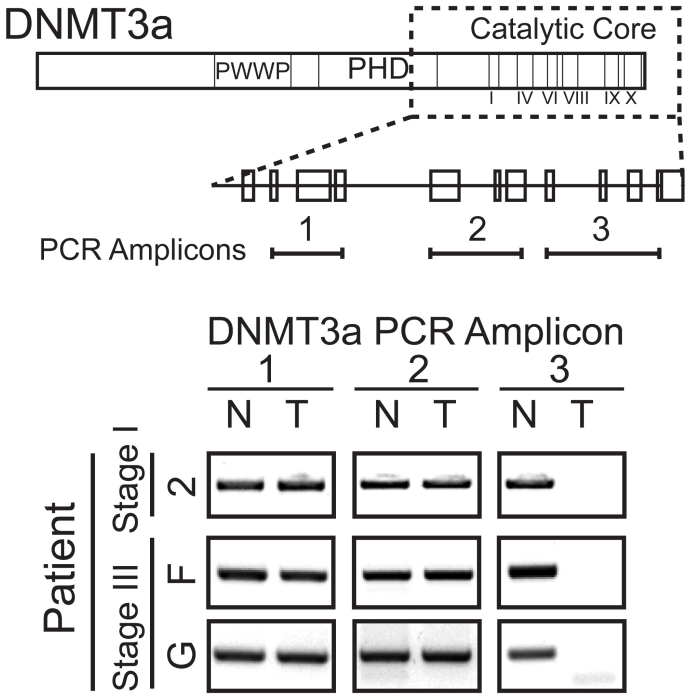


Figure 7

Figure 7. Gross deletion of DNMT3a in RCC: Primary tumour samples were screened for DNMT3a mutations with primers specifically targeting the sequence coding for the N-terminal catalytic core. A schematic diagram of the DNMT3a protein (top) indicates the genomic organization of the catalytic core-coding region of DNMT3a, which is expanded below to highlight the location of the PCR amplicons screened. (Bottom) RT-PCR of the DNMT3a catalytic core, using the indicated primer sets, was used to screen normal adjacent kidney and stage I and III renal cell carcinoma (RCC) tumour tissue samples for gross truncations. PHD, plant homeodomain, PWWP, proline and tryptophan rich domain.

Next we tried to reconcile these findings in RECs and three established RCC cancer cell lines by comparing their respective DNMT3a levels. As expected, compared to the primary culture of REC cells, all three renal lines (786-O, KTCL and RCC4) displayed lower DNMT3a mRNA and protein levels (**Figure 8A-C**). Again here, when DNMT3a, DNMT3b, DNMT1 and MBD2b expression was assessed, only DNMT3a was systematically downregulated in all RCC lines tested compared to RECs (**Figure 8A**). Importantly, the depletion of DNMT3a correlated with marked hypomethylation in the *EPAS1* promoter (**Figure 8, Figure 5B**) and upregulation of HIF-2α mRNA levels as detected by qPCR (**Figure 1B**) and *in situ* hybridization (**Figure 1C**) in the renal cancer cells. Our analyses of primary tumours and adjacent normal tissues were reproduced in RECs and corresponding cancer cells, supporting that loss of DNMT3a expression could be a founding factor in the alteration of *EPAS1* epigenetic silencing in cancers.

The DNMT3a gene encodes a full length and a shorter mRNA transcribed from an alternative promoter each producing two gene products DNMT3a1 and DNMT3a2, respectively (Chen, et al., 2002). Studies have shown DNMT3a2 to be enriched in embryonic tissues and stem cells (Weisenberger, et al., 2002; Chen et al., 2002). Since our qPCR and protein expression analyses either measured pan or DNMT3a1-specific expression, respectively, we also tested for isoform specific expression of DNMT3a in RECs and RCC using DNMT3a1 and DNMT3a2-specific primers in parallel to western blot analysis using an antibody recognizing both peptides (Chen et al., 2002; **Figure 8B, 8C**).

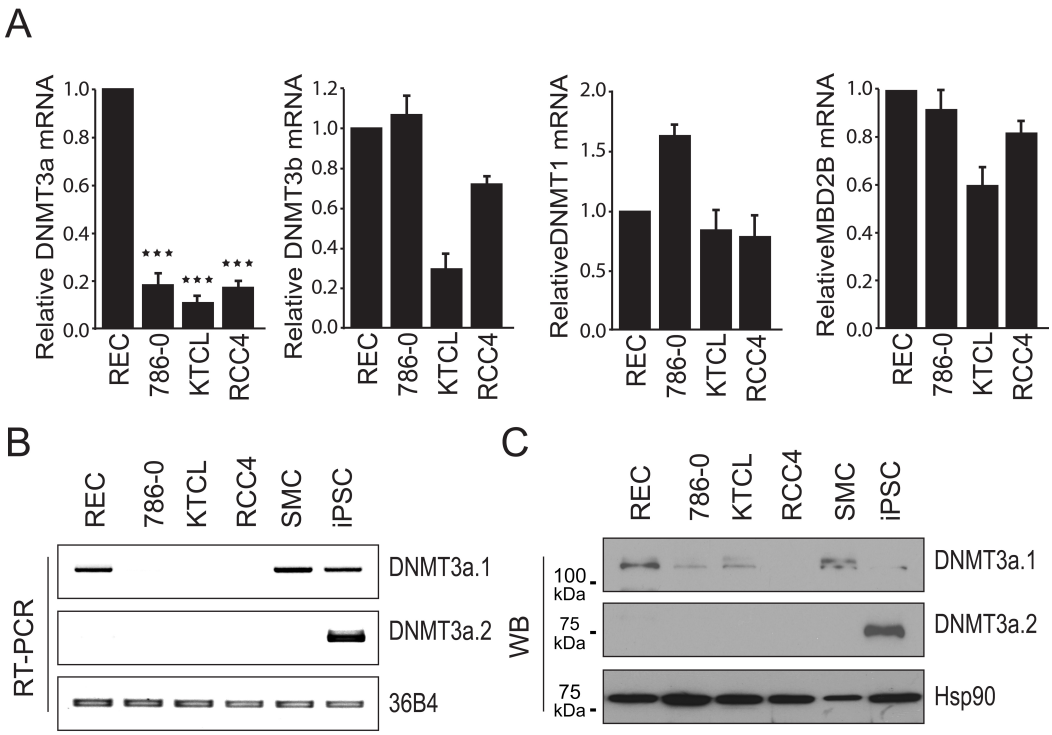


Figure 8

Figure 8. Loss of DNMT3a in RCC cell lines: (A) Relative gene expression of several components involved in DNA methylation was measured by qPCR in three renal cell carcinoma (RCC) cell lines; 786-0, KTCL, RCC4 and relative expression was normalized to primary cells. Genes tested were DNMT3a (left), DNMT3b (Middle left), DNMT1 (Middle right) and MBD2B (right). Total RNA (B) and protein (C) extractions from RECs and three RCC cell lines, 786-0, KTCL, and RCC4, were analyzed by RT-PCR (B) and Western blot (C) for DNMT3a1 and DNMT3a2 expression in the samples from A). DNMT3a.1 and DNMT3a.2-positive cell lines, smooth muscle cells (SMCs), and induced pluripotent stem cells (iPSCs), respectively, were used as positive controls in these experiments.

We found DNMT3a1 to be the main DNMT3a isoform expressed in primary cells and both DNMT3a1 mRNA and polypeptide to be downregulated in RCC compared to primary human renal epithelial cells (**Figure 8B, 8C**). Altogether, we found consistent downregulation of DNMT3a and loss of *EPAS1* epigenetic silencing in renal tumour tissues compared to normal epithelia. We further matched these findings in RCC cell lines and primary cells supporting that this *in vitro* system can be utilized to test the relevance of DNMT3a loss with regards to HIF-2α-related cancer phenotypes.

2.1. Expression of DNMT3a is sufficient to repress HIF-2α expression in human RCC cells

Given the significant reduction in DNMT3a levels observed in human renal carcinomas and cell lines (**Figure 9A**), we decided to study the consequence of re-introducing DNMT3a expression in renal cancer cells. First we specifically asked whether *EPAS1* epigenetic silencing could be restored upon stable re-introduction of DNMT3a alone in 786-0 cells. First, the exogenously-expressed GFP-DNMT3a fusion protein was capable of associating with both the *EPAS1* promoter and the DNMT3a-specific target Eye absent homolog 4 (*EYA4*) (Choi et al., 2011), supporting that the mechanisms targeting DNMT3a to the DNA were functional in these cancer cells and could be harnessed to restore normal *EPAS1* epigenetic silencing (**Figure 9B**). Next, we tested the epigenetic status of *EPAS1* in DNMT3a-reintroduced 786-0 cells and MSRPCR revealed that DNA methylation returned at levels similar to that observed in primary REC (**Figure 9C**). This restoration in the *EPAS1* methylation also correlated with a reduction in HIF-2α mRNA close to levels of

primary cells (**Figure 9D**). These reductions in HIF-2α mRNA perfectly correlated with reduced protein levels in the cell lines tested above (**Figure 9E**).

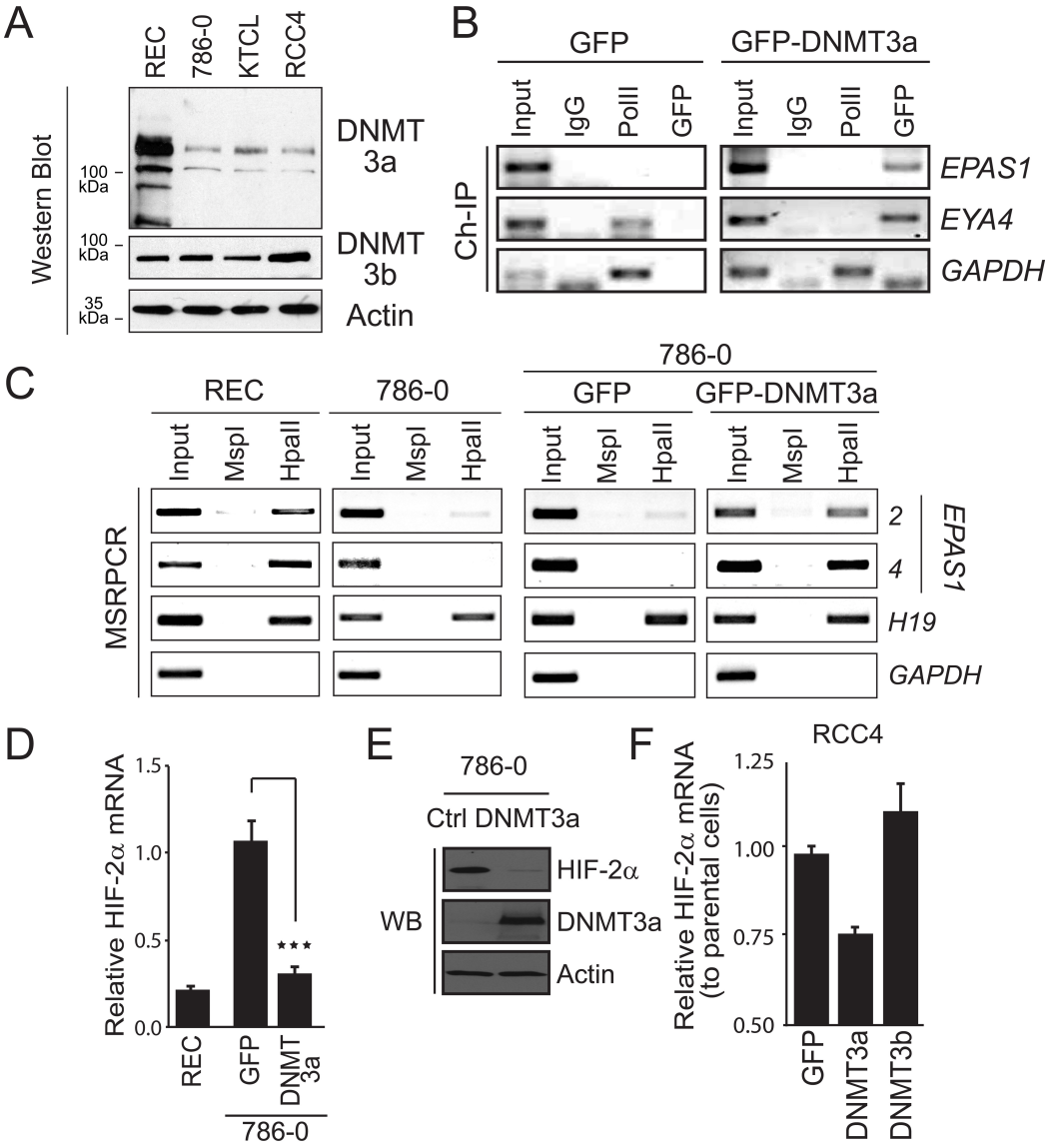


Figure 9

Figure 9. DNMT3a is sufficient to repress HIF-2α expression: (A) Primary renal epithelial cells (REC) and renal cell carcinoma (RCC) cell lines (786-0, KTCL, RCC4) were tested for DNMT3a protein levels by immunoblotting total cell extracts with a DNMT3a-specific antibody. DNMT3b and actin were used as controls. (B) Chromatin immunoprecipitation (ChIP) technique tested for proper targeting of recombinant GFP-DNMT3a by using GFP-trap beads, RNA polymerase II and IgG antibody. PCR amplification of bound chromatin was done using *EPAS1* (set 4 in **Figure 1A**) and *EYA4* specific primers were used as an independent positive control. (C) GFP-DNMT3a reintroduced and GFP control cells were tested for *EPAS1* DNA methylation using MSRPCR and the primers depicted in **Figure 1A**. (D) qPCR analysis of HIF-2α mRNA levels correlated changes in DNA methylation with *EPAS1* expression and DNMT3a. HIF-2α mRNA levels in reintroduced DNMT3a and control stable 786-0 clones were normalized to parental 786-0 cells. (E) Protein levels of HIF-2α and DNMT3a in 786-0 stably reintroduced with GFP-DNMT3a. (F) *EPAS1* differential expression was also tested by qPCR in RCC4 after transient expression of GFP, GFP-DNMT3a and GFP-DNMT3b.

We used another RCC cell line (RCC4) that was not expressing DNMT3a to transiently reintroduce GFP-DNMT3a expression and found that HIF-2α mRNA was also downregulated similarly to the 786-0 stable cell lines (**Figure 9F**). Interestingly expression of GFP-DNMT3b did not affect expression of HIF-2α mRNA in these conditions (**Figure 9F**). On the whole, this data identifies DNMT3a as a critical regulator of *EPAS1* epigenetic silencing and supports that loss of DNMT3a activity causes constitutive *EPAS1* expression that is observed in cancers.

2.2. DNMT3a regulates the expression of HIF-2α in normal human renal epithelial cells.

Having established that ectopic expression of DNMT3a was sufficient to restore normal epigenetic silencing of *EPAS1* in cancer cells, we tested if DNMT3a was directly implicated in regulating *EPAS1* epigenetic silencing in normal renal epithelial cells. We first performed a chromatin immunoprecipitation (ChIP) assay to test the physical interaction of DNMT3a at the *EPAS1* locus using a standard protocol (Svotelis et al., 2009). ChIP revealed a strong and specific binding for DNMT3a, but not DNMT3b at the *EPAS1* locus (**Figure 10A**), suggesting that DNMT3a could be responsible for regulating HIF-2α expression in a normal context. DNMT3b was efficiently pulled-down during the experiment and bound the *H19* locus as expected (Linhart et al., 2007).

To further test the functional implication of DNMT3a in regulating *EPAS1* expression in normal human epithelial cells, we used two different lentiviral shRNA-expressing constructs (DNMT3a(1) and DNMT3a(2)) to knockdown endogenous DNMT3a. We were capable of targeting DNMT3a mRNA and protein levels in RECs and significantly reducing its expression in the primary human cells (**Figure 10B**). Cells with reduced or no DNMT3a expression, displayed increased sensitivity to HpaII cleavage at the *EPAS1* locus, but not the imprinted *H19* locus, the latter used as an independent DNA methylation control (**Figure 10C**). Primary cells depleted of DNMT3a also possessed high levels of HIF-2α both at the mRNA (**Figure 10D)** and hypoxic protein levels (**Figure 10E**) while the expression of HIF-1α remained generally unchanged (**Figure 10E**). *EYA4* transcripts, a

known DNMT3a regulated gene (Choi et al., 2011) were also upregulated following DNMT3a knockdown (**Figure 10F**). Finally, silencing of DNMT3b (**Figure 10G, 10H**) had no effect on the methylation status of *EPAS1* (**Figure 10C**) or HIF-2α expression (**Figure 10D**). These data support that the epigenetic silencing of HIF-2α in normal renal epithelial cells requires intact DNMT3a function.

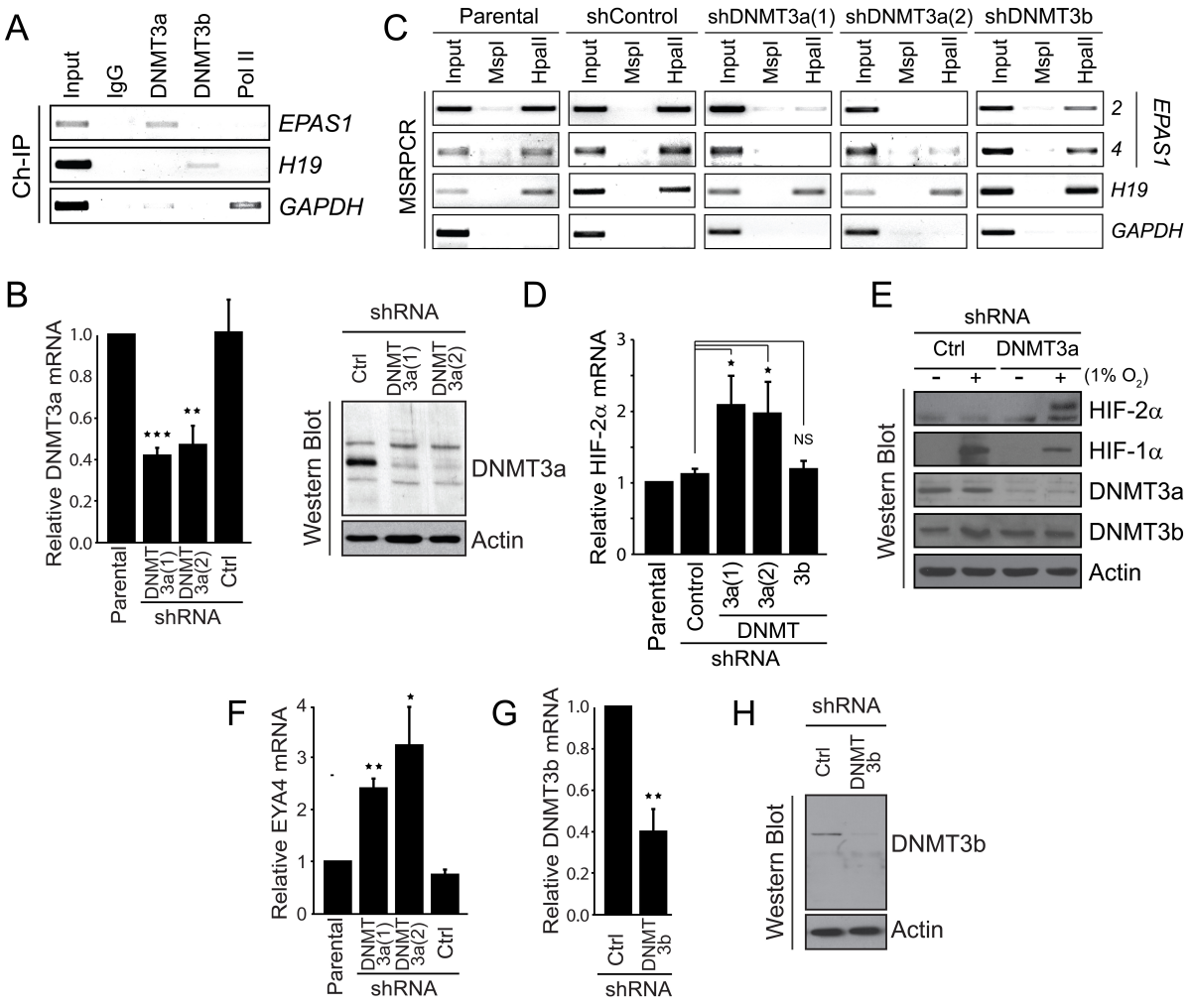


Figure 10

Figure 10. DNMT3a represses HIF-2α expression in normal renal cells: (A) Endogenous DNMT3a, DNMT3b, RNA polymerase II (PolII) and negative control IgG were immunoprecipitated from crosslinked chromatin samples of primary renal epithelial cells (REC) using specific antibodies. *EPAS1* (set 4), H19 (DNMT3b positive control) and GAPDH (PolII positive control) primers were used to amplify the DNA pulled down with the antibodies. (B) RECs were transduced with lentiviral shRNA constructs targeting two different regions of DNMT3a mRNA (sh3a(1) and sh3a(2)) and a control scramble sequence (shCtrl.). DNMT3a knockdown was confirmed by qPCR (left) and western blotting (right) total protein extracts using specific antibodies against DNMT3a and loading control actin. (C) REC genomic DNA samples corresponding to B) were extracted and tested for DNA methylation with MSRPCR using the same primers as before targeting *EPAS1* CpG Island (**Figure 1A**) and *H19* and *GAPDH* specific primers for positive and negative controls of DNA methylation, respectively. (D) Total RNA was extracted from sh3a(1), sh3a(2) and scramble shRNA transduced RECs along with the untreated control. *EPAS1*, *DNMT3a* and *EYA4* mRNA levels were measured by qPCR. (E) Total protein extracts of RECs exposed or not to 1% oxygen atmosphere were immunoblotted for the expression of HIF-2α, HIF-1α, DNMT3a, DNMT3b, and actin as a control. (F) The DNMT3a target EYA4 is up-regulated during DNMT3a knockdown. REC RNA extracts from B) were assessed for EYA4 mRNA expression by qPCR. (G) Silencing of DNMT3b in RECs. RECs infected with control or lentiviral shRNA targeting DNMT3b were monitored for DNMT3b mRNA (Left) and protein (Right) expression by qPCR and Western blot, respectively. Columns, mean (n=3). Error bars represent SEM. Significance was measured by Student t test; *$P < 0.05$. **$P < 0.01$, ***$P < 0.001$. NS, not significant.

3. Biological consequence of DNMT3a loss in RCC

3.1. DNMT3a-depleted human renal epithelial cells are incapable of growing autonomously.

We suspected that silencing of DNMT3a could be conferring a positive gain of function since DNMT3a-silenced renal primary cells consistently supported more passages and looked morphologically better in prolonged culture than control shRNA expressing REC cells (**Figure 11A**). The capacity to self-sustain proliferation is a hallmark conferred by the loss of VHL and the activation of the HIF-2α oncogenic axis in renal cancer cells (Pause et al., 1998; de Paulsen et al., 2001; Smith et al., 2005). Studies have shown that HIF-2α is specifically required for RCC tumour growth *in vivo* (Kondo et al., 2002). Since depletion of DNMT3a in primary cells was sufficient to cause the expression of HIF-2α in hypoxia we tested whether loss of DNMT3a-mediated epigenetic silencing was sufficient to bestow growth autonomy properties to human renal epithelial cells. The assay to test the capacity of primary cells to grow without serum, a key property of tumour cells (de Paulsen et al., 2001) as described before, was done by incubating cells in media without growth factors and supplemented with insulin-transferrin-selenium (ITS) instead of fetal bovin serum (FBS). Proliferation of ITS-starved cells was measured via BrdU incorporation in normoxia and hypoxia in parallel. As expected, untreated primary cells were unable to grow without serum and growth factors but so were DNMT3a-depleted cells irrespectively of oxygen concentrations (**Figure 11B**). It thus appeared that inducing the endogenous expression of HIF-2α, by targeting DNMT3a in normal renal cells did not confer growth autonomy,

suggesting that additional alterations are required to drive HIF-2α-dependent autonomous growth. To test the relevance of DNMT3a to different cancer growth-related phenotypes, we tested the capacity of RECs to form three-dimensional multicellular spheroids masses and grow in suspension and in soft agar conditions. Silencing DNMT3a did not allow primary human epithelial cells to growth in multicellular masses either in suspension or in soft agar (**Figure 11C, D**). In comparison, the RCC cell line 786-0 formed multicellular structures in both assays (**Figure 11C, D**). Furthermore, reintroduction of DNMT3a in transformed renal cancer cells (786-0) reduced their capacity to form multicellular masses in suspension and in soft agar (**Figure 11C, D**). It thus appeared that the expression of HIF-2α via DNMT3a-loss was not sufficient to cause cancer-like phenotypic growth in normal renal epithelial cells but was required for the growth of transformed renal cells.

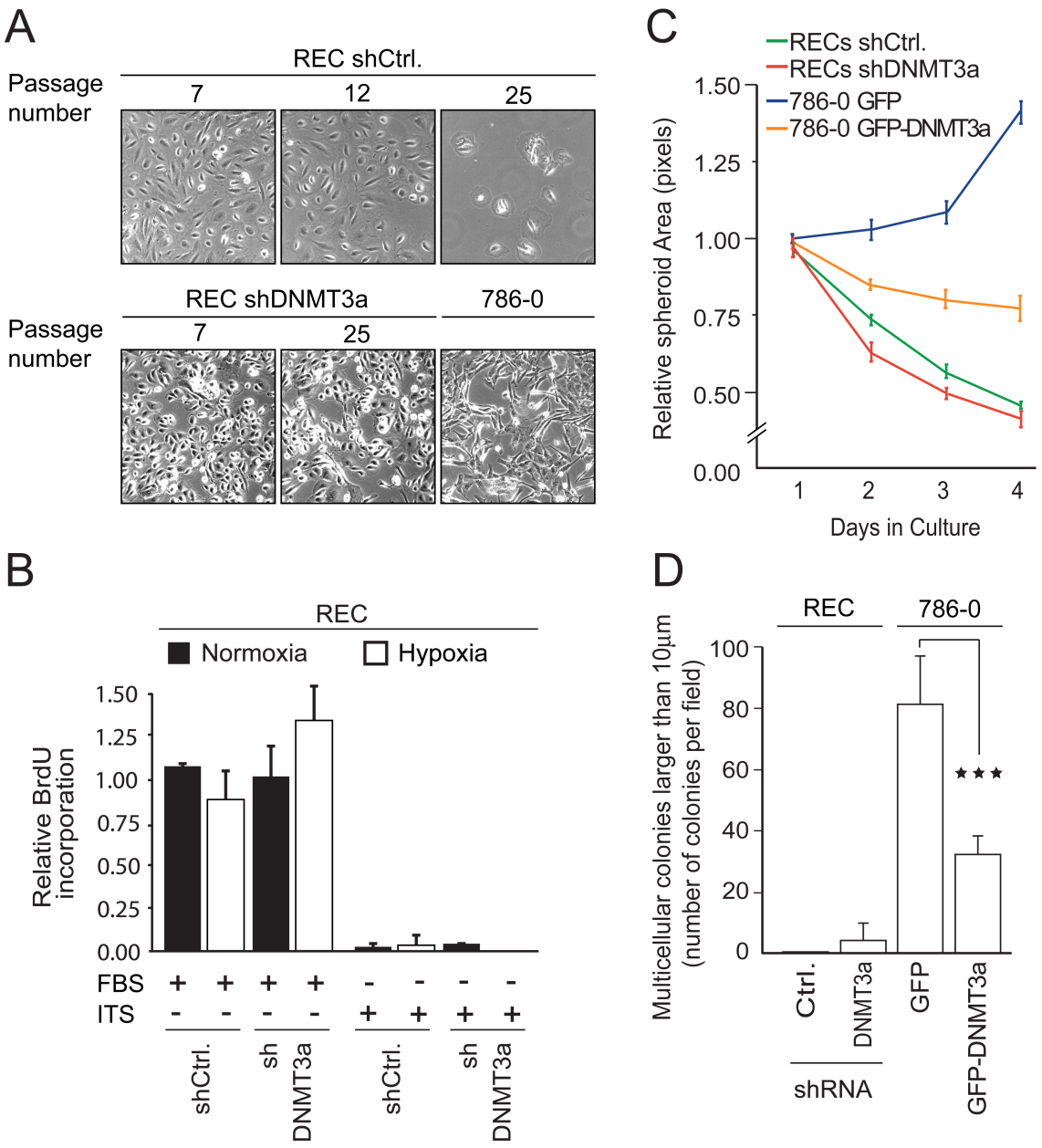

A
REC shCtrl.
Passage number
7
12
25
REC shDNMT3a
786-0
Passage number
7
25
C
RECs shCtrl.
RECs shDNMT3a
786-0 GFP
786-0 GFP-DNMT3a
Relative spheroid Area (pixels)
1.50
1.25
1.00
0.75
0.50
0.00
1
2
3
4
Days in Culture
B
REC
Normoxia
Hypoxia
Relative BrdU incorporation
1.50
1.25
1.00
0.75
0.50
0.25
0.00
FBS + + + + - - - -
ITS - - - - + + + +
shCtrl.
sh DNMT3a
shCtrl.
sh DNMT3a
D
REC
786-0
Multicellular colonies larger than 10µm (number of colonies per field)
100
80
60
40
20
0
★★★
Ctrl.
DNMT3a
GFP
GFP-DNMT3a
shRNA

Figure 11

Figure 11. DNMT3a represses abnormal growth of cancer cells but its depletion is not sufficient to convert normal cells into multicellular masses *in vitro*: (A) Bright field imaging of control (top row) or DNMT3a-depleted (bottom row) primary renal epithelial cells (REC) cells in normal growth conditions and compared between passages. The morphology of RECs is also compared to simultaneous cultures of 786-0 cells, a tumourigenic renal cell carcinoma cell line (bottom-right image). (B) BrdU incorporation assay testing the effect of shRNA silencing DNMT3a in serum supplemented (FBS) or serum starved (ITS) RECs for both normoxic and hypoxic cellular proliferation. BrdU was labelled for the last 2h of treatment and the data is presented as BrdU:Hoescht ratio relative to parental (unaltered) cells. (C) $1x10^4$ cells in suspension were plated and agglomerated to form multicellular spheroid in culture and growth was measured by acquiring bright field images and measuring the relative pixel density. REC expressing both a control and a DNMT3a-targeting shRNA were tested with 786-0 RCC cells expressing GFP or GFP-DNMT3a. (D) $2x10^3$ single cells from the same samples as in C) were plated in soft agar and multicellular colonies with diameters of 10mM or greater were counted after 14 days of growth.

3.2. Reintroduction of DNMT3a in Renal cancer cells phenocopies HIF-2α silencing or VHL reintroduction and suppresses normoxic autonomous growth and tumour formation.

Cancer cells have the ability to stimulate their own proliferation, a core hallmark of cancers (Hanahan and Weinberg, 2011). Going back to our renal cancer system, we decided to test whether DNMT3a-mediated silencing of *EPAS1* could have a dominant effect on the proliferation of tumourigenic renal cancer cells that already have the ability to grow autonomously. Suppression of HIF-2α expression is known to inhibit tumour growth of RCC and many other cancers (Kondo et al., 2002; Franovic et al., 2009), therefore, we sought to assess the ability of DNMT3a to impair autonomous growth of tumourigenic RCC cells. Under standard growth conditions, the proliferation rates of 786-0 cells stably-expressing GFP-DNMT3a or a control GFP protein were not significantly different from the empty vector control lines (**Figure 12A-left panel**). We then compared the effect of reintroducing the expression of DNMT3a to HIF-2α shRNA-mediated silencing or reintroducing VHL tumour suppressor gene (Pause, et al., 1998; Smith et al., 2005) using BrdU incorporation in ITS (**Figure 12A**). WT7 cells are a stably-expressing VHL cell line established from 786-0 and described before (Iliopoulos et al., 1995). As expected, re-introduction of DNMT3a in renal cancer cells caused a marked reduction in the proliferation of 786-0 cells in low serum conditions (**Figure 12A, left panel, white bars**). This effect of DNMT3a on ITS-dependant growth was almost identical to either the direct targeting of HIF-2α via shRNA or the reintroduction of wild type VHL function (**Figure 12A middle and right panel**).

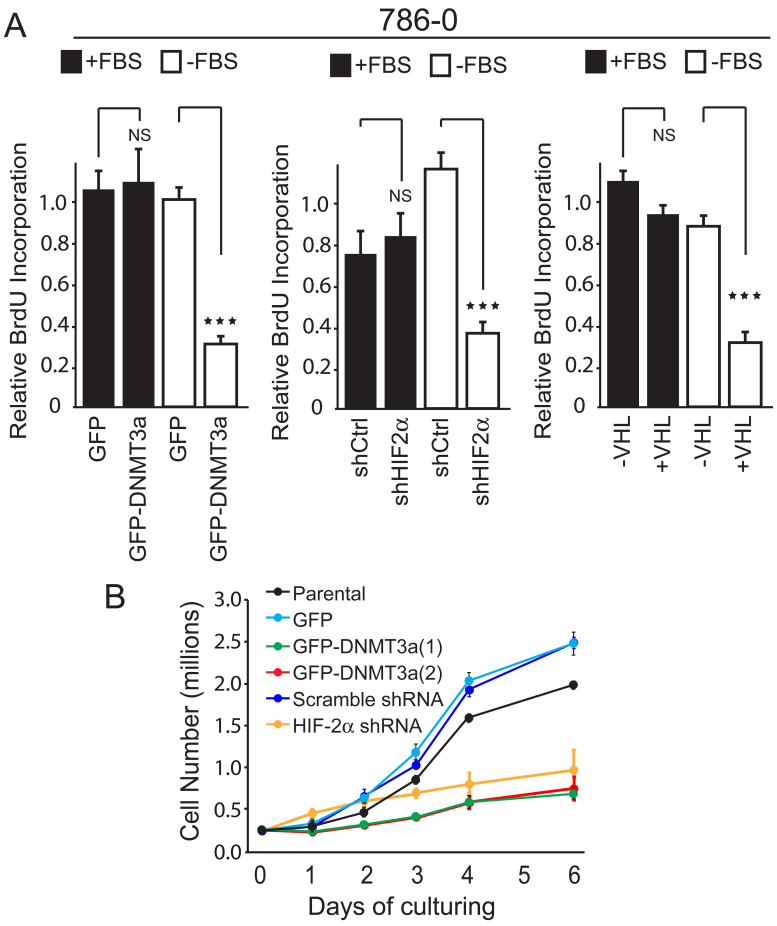


Figure 12

Figure 12. Reintroduction of DNMT3a represses autonomous growth of RCC: (A) 786-0 cells expressing controls (GFP and shCtrl), GFP-DNMT3a (Left panel), shRNA targeting HIF-2α (middle panel) or VHL (right panel) were incubated in normal (FBS) or serum free (-FBS) media before performing a BrdU incorporation assay. Serum-free growth was tested by incubating cells in ITS media for 48h followed by 2h BrdU incorporation and immunochemical detection of the BrdU adduct in the DNA. Data is presented as BrdU:Hoescht ratio relative to parental (unaltered) cells. (B) Serum-free growth of 786-O cells stably expressing GFP-DNMT3a was evaluated by cell counts. $2.5x10^5$ cells were initially seeded in serum-supplemented media and switched to serum free media. Cells were left in culture for 6 days and three different samples counted in triplicate every day.

To complement the BrdU assay, proliferation in ITS-supplemented media was also measured by counting the cells each day during 6 days of culture. Two independent DNMT3a-reintroduced clones displayed the same growth impediment to the 786-0 cells expressing HIF-2α shRNA while control cells all grew normally (**Figure 12B**). These independent assays confirmed the severe growth defects of DNMT3a-expressing 786-0 cells and mirrored the effects of directly manipulating the expression of RCC cancer-related genes *VHL* and *EPAS1*. Expectedly, DNMT3a-reintroduced 786-0 failed to form tumours *in vivo* (**Figure 13**) an effect similar to shRNA silencing of HIF-2α (Kondo et al., 2003) or VHL-reintroduction (Iliopoulos et al., 1995; Smith et al., 2005). These data suggested that the restoration of the DNMT3a epigenetic program, including the silencing of *EPAS1*, was sufficient to abolish the tumourigenic hallmarks of human renal cancer cells.

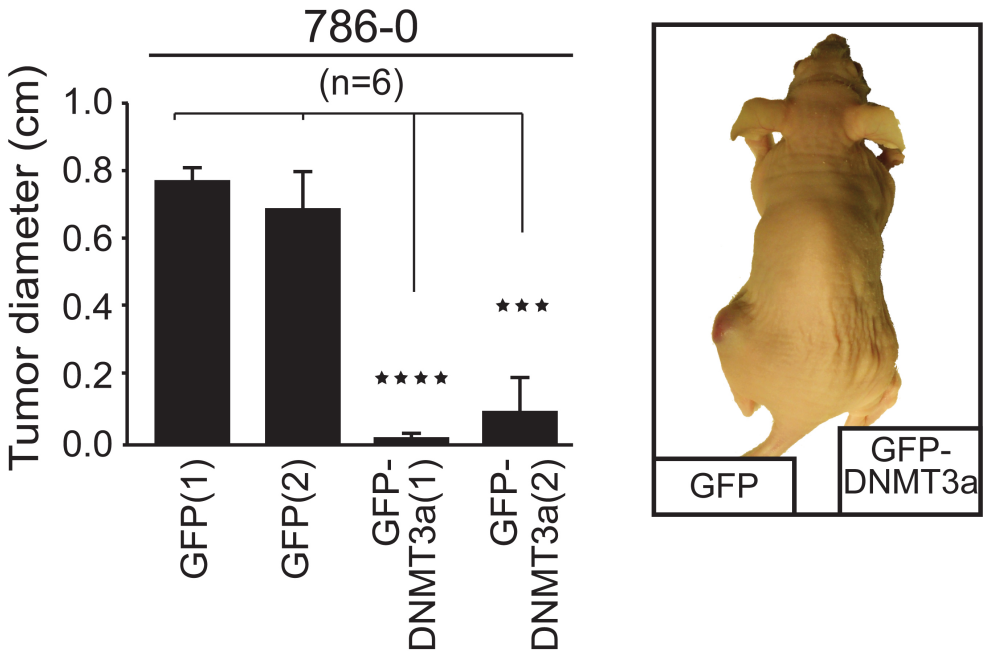


Figure 13

Figure 13. DNMT3a reintroduction repressed 786-0 tumour growth: Two independent clones of 786-0 cells stably expressing GFP or GFP-DNMT3a were injected in the flank of immunodeficient mice and tumour size at end point is shown as tumour diameter in cm (Left panel). A representative image of the experiment is shown (Right panel).

3.3. Transient expression of HIF-2α is sufficient to rescue growth autonomy defect of DNMT3a-expressing RCC cells and EGFR signaling.

To demonstrate the functional importance of HIF-2α expression amid the DNMT3a-directed DNA methylation epigenetic program we decided to ectopically express HIF-2α in DNMT3a-reintroduced cells. We thus transfected a HIF-2α cDNA construct in DNMT3a-reintroduced 786-0 cells aiming to test for the restoration of growth autonomy and tumour formation capacity *in vivo*. Unfortunately we were not able to stably express HIF-2α in DNMT3a-expressing 786-0 cells since cells died during the antibiotic selection and the limited number of resistant clones did not express HIF-2α.We were able to circumvent this limitation by transiently expressing HIF-2α (**Figure 14A**) and decided to test the effect of HIF-2α for the growth of DNMT3a-reintroduced 786-0 cells *in vitro* (**Figure 14B**). HIF-2α re-expression had no effect on serum growth (**Figure 14B-top panel**) but fully restored the autonomous growth defect of DNMT3a-expressing cells (**Figure 14B-bottom panel**). Since several lines of evidence pinpointed the EGFR growth axis to be a crucial oncogenic

pathway activated by HIF-2α in RCC (de Paulsen et al., 2001; Smith et al., 2005; Zhao et al., 2014) we decided to target EGFR signalling using gefinitib (Iressa), a specific EGFR tyrosine kinase inhibitor used for cancer therapy (Roskoski R, 2014). As expected in DNMT3a-expressing 786-0 cells, HIF-2α was incapable of restoring autonomous growth when cells were treated with Iressa (**Figure 14C**).

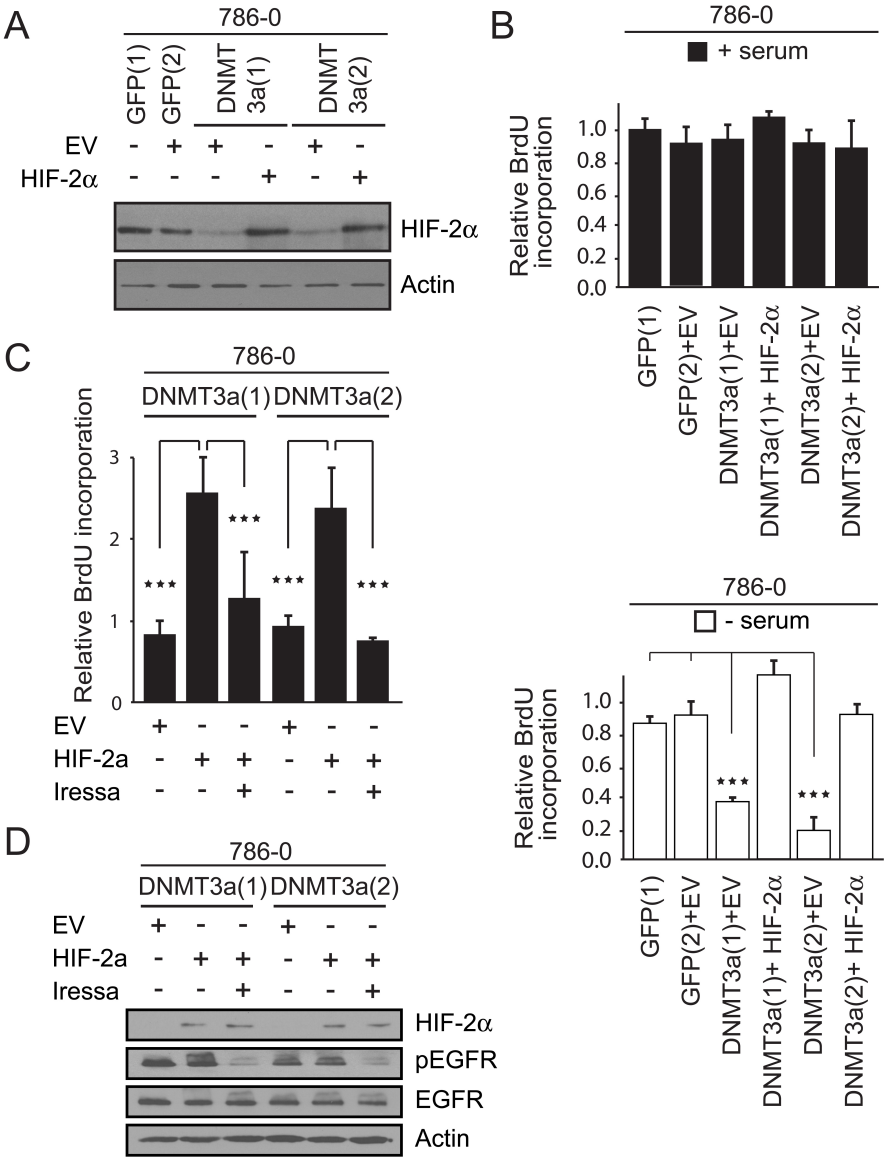


Figure 14

Figure 14. Transient expression of HIF-2α rescue DNMT3a-mediated autonomous growth defect of 786-0 cells: (A) Western blot analysis of HIF-2α protein levels in two independent 786-0 clones with the stable reintroduction of DNMT3a. Cells were transiently transfected with a vector expressing HIF-2α in parallel with a control empty vector (EV). (B) Serum-free growth was tested by incubating DNMT3a-reintroduced 786-0 cells transiently expressing HIF-2α in ITS media (Bottom panel –serum) for 48 h followed by 2 h BrdU incorporation and immunochemical detection of the BrdU adduct in the DNA. The data is presented as a ratio of BrdU:Hoescht normalized to parental cells. Normal serum-supplemented growth was tested in parallel (top panel +serum). (C) DNMT3a-reintroduced 786-0 cells were transiently transfected with control (EV) and HIF-2α expressing plasmids and tested for the EGFR-dependent serum-free growth. BrdU incorporation experiments in ITS and in gefitinib (Iressa), a specific inhibitor of EGFR tyrosine kinase activity, are shown. (D) Western blot controls matching the analysis of EGFR autophosphorylation and inhibition of EGFR activity with Iressa in 786-0 cells corresponding to C).

Immunoblotting of HIF-2α expressing and DNMT3a reintroduced 786-0 cells treated with Iressa confirmed the total loss of EGFR phosphorylation from the samples corresponding to **Fig. 14C** (**Figure 14D**). Altogether, these experiments provided additional support that DNMT3a was functionally connected to the regulation of HIF-2α and its oncogenic properties in renal cancer cells.

4. DNMT3a-mediated epigenetic silencing of *EPAS1* in non-VHL cancers.

4.1 Genetically different cancers display alterations in DNMT3a.

HIF-2α is expressed in a great proportion of human cancers independently of VHL status but mainly as a consequence of the toxic tumour microenvironment (Pietras et al., 2010). HIF-2α expression correlates with worsened prognosis, tumour resistance and recurrence, altogether supporting a clinically relevant role for HIF-2α expression in tumour cells (reviewed in Qing and Simon, 2009). Several lines of evidence suggested that genetically diverse cancer could require HIF-2α expression to grow in the hypoxic tumour microenvironment (reviewed in Franovic and Lee, 2010). Independent research groups reported an action of HIF-2α in inducing the expression of multiple RTK and some of their ligands involved in facilitating unrestricted growth and the survival of genetically diverse tumour cells (Burkitt et al., 2009; Franovic et al., 2009; Wang and Schneider, 2010; Chiavarina et al., 2012; Xu et al., 2014). Thus, shRNA silencing experiments provided a direct link between HIF-2α expression and growth of cancer cells (Li et al., 2009; Franovic et al., 2009; Bordoli et al., 2011; Xue et al., 2012). It is not clear however if DNMT3a-mediated epigenetic silencing of *EPAS1* is implicated in regulating the expression of HIF-2α outside renal cancers.

Interestingly, analysis of cancers and matching normal tissues from the protein atlas databank showed that DNMT3a expression was down in several different human tumours that are not known to be VHL dependent and either require HIF-2α for growth (Franovic et

al., 2009) (**Figure 15**) or are not known to require HIF-2α for tumourigenesis in preclinical models (**Figure 16**). These results reflected on our analysis of renal primary tumour samples (**Figure 6**) and showed that DNMT3A was downregulated in a number of different cancer samples and unrelated malignancies. For this reason, we decided to directly test the effect of expressing DNMT3a in VHL wild type cancer cells (**Figure 17**). We selected three cell lines that several independent groups showed required HIF-2α for tumour growth (A549, HCT116, U87MG) and three more cell lines for which a role of HIF-2α is debated (MDA-MB-231, SKOV3, PC3). Lentiviral-mediated transient expression of DNMT3a in these unrelated cancer cell lines caused a significant downregulation of HIF-2α mRNA albeit that was not the case for the breast cancer cells (**Figure 17**). From these experiments we selected three HIF-2α-dependent cell lines and derived cell clones with stable DNMT3a expression that were later tested for tumourigenesis *in vivo* (**Figure 18**).

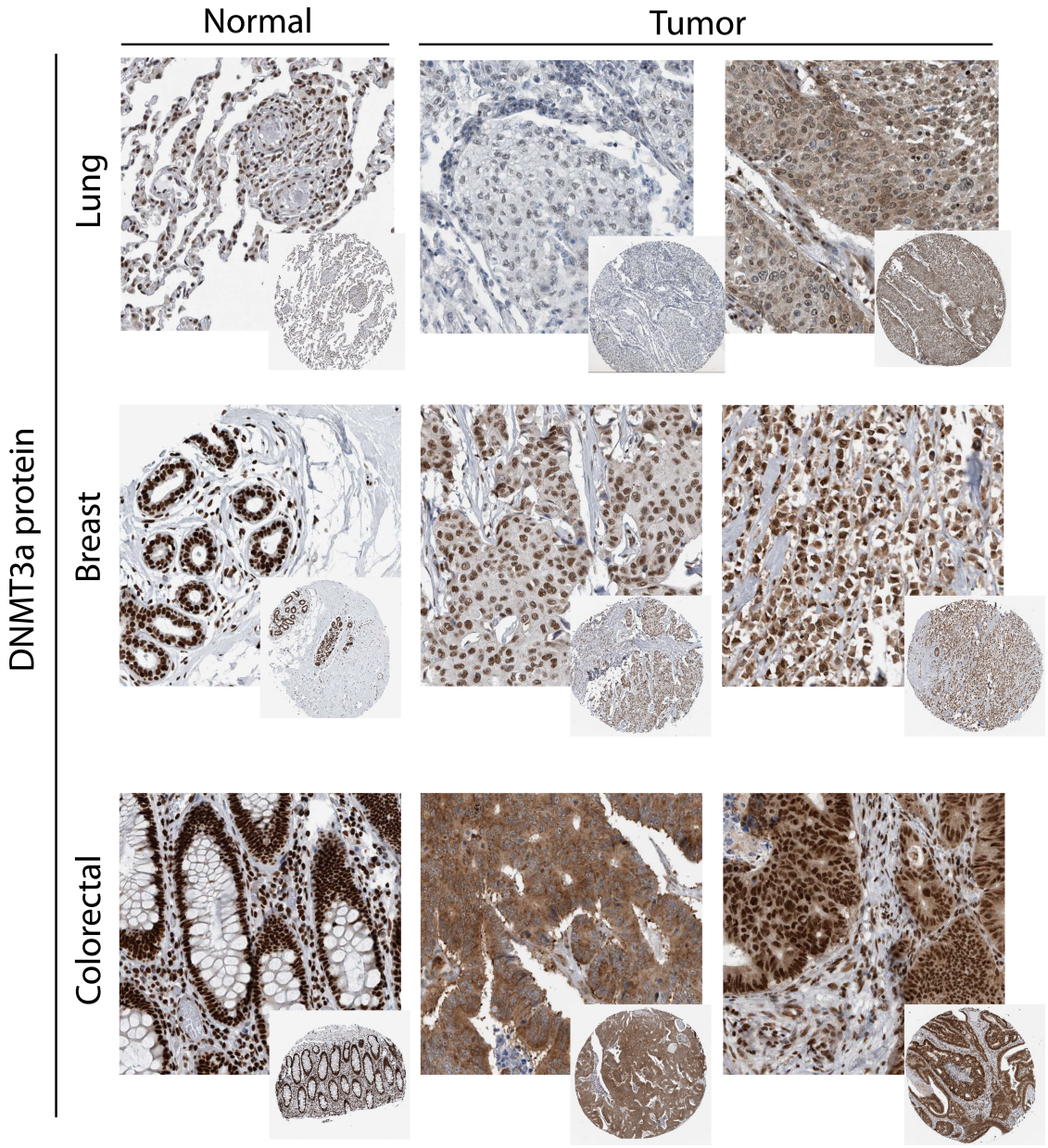


Figure 15

Figure 15. DNMT3a alterations in HIF-2α-dependent non-VHL cancers: The protein atlas databank was interrogated for the expression of DNMT3a in normal and tumour samples from lung (top row), breast (middle row) and colorectal (bottom row) cancer cases stained with DNMT3a antibody. Representative samples are shown.

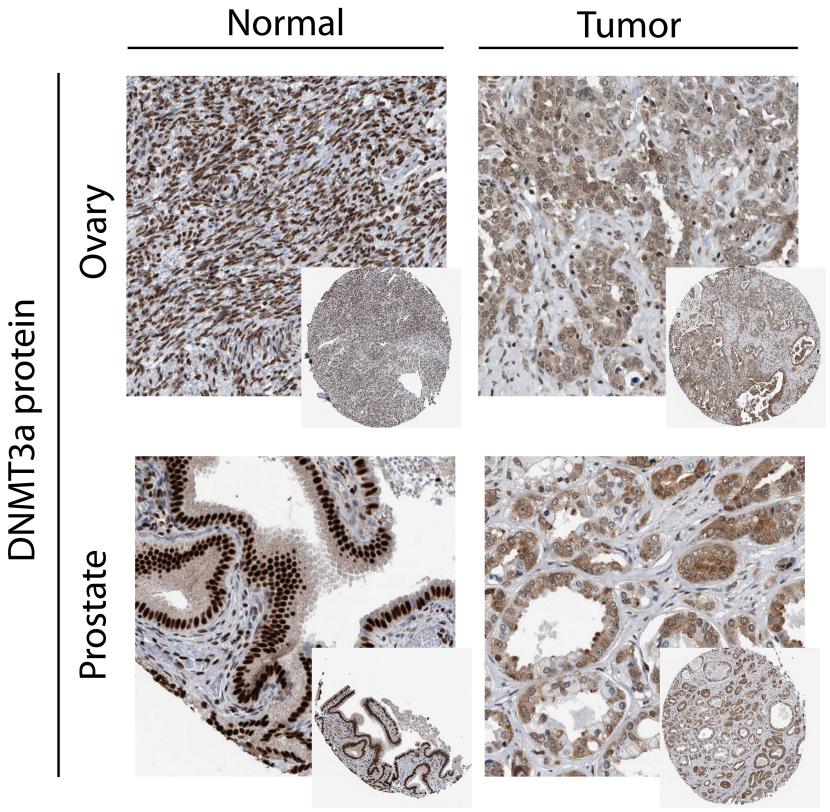


Figure 16

Figure 16. DNMT3a alterations in other non-VHL cancers: The protein atlas databank was interrogated for the expression of DNMT3a in normal and tumour samples from ovarian (top row) and prostate (bottom row) cancer cases stained with DNMT3a antibody. Representative samples are shown.

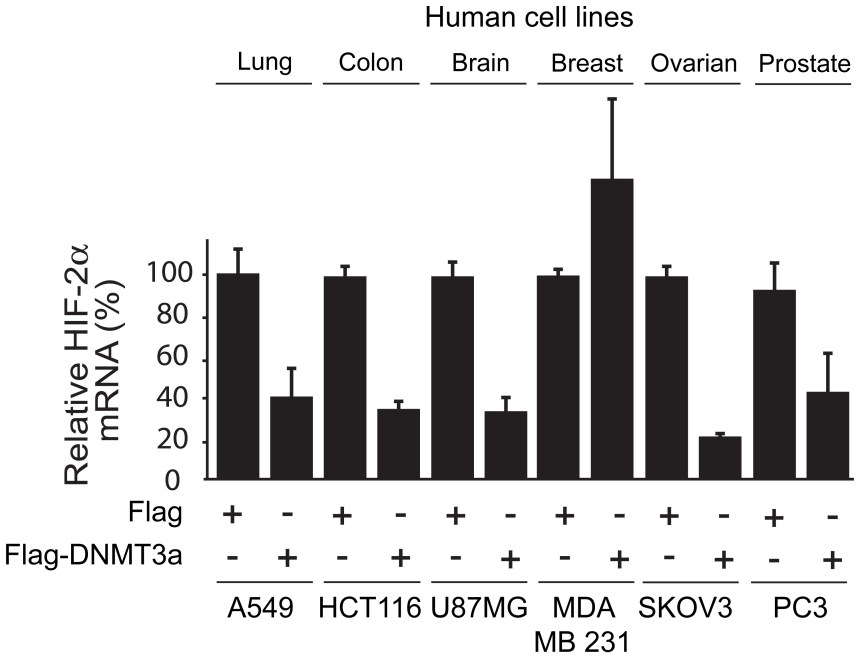


Figure 17

Figure 17. Overexpression of recombinant DNMT3a represses HIF-2α expression in non-VHL cancer cells: qPCR analysis of HIF-2α mRNA levels from various unrelated cancer cell lines infected with control or DNMT3a-expressing lentiviruses. Lung (A549), colorectal (HCT116), brain (U87MG), breast (MDA MB 231), ovarian (SKOV3) and prostate (PC3) tumourigenic cells were tested for HIF-2α expression and samples were normalized to the corresponding parental cells.

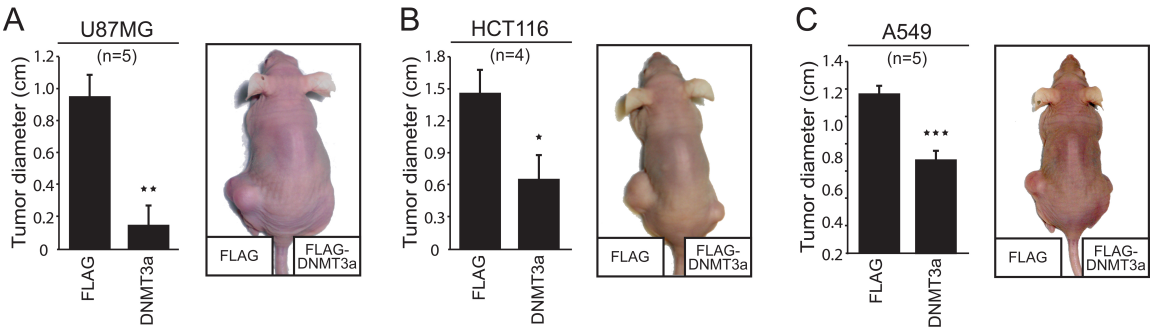


Figure 18

Figure 18. Overexpression of recombinant DNMT3a represses tumour formation in non-VHL cancer cells: (A) Brain (U87MG), (B) colorectal (HCT116) and (C) lung (A549) stable cell lines expressing DNMT3a or a control empty vector (Flag) were injected in the flank of immunodeficient mice. Tumour diameter (cm) at end point is shown (left panel for each) and representative images of the experiments are shown (right panel for each).

We found that all DNMT3a-expressing cell lines had a significant reduction in tumour formation capacity *in vivo* (**Figure 18A, B, C**) with the U87MG cell line having the more severe defect (**Figure 18A**). Since these three different cells lines were known to specifically require HIF-2α for autonomous growth and we had previously established the autonomous growth phenotype of DNMT3a-reintroduced 786-0 cells (**Figure 12, 13**), we tested the effect of reintroducing DNMT3a on the capacity of brain (U87MG) (**Figure 19A, D**), colorectal (HCT116) (**Figure 19B, E**) and lung (A549) (**Figure 19C, F**) cancer cells to grow without serum (**Figure 19**). For all three tumourigenic cell lines, the BrdU incorporation data supported a dominant negative effect of DNMT3a expression on autonomous growth and provided an explanation to why tumour formation capacity was reduced *in vivo* (**Figure 19**). Interestingly, the reduction in the growth capacity of the DNMT3a-expressing cells in ITS was matching the effect of directly targeting HIF-2α via shRNA silencing, supporting that DNMT3a and HIF-2α were functionally redundant for growth autonomy in these three unrelated cancers (**Figure 19**). We confirmed the repression of HIF-2α expression in U87MG, HCT116, and A549 cell lines stably expressing DNMT3a by measuring the mRNA levels by qPCR (**Figure 20A**) and protein expression in hypoxic samples using western blot (**Figure 20B**).

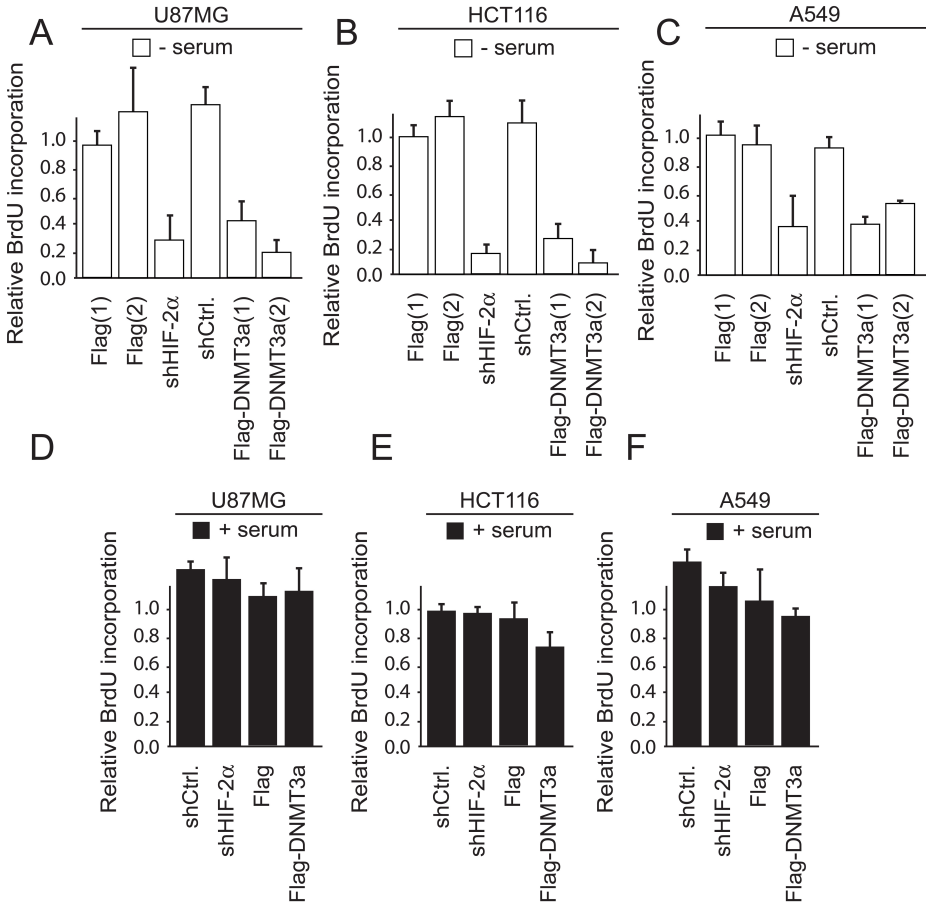


Figure 19

Figure 19: DNMT3a regulates autonomous growth of non-VHL cancer cells: (A) U87MG, (B) HCT116 and (C) A549 cells expressing controls (Flag and shCtrl), Flag-DNMT3a or shRNA targeting HIF-2α were incubated in serum free media before performing a BrdU incorporation assay. Serum-free growth was tested by incubating cells in ITS media for 48h, including the last 24h incubated in low oxygenated atmosphere (1%) followed by 2h BrdU incorporation and immunochemical detection of the BrdU adducts in the DNA. Data is presented as BrdU:Hoescht ratio relative to parental (unaltered) cells. Similar experiments as in A), B) and C) were performed in (D) U87MG, (E) HCT116 and (F) A549 cells stably expressing Flag-DNMT3a, shRNA targeting HIF-2α or controls (Flag and shCtrl.) but in normal serum-supplemented media instead.

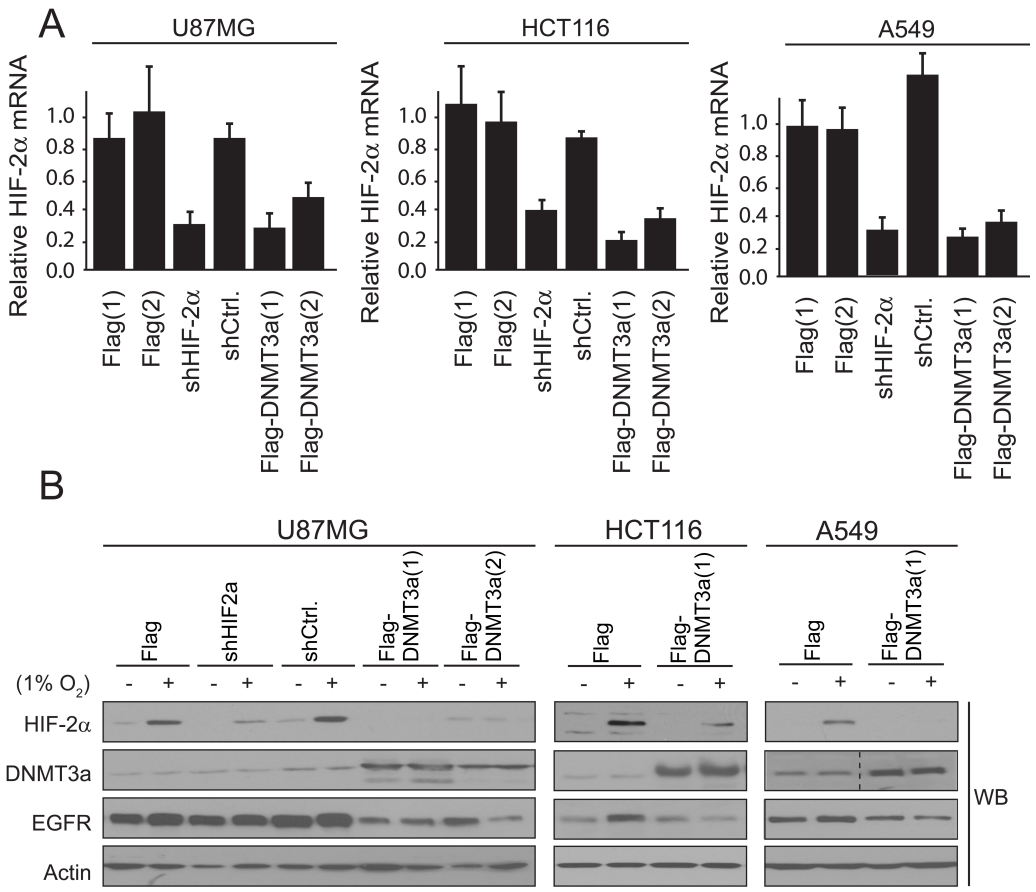


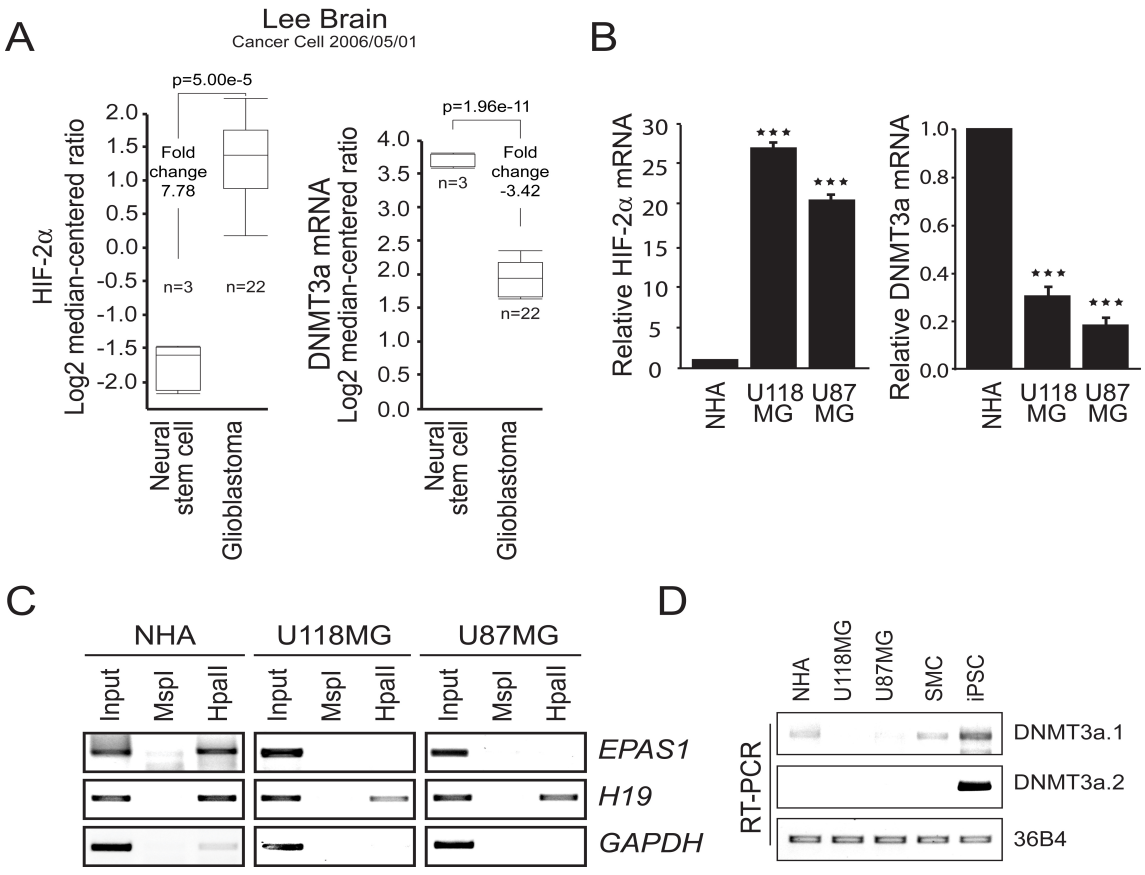
Figure 20

Figure 20. DNMT3a supresses HIF-2α expression in non-VHL cancer cells: (A) QPCR analysis of HIF-2α mRNA levels in U87MG (left), HCT116 (middle) and A549 (right) cells expressing controls (Flag and shCtrl), Flag-DNMT3a or shRNA targeting HIF-2α. (B) Western blot analysis of HIF-2α protein levels in hypoxia-treated U87MG (left), HCT116 (middle) and A549 (right) cells expressing controls (Flag and shCtrl), Flag-DNMT3a or shRNA targeting HIF-2α. EGFR hypoxic protein levels were tested in parallel with DNMT3a and actin loading control.

As expected, HIF-2α mRNA and protein expression in U87MG, HCT116 and A549 DNMT3a-expressing cells were significantly reduced and comparable to levels achieved via direct shRNA silencing when compared altogether and to control cells (**Figure 20**). So far, we gathered evidence that the ectopic expression of DNMT3a was sufficient to silence HIF-2α mRNA expression in tumourigenic cell lines from different cancer types, further suggesting that loss of DNMT3a-mediated epigenetic silencing of *EPAS1* could be implicated in non-VHL cancer.

4.2. Epigenetic silencing of *EPAS1* is down in brain cancer cell lines

We decided to get insight to the relevance of DNMT3a epigenetic silencing in human cancers by interrogating the Oncomine curated gene expression database for the expression of *EPAS1* (www.oncomine.org). An inverted correlation was observed between the expression of DNMT3a and HIF-2α mRNAs in a published subset of brain tumours (Lee et al., 2006). Interestingly, HIF-2α mRNA was 7.8 fold higher in tumours (**Figure 21A left panel**) and inversely correlated with DNMT3a mRNA expression which was 3 fold downregulated in tumour samples (**Figure 21A right panel**), raising the possibility that the epigenetic reprogramming of HIF-2α via loss of DNA methylation might not be restricted to renal cancers. We deemed the published data on primary brain cancer was providing an opportunity to further test our hypothesis that DNMT3a epigenetic silencing was lost in another cancer cell system. We thus performed a series of experiments consisting of comparing normal human astrocytes (NHA) and their transformed derivative astrocytoma lines (U118MG and U87MG) hoping to reconcile our findings on REC and RCC lines.


A
Lee Brain
Cancer Cell 2006/05/01
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Fold change 7.78
n=3
n=22
HIF-2α Log2 median-centered ratio
Neural stem cell
Glioblastoma
p=1.96e-11
Fold change -3.42
n=3
n=22
DNMT3a mRNA Log2 median-centered ratio
B
Relative HIF-2α mRNA
Relative DNMT3a mRNA
NHA
U118 MG
U87 MG
C
NHA
U118MG
U87MG
Input
MspI
HpaII
EPAS1
H19
GAPDH
D
NHA
U118MG
U87MG
SMC
iPSC
RT-PCR
DNMT3a.1
DNMT3a.2
36B4

Figure 21

Figure 21. *EPAS1* is methylated and inversely correlates with DNMT3a expression in human brain cancers: (A) Oncomine database was interrogated for HIF-2α expression by keyword search. Analysis was filtered for mRNA data of brain tumour samples, tested between tumour and control normal with a p value of 0.05 or higher. Only the study by Lee et al., 2006 had HIF-2α mRNA (left panel) and DNMT3a (right panel) analysed in parallel. The data and the statistics from the oncomine meta-analysis are shown for each gene. (B) qPCR analysis of HIF-2α and DNMT3a expression was tested from total RNA extracted of normal human astrocytes (NHA), U118MG and U87MG astrocytoma cells. HIF-2α expression was normalized to U87MG and DNMT3a to NHA. (C) Genomic DNA extracted from NHA and corresponding astrocytoma cell lines U118MG and U87MG was tested for *EPAS1* DNA methylation using MSRPCR. The same primers (set 4) as before were used including positive and negative DNA methylation control genes *H19* and *GAPDH*, respectively (see **Figure 1A**). (D) Total RNA extractions from NHA and tumourigenic cell lines U118MG and U87MG were analyzed by RT-PCR for DNMT3a.1 and DNMT3a.2 isoform expression in parallel to housekeeping gene *36B4*. DNMT3a.1- and DNMT3a.2-positive cell lines, smooth muscle cells (SMCs), and induced pluripotent stem cells (iPSCs), respectively, were used as positive controls.

We first decided to confirm the results found in the Oncomine databank by comparing the expression of HIF-2α mRNA between NHA and two different astrocytomas cell lines U118MG and U87MG using qPCR. In parallel experiments, we tested the DNA methylation levels of *EPAS1* between normal and brain cancer cells and found that these were inversely proportional to the expression of HIF-2α mRNA (**Figure 21B left panel, C**), supporting that DNMT3a-mediated epigenetic silencing might be lost in astrocytomas. Consequently, we also measured the differential expression of DNMT3a mRNA and protein using our *in vitro* cell culture system of NHA and tumourigenic brain cell lines U87MG and U118MG. As expected, tumourigenic cells displayed a significant reduction of both mRNA and protein levels of DNMT3a (**Figure 21B right panel, 22A**). In addition, isoform analysis revealed DNMT3a1 compared to DNMT3a2 was the most abundantly expressed isoform of DNMT3a in NHA (**Figure 21D**). Altogether, the analysis of primary astrocytes (NHA) and astrocytomas lines (U118MG and U87MG) revealed a pattern identical to renal cell lines, with hypomethylation of *EPAS1* (**Figure 5B**, **Figure 21C**), upregulation of HIF-2α (**Figure 1B**, **Figure 21B left panel**) and DNMT3a mRNA repression (**Figure 8**, **Figure 21B right panel**). Based on these evidences we evaluated the possibility that similarly to the renal cancer system, loss of DNMT3a could be directly implicated in the epigenetic reprogramming of HIF-2α in brain cancer cells.

4.3. DNMT3a expression is sufficient to silence HIF-2α in brain cancer cells

The next important question consisted of testing if DNMT3a expression alone was capable of restoring the normal epigenetic status and expression of *EPAS1* in tumourigenic brain cells. For this purpose we used a lentiviral construct expressing a recombinant DNMT3a protein and created U87MG cells with stable expression of DNMT3a as described before (**Figure 22A**). We were able to reintroduce DNMT3a in U87MGs to levels resembling those of NHAs (**Figure 22A**).

DNMT3a reintroduction in U87MG tumourigenic cells re-established the DNA methylation pattern of *EPAS1* that was seen in NHA when tested using MSRPCR assays on two separate segments of the *EPAS1* CpG Island (**Figure 22C, D**). When we tested the levels of HIF-2α mRNA using qPCR, we found a significant reduction in two independent DNMT3a-expressing clones compared to controls (**Figure 20A**). In addition the mRNA levels of HIF-2α in these stable cell lines were almost identical to the levels of NHA non-transformed cells (**Figure 22B**). From this, we decided to explore further the possibility that DNMT3a-epigenetic silencing could be implicated in the growth and survival of hypoxic astrocytomas cells and conducted a series of experiments to test this idea.

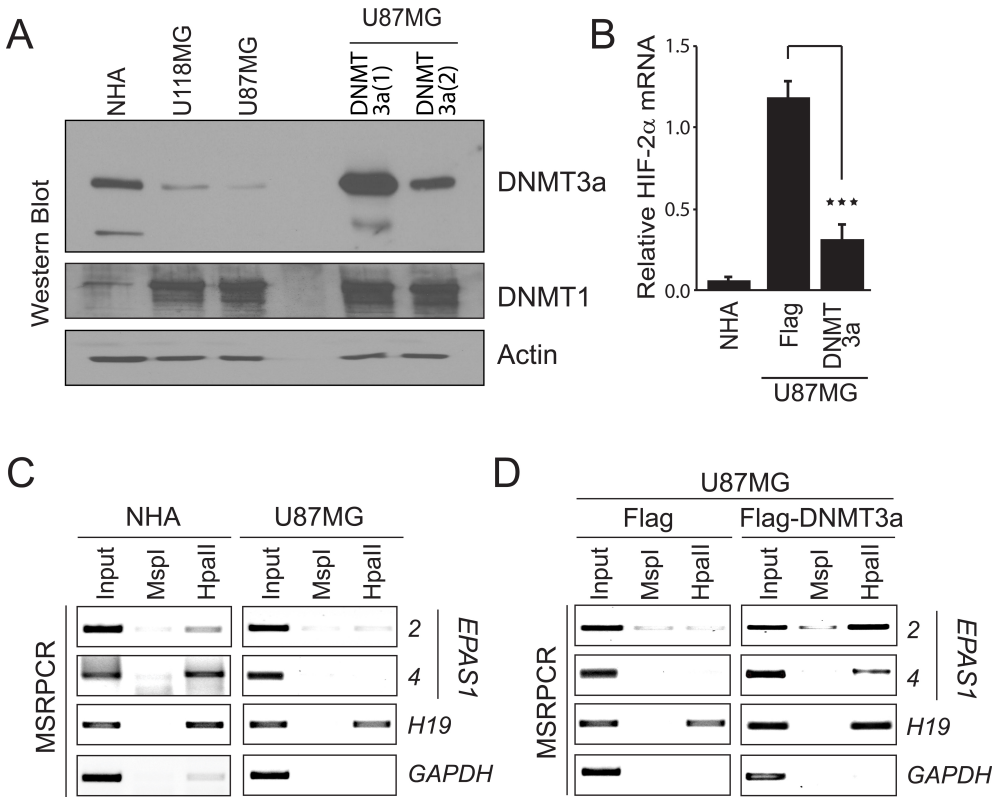

Figure 22

Figure 22. Reintroduction of DNMT3a in astrocytomas cells: (A) Western blot testing the levels of DNMT3a, DNMT1 and loading control actin in normal human astrocytes (NHA), two parental astrocytomas cell lines (U118MG and U87MG) and two independent stable U87MG clones with reintroduced DNMT3a expression. (B) QPCR was used to measure the mRNA levels of HIF-2α in NHA and U87MG cells stably reexpressing DNMT3a or a control lentivirus (Flag). (C) MSRPCR analysis of *EPAS1* DNA methylation in genomic DNA samples extracted from NHA and U87MG cells. Two regions of the *EPAS1* CpG island were tested (see **Figure 1A**), in parallel to *H19* and *GAPDH* positive and negative DNA methylation controls, respectively. (D) MSRPCR analysis of *EPAS1* DNA methylation in genomic DNA samples extracted from U87MG cells stably expressing DNMT3a or a control lentivirus (Flag).

4.4. Reintroduction of DNMT3a represses cell growth and survival in hypoxia

A significant amount of data supports the direct requirement of HIF-2α oncogenic axis for brain cancer malignant growth and survival in the hypoxic tumour microenvironment (Li et al., 2009; Franovic et al., 2009; Seidel et al., 2010). Our experiments confirmed that reintroduction of DNMT3a negatively regulated HIF-2α protein expression in hypoxic brain cancer cells (**Figure 20B, left panel**). Logically, we decided to test the effect of restoring the normal epigenetic silencing of *EPAS1* on the capacity of U87MG cancer cells to survive in a hypoxic atmosphere. We thus tested the growth capacity of U87MG stable cells using the BrdU incorporation assay and in parallel cellular viability using a trypan blue exclusion assay in control normoxic and prolonged hypoxic conditions. As predicted, DNMT3a-expressing glioblastoma (U87MG) cells showed repressed proliferation and viability, compared to empty Flag vector control, when grown in culture at low oxygen tension (**Figure 23**).

Since our previous renal cancer model supported the involvement of HIF-2α in the growth of early cancerous lesions we wanted to measure the implication of DNMT3a loss to the capacity of early tumour mass to adapt and grow past the oxygen limitation barrier. For this purpose we tested the effect of triggering hypoxia as a result of tumour cell growth and not from a change in oxygen partial pressure of the atmosphere. *In vivo* labelling and direct measurement of oxygen levels suggested that oxygen could not diffuse deeper than 100-200 microns into tissues thereby causing the cells in the tumour core to become hypoxic (**Figure 24A**). The multicellular spheroid assay (MSA) consisting of growing clusters of

tumourigenic cells in suspension allows the recapitulation of the transition between normoxic and hypoxic growth *in vitro* (Sutherland et al., 1986, **Figure 24B**). We decided to utilise MSA to test the effect of reintroducing DNMT3a expression on the capacity of cancer cells to reach a diameter exceeding 100-200 microns and pass the oxygen diffusion limit.

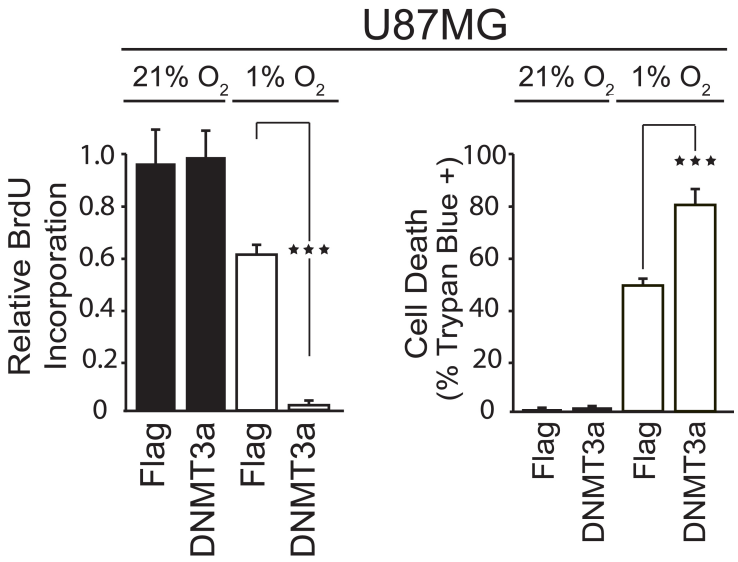

Figure 23

Figure 23. DNMT3a represses growth and survival of brain cancer cells in low oxygen: U87MG cells with reintroduced DNMT3a or control (Flag) were grown for 96h in normal (21% O_2) or hypoxic atmosphere (1% O_2). BrdU incorporation was done during the last 2h of treatment and measured by immunohistochemistry. The ratio of BrdU:Hoescht positive cells was normalized to unaltered parental U87MG cells (left panel). DNMT3a-reintroduced U87MG cells were incubated in a normal (21% O_2) or hypoxic (1% O_2) environment and cell death was measured using trypan blue viability dye. The percentage of trypan blue positive cells (dead cells) was calculated over the total number of cell in each sample (right panel).

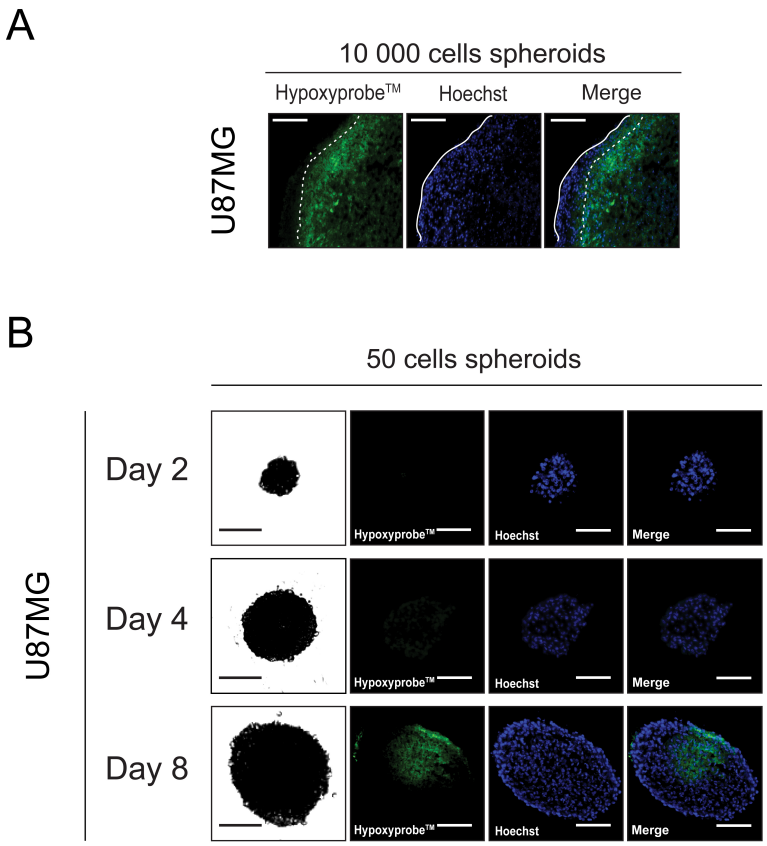

Figure 24

Figure 24. Multicellular spheroid masses transition from normoxic to hypoxic growth: (A) $1x10^4$ U87MG single cells were plated and agglomerated to form multicellular spheroid masses. 96h post-plating spheroids were incubated with media containing pimonidazole (hypoxyprobe), harvested, fixed and stained for the cellular incorporation hypoxyprobe and Hoescht to visualize all cells. Scale bars represent 200 microns. (B) 50 U87MG single cells were plated and agglomerated to form multicellular spheroid masses. Two, four and eight days post-plating spheroids were incubated for 1 h with media containing pimonidazole (hypoxyprobe), harvested, fixed and stained for the cellular incorporation hypoxyprobe (green) and Hoescht (blue) to visualize hypoxic and total cells, respectively. Scale bars represent 100 microns.

4.5. DNMT3a-expressing cells cannot form a hypoxic tumour cell mass *in vitro*.

Reports have shown that in three-dimensional growth, cancer cell spheroids masses display the same oxygen diffusion range as *in vivo* tumour models (Krohn et al., 2008; Rijken et al., 2000). To mimic the early stages of tumour growth and study the transition between normoxic and hypoxic states of growth, we plated a low number of cells (50) and followed spheroid formation over a period of 8 days. Importantly, we were able to observe the transition between normoxic and hypoxic spheroid growth *in vitro* using Hypoxyprobe^{TM}, an hypoxia tracer that stains cells exposed to less than 2% O_2 (Chou et al., 2004), (**Figure 24B**). In this system, hypoxia occurred predictively after the multicellular masses reached a radius greater than the approximated oxygen diffusion limit (**Figure 24B**). Interestingly, U87MG spheroids stably expressing DNMT3a were unable to grow to a diameter greater than ~100mm, in accordance with the diffusion limit of oxygen in tumours xenograft and spheroids (Rijken et al., 2000; Lambrechts et al., 2013) (**Figure 25A**). In parallel experiments, control cells formed large spheroid masses (**Figure 25A, B**) that were hypoxic since HIF-2α protein could be detected by western blot (**Figure 25C**). RT-PCR analysis confirmed that HIF-2α mRNA expression was reduced in DNMT3a-expressing spheroids (**Figure 25C**), establishing that the epigenetic silencing of *EPAS1* was acting upstream of the hypoxia response.

In addition, by forcing the formation of hypoxia by generating large spheres of astrocytoma (U87MG) cells (10^5) that were incubated with Hypoxyprobe our analysis revealed that expression of DNMT3a impaired the formation of hypoxic cores (**Figure 25D**). These data suggest that restoration of DNMT3a epigenetic program, including the silencing of *EPAS1*, is sufficient to abolish the tumourigenic potential by inhibiting the hypoxic cell growth.

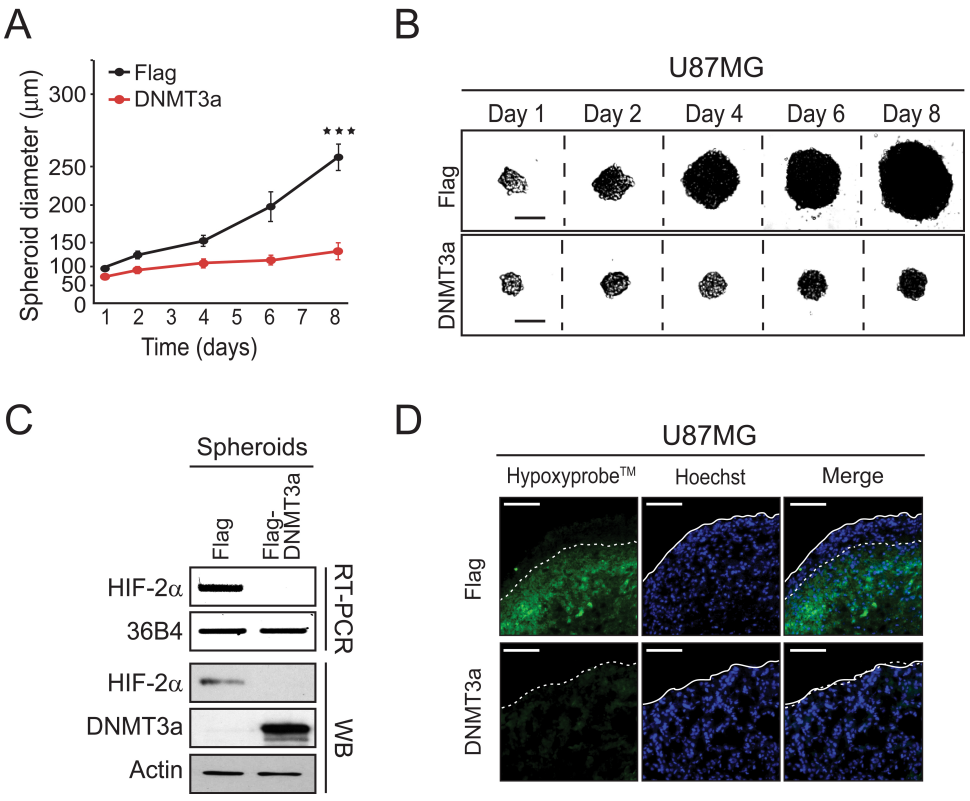

Figure 25

Figure 25. DNMT3a restrains the normoxic-to-hypoxic growth transition of U87MG multicellular masses: (A) Quantification of spheroid diameter from Flag- or Flag-DNMT3a–expressing U87MG cells (50 cells per well) grown for the indicated times. (B) Representative spheroids from Flag- and Flag-DNMT3a–expressing U87MG cells (50 cells per well) grown for the indicated times. Scale bars represent 100 microns. (C) Flag- and Flag-DNMT3a–expressing 8 days old U87MG spheroids were lysed and HIF-2α or DNMT3a mRNA/protein levels were assessed by RT-PCR or Western blotting (WB). (D) U87MG cells (10^5) with or without DNMT3a stably expressed were plated and grown for 96 h before the addition of Hypoxyprobe to the media for 1h. Spheroids were then prepared for cryosectioning, Hoechst staining (blue), and immunofluorescence detection of Hypoxyprobe (green)-positive cells. Scale bars represent 200 microns.

4.6. Expression of HIF-2α is sufficient to restore cellular adaptation to hypoxia

We hypothesized that re-expression of HIF-2α alone could be sufficient to restore some of the capacity of DNMT3a expressing cells to survive and grow in hypoxia. To confirm that the growth properties imparted by DNMT3a expression are mediated by the silencing of HIF-2α, and no other epigenetic targets, we transduced U87MG cells stably expressing exogenous DNMT3a with a HIF-2α construct whose expression was unfettered by the CpG island found at the endogenous *EPAS1* locus. This allowed the restoration of HIF-2α mRNA and protein expression (**Figure 26 A, B, C**), while methylation of the endogenous *EPAS1* promoter was maintained (**Figure 27**). Ectopic HIF-2α was capable of restoring BrdU incorporation to wild type levels during hypoxic growth (**Figure 28-left panel**). In addition the cellular viability in prolonged low oxygen conditions was also fully back to that of parental cells (**Figure 28-right panel**) and thus confirmed that from all the genes under the regulation of DNMT3a epigenetic silencing, HIF-2α was a central factor for the regulation of growth and survival in low oxygen.

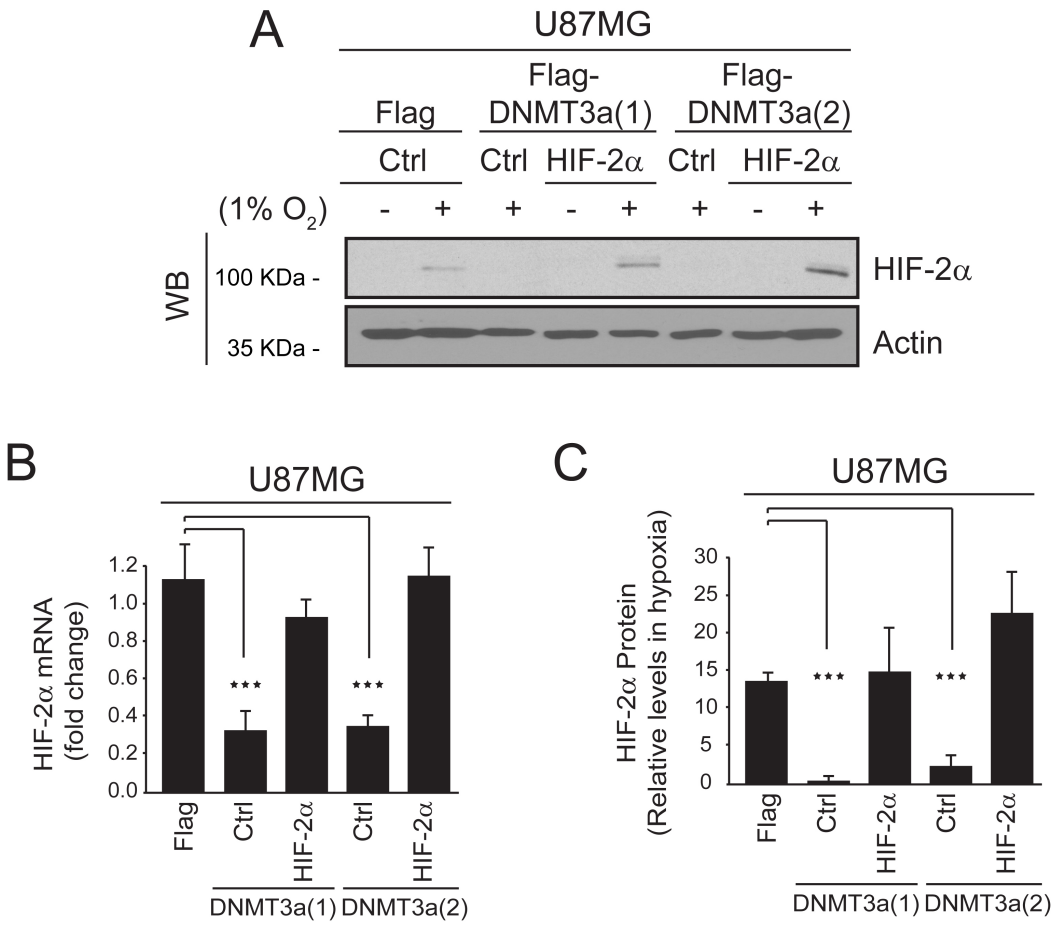

Figure 26

Figure 26. Rescue of HIF-2α expression in DNMT3a-reintroduced U87MG tumourigenic cells: (A) HIF-2α expression levels were compared between U87MG cells stably expressing an empty Flag vector and two clonal Flag-DNMT3a lines transduced with the control or HIF-2α-expressing lentivirus. U87MG cells were grown at the indicated oxygen tension before total protein extraction and Western blot for HIF-2α or the loading control actin. (B) qPCR analysis of HIF-2α mRNA expression level in total RNA extracted from cells corresponding to A). (C) Quantification of HIF-2α protein levels from the Western blots in A).

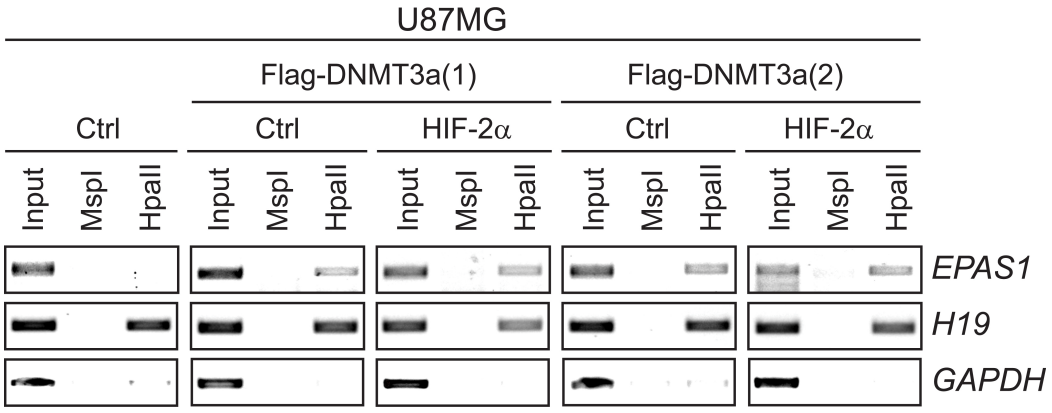

Figure 27

Figure 27. Epigenetic silencing of *EPAS1* is maintained in DNMT3a-reintroduced U87MG cells expressing exogenous HIF-2α: Exogenous HIF-2α expression does not affect DNA methylation at the *EPAS1* locus. MSRPCR analysis of genomic DNA extracted from two stables Flag-DNMT3a–expressing U87MG clones stably coexpressing control empty vector or HIF-2α lentivirus constructs. *EPAS1* (primer set 4), *H19* (positive DNA methylation control), and *GAPDH* (negative DNA methylation control) were amplified following MspI/HpaII restriction digestions.

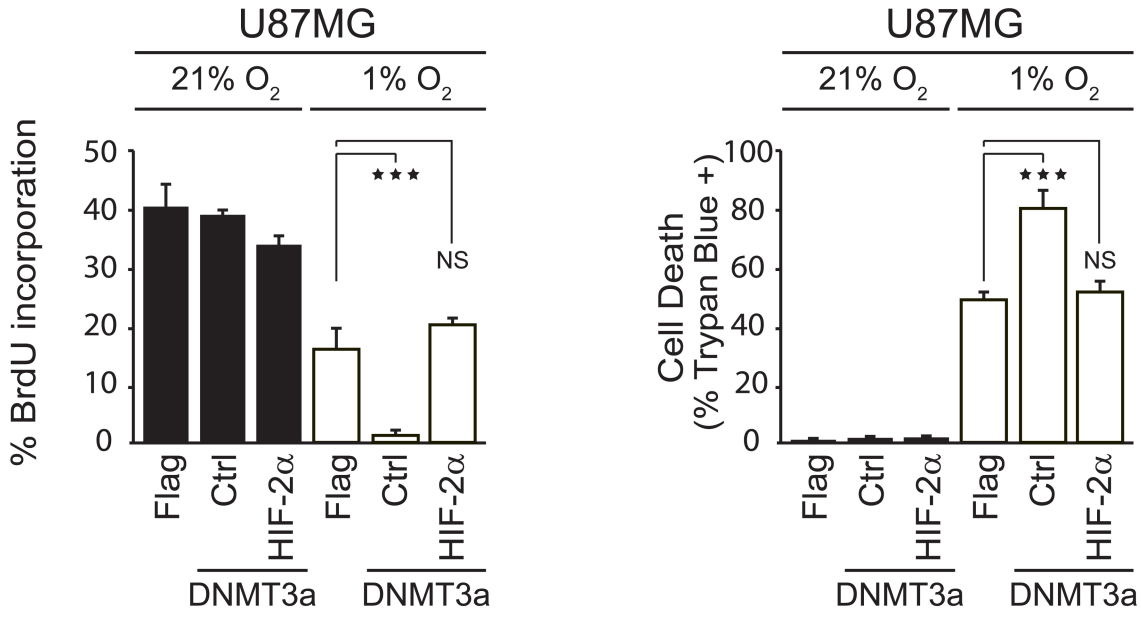

Figure 28

Figure 28. HIF-2α expression is sufficient to restore hypoxic growth and survival of DNMT3a-reintroduced U87MG tumourigenic cells: U87MG astrocytomas stably expressing Flag or Flag-DNMT3a were infected with an empty vector (Ctrl) or a HIF-2α cDNA–containing lentivirus and grown for 96 h in a normoxic (21% O_2) or hypoxic (1% O_2) atmosphere in parallel. Cell proliferation was measured by incorporating BrdU during the last hour of normoxic or hypoxic treatment. Immunohistochemistry was used to detect the BrdU-labelled cells and the percentage of BrdU:Hoescht is shown (left). Cell viability was measured using trypan blue staining on U87MG cells stably expressing Flag or Flag-DNMT3a and with the rescue of HIF-2α or a control plasmid. In parallel experiments, cells were incubated in normal (21% O_2) or hypoxic (1% O_2) atmosphere for 96 h (Right).

4.7. Expression of HIF-2α is sufficient to form a hypoxic tumour core *in vitro* and bestow full tumourigenic potential to DNMT3a-expressing U87MG cells.

Contrarily to 786-0 renal cancer cells, we were able to establish long term cultures of DNMT3a-expressing U87MG cells with stable ectopic expression of HIF-2α using lentivirus (**Figure 26**). For that reason, we used two independent U87MG clones expressing DNMT3a and a polyclonal population of HIF-2α rescued cells that we injected in the flank of immunocompromised mice in parallel to control HA-expressing lentivirus to test tumour formation *in vivo*. While cells with re-introduced DNMT3a failed to undergo tumourigenesis in the nude mouse xenograft assay (**Figure 29 A, B, C**), exogenously-expressed HIF-2α restored tumour growth to levels matching the empty vector (Flag) control cell lines (**Figure 29 A, C**).

Analysis of *in vitro* spheroid growth demonstrated that ectopic-expression of HIF-2α restored the ability of DNMT3a-expressing cells to grow past the point of oxygen limitation (**Figure 30 A, B)** and form hypoxic cores (**Figure 30 C, D**). Put together, these results rallied the role of HIF-2α oncogenic program with the gatekeeping function of DNMT3a-mediated epigenetic silencing in the prevention of hypoxic tumour growth.

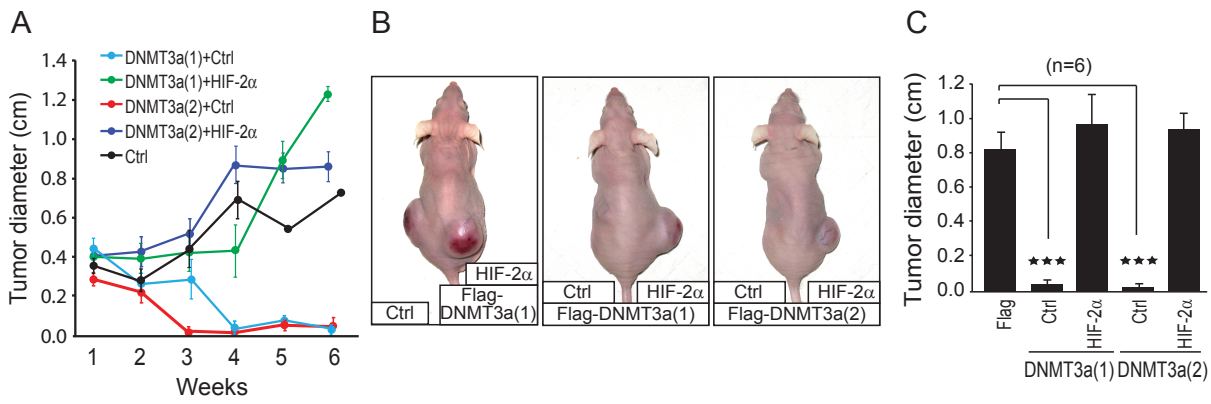

Figure 29

Figure 29. HIF-2α ectopic expression rescue tumour growth defect of DNMT3a-reintroduced U87MG cells: (A) Growth kinetics of U87MG xenograft tumours corresponding to Fig. 26. U87MG cells stably expressing Flag-DNMT3a and coexpressing control empty vector (Ctrl) or HIF-2α lentivirus constructs were injected into nude mice and the diameter of the tumours measured every week. (B) Representative images of the *in vivo* tumour experiment. (C). Xenograft diameters in cm were measured at the end point. Significance was measured by a Student t test; *P < 0.05, **P < 0.01, ***P < 0.001. NS, not significant.

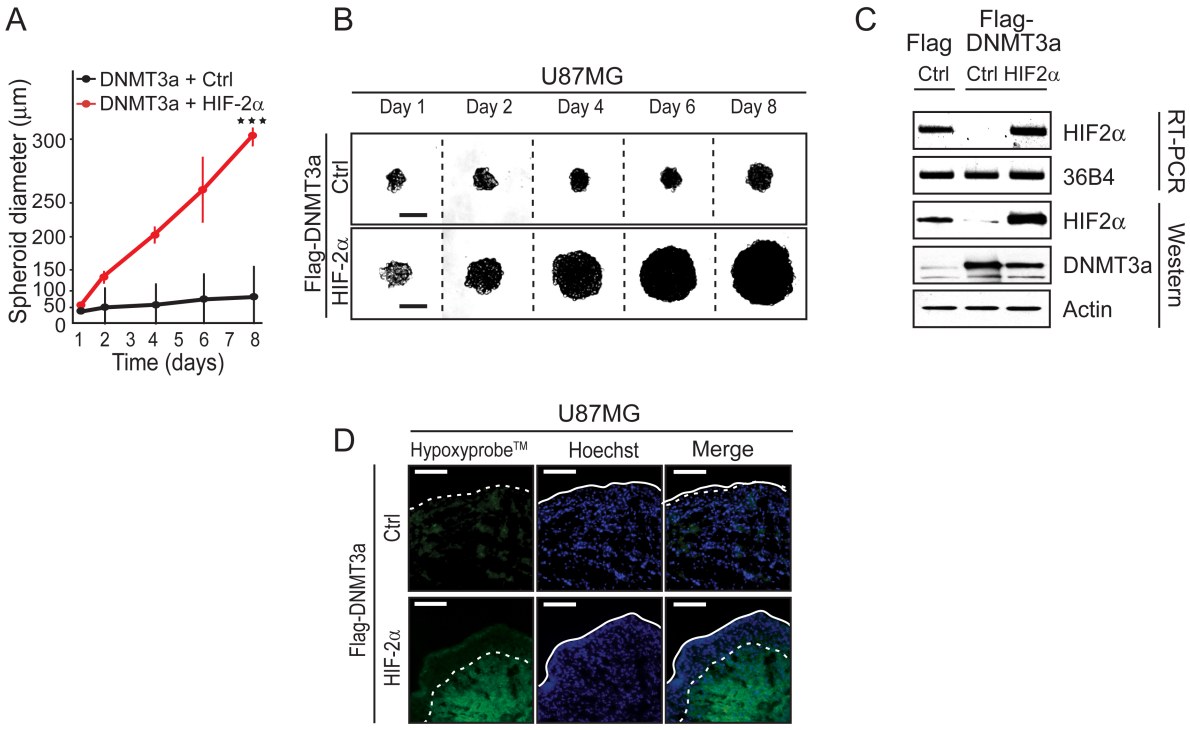

Figure 30

Figure 30. Restoration of HIF-2α expression is sufficient to allow DNMT3a-reintroduced U87MG cells to pass the hypoxic barrier: (A and B) Fifty cells per well of U87MG with Flag-DNMT3a and stably coexpressing control empty vector or a HIF-2α lentivirus construct were plated and their growth was quantified (A), and representative (B) spheroids are presented from the indicated times. (C) Eight-day-old U87MG spheroids with stably expressed Flag, Flag-DNMT3a, and Flag-DNMT3a coexpressing an empty vector (Ctrl) or HIF-2α cDNA were lysed and HIF-2α or DNMT3a mRNA/protein levels were assessed by RT-PCR or Western blotting. (D) U87MG cells (10^5) with Flag-DNMT3a and empty vector or HIF-2α cDNA were Hoechst-stained (blue) and stained for Hypoxyprobe (green)-positive cells.

5. DNMT3a-mediated epigenetic silencing of *EPAS1* and the systemic response to hypoxia

5.1 HIF-2α supports protein translation in a variety of hypoxic cancer cells.

The experiments we conducted so far led us to appreciate the role of HIF-2α expression for cellular adaptation to hypoxia. Quite surprisingly, among the multitude of genes under DNMT3a-mediated epigenetic silencing, HIF-2α appeared to be central for many hypoxia-related mechanisms including hypoxic tumour growth. The central connection between *EPAS1* and human adaptation to low oxygenation also seemed to suggest that HIF-2α was part of a systemic response to hypoxia. As discussed in the introduction, the adaptation to low oxygenated environment necessitates a coordinated and systemic cellular response (**Section 3.2**) that can only be partially explained based on HIF-2α-directed transcription. As mentioned before, the canonical response to low oxygen is the transcriptional induction of selected genes implicated in low oxygen survival (**Section 3.2.2**). It is well understood that hypoxia has a negative effect on the rate of protein translation in mammalian cells (Wouters et al., 2005). It is therefore unclear why cells exposed to low oxygen would choose to produce several hypoxia-related transcripts without having the possibility to produce the corresponding gene product. We thus suspected that HIF-2α could be implicated in transcribing and translating a subset of genes required for the growth of hypoxic cancer cells.

In parallel to the main findings on DNMT3a-mediated epigenetic regulation of *EPAS1*, some work I have done contributed to the discovery of a novel translational function of HIF-2α in low oxygenated cells and its downstream effect on growth and survival of hypoxic cells (Uniacke et al., 2012). Previous work from our laboratory led to the discovery that low oxygen was triggering the recruitment of EGFR mRNA to polysomal fractions (Franovic et al., 2009). ^{35}S labelling experiments also corroborated these findings and showed that the direct synthesis of EGFR in hypoxia was increased (Landeroute et al., 1992). Additional experiments suggested that the overexpression of HIF-2α was capable of catalyzing further the recruitment of EGFR mRNA to the actively translated intracellular pool of RNAs (Franovic et al., 2009). It was therefore hypothesized that HIF-2α could be directly stimulating the hypoxic translation of EGFR or other growth-promoting genes. To test this idea, we looked for the direct interaction of HIF-2α with endogenous EGFR mRNA by performing a series of RNA Immunoprecipitation experiments (RIP). These experiments showed that HIF-2α and not HIF-1α was specifically recruited to a 3'untranslated region (UTR) of EGFR mRNA in hypoxia-treated brain (U87MG), breast (MDA-MB 231) and prostate (PC3) cell lines (**Figure 31A**, Uniacke et al., 2012). A series of luciferase reporter constructs were used to further map the regulatory region conferring translation activity in low oxygen (**Figure 31B**). We found that a 200nt 3'UTR sequence from EGFR mRNA was required for the hypoxic translation of the luciferase gene and sufficient to confer full hypoxic translational activity to hypoxia-unrelated gatekeeping mRNAs such as actin or lamin (**Figure 31C**).

This ribonucleic hypoxia response element (rHRE) sequence was further shown to function in conditions of RNA polymerase inhibition since the hypoxic expression of the luciferase transgene occurred in actinomycin D pre-treated cells (Uniacke et al., 2012). We corroborated these findings at the endogenous level and EGFR protein expression was reliably induced in Actinomycin D-treated U87MG cells or when silencing of HIF-1β blocked HIF transcriptional activity (**Figure 31D**). Together, these experiments documented a novel hypoxic translation system that was independent of the canonical HIF-2α transactivation activity.

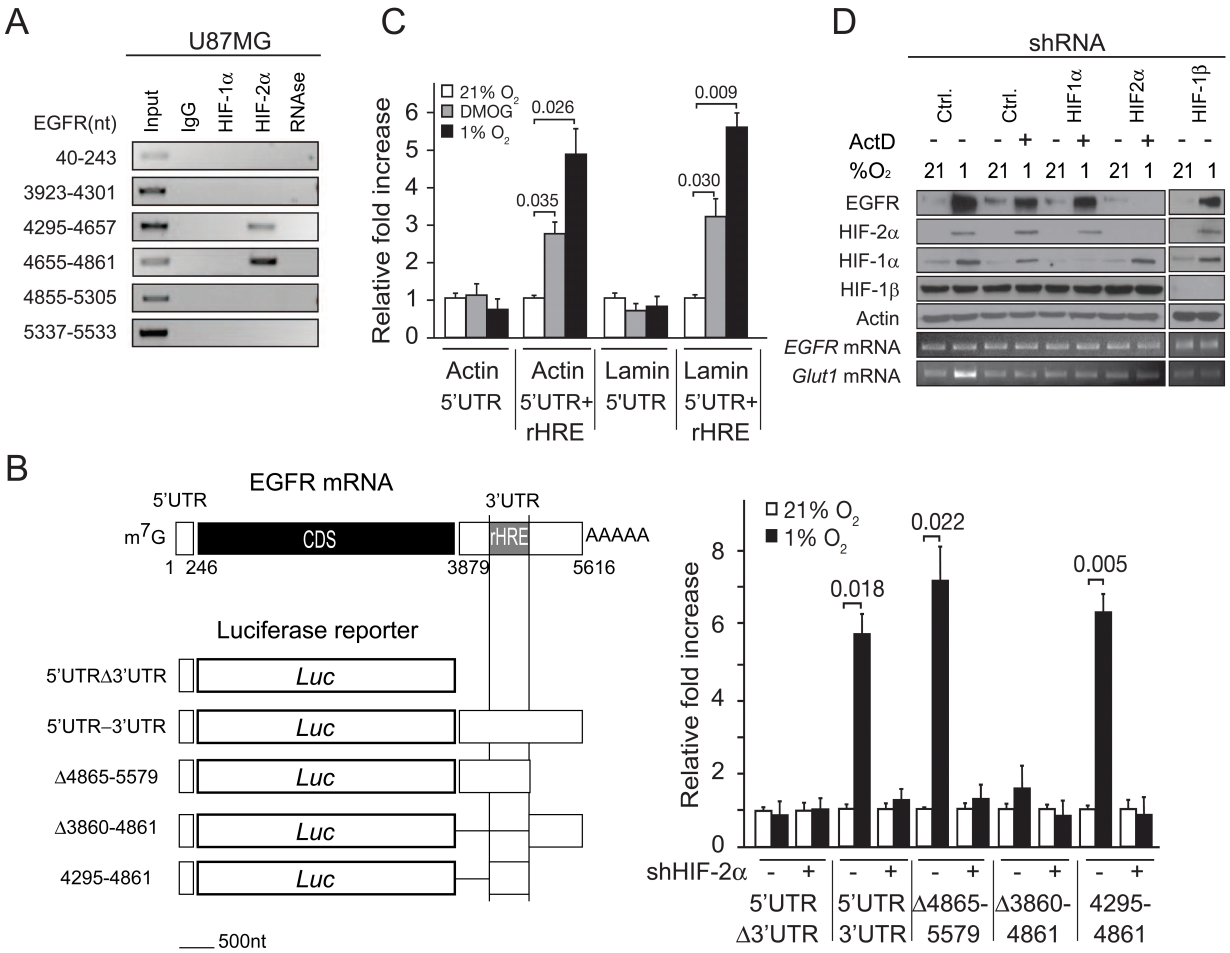

Figure 31

Figure 31. HIF-2α binds and regulates the EGFR 3'UTR mRNA ribonucleic hypoxia response element (rHRE) conferring hypoxic translation: (A) RNA immunoprecipitation of HIF-1α and HIF-2α. IN, input; RN, RNase-treated; nt, nucleotides. (B) Regions of the EGFR 3'UTR were cloned into a luciferase reporter vector. The highlighted region indicates the HIF-2α binding site (rHRE) observed by RNA immunoprecipitation in (A) (left). Dual luciferase assays in cells transfected with EGFR 3'UTR reporter constructs (right). (C) Dual luciferase assays in cells transfected with reporter constructs containing the 5'UTR of actin or lamin a/c with or without a 3'rHRE. DMOG, dimethyloxalylglycine. (D) Western blot of EGFR protein and mRNA in HIF-2α, HIF-1α or HIF-1β knockdown cells in the presence of actinomycin D (ActD). GLUT1 (also known as SLC2A1) was used as a control. Cont, control; shRNA, short hairpin RNA. Significances of fold changes (Student's t-test) are shown. Results are means and s.e.m. (n=3). From Uniacke et al., 2012.

Coimmunoprecipitation of HIF-2α and mass spectrometry sequencing of the binding partners further supported the novelty of this protein translation pathway since protein bound to HIF-2α and the rHRE were not the canonical HIF transcriptional cofactors. RNA-binding protein 4 (RBM4), a protein implicated in mRNA splicing (Markus and Morris, 2009) and Eukaryotic translation initiation factor 4E isoform 2 (eIF4E2) (an homolog of the eIF4E cap binding protein and catalytic unit of the translation initiation complex eIF4F) and eIF4A (a component of the eIF4F complex with RNA helicase function) were found to specifically interact with HIF-2α in hypoxic U87MG cells (Uniacke et al., 2012). Albeit it was not clear if the translation activity of HIF-2α was relevant for life in hypoxia, ^{35}S

labelling experiments in HIF-2α, RBM4 or eIF4E2-siRNA silenced U87MG cells later suggested that a great proportion of the hypoxic protein synthesis activity was dependent on this pathway (**Figure 32A, B**, Uniacke et al., 2012). To reconcile the contribution of HIF-2α-mediated translation to the early stages of cancer cell growth we conducted another set of experiments to test the requirement of hypoxic cancer cells on eIF4E2 expression specifically.

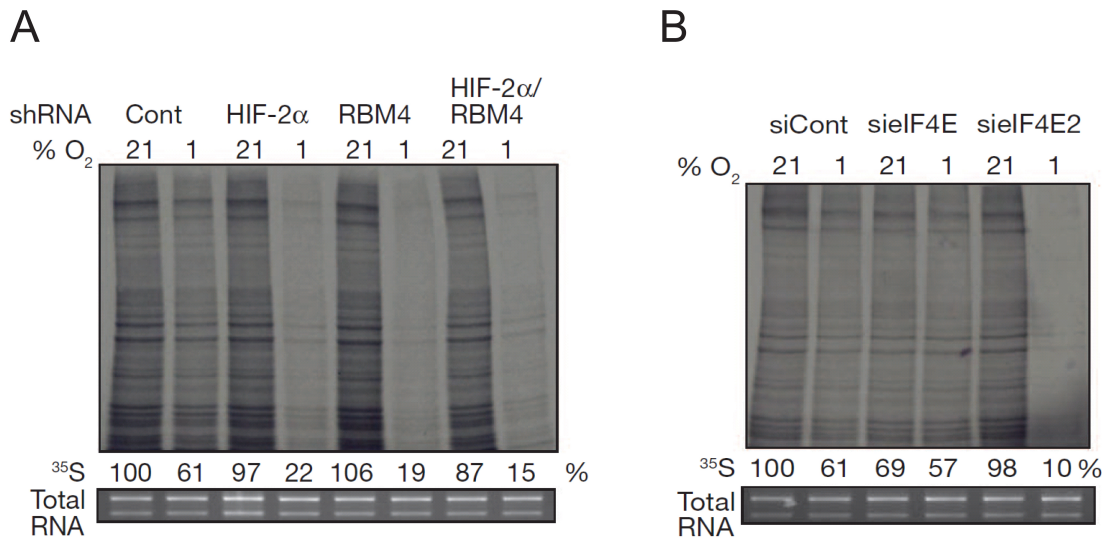

Figure 32

Figure 32. The HIF-2α/RBM4/eIF4E2 complex regulates protein synthesis in low oxygen: (A) Global translation rates in normoxic or hypoxic HIF-2α and/or RBM4 knockdown U87MG cells measured by ^{35}S incorporation. Total RNA is used as a loading control and the percentage of ^{35}S incoporation is shown on top of it. (B) ^{35}S global translation rates in normoxic or hypoxic eIF4E or eIF4E2 knockdown U87MG cells. Total RNA is used as a loading control and the percentage of ^{35}S incorporation is shown on top of it. From Uniacke et al., 2012.

5.2. The hypoxic translation machinery is required for traversing the hypoxia barrier and tumour growth.

We used our *in vitro* tumour multicellular spheroid assay to evaluate the importance of the oxygen-regulated protein synthesis machinery for the early stages of hypoxic growth in the tumourigenic astrocytomas cell line U87MG. We used U87MG cells stably depleted of eIF4E2 (Uniacke et al., 2014) and compared their growth in parallel assays to control shRNA lentivirus expressing cells. For the *in vitro* tumour growth assay, eIF4E2-depleted U87MG cells were plated as low-density clusters of 50 cells in suspension with control expressing cells in parallel. Over time eIF4E2 knockdown cells failed to form multicellular masses beyond a diameter of 100-200 microns while control cells readily grew beyond this size (**Figure 33**). Interestingly, these experiments matched perfectly the phenotype acquired by reintroducing DNMT3a-mediated epigenetic silencing of HIF-2α (**Figure 25A, B** and **Figure 30A, B**) and showed that eIF4E2 expression was required to shift cancer cell's growth from normoxic to hypoxic conditions (**Figure 33A**). Since eIF4E2 protein is expressed via two different protein species on western blot and each is selectively targeted by the various shRNAs, we also tested the effect of specifically reintroducing eIF4E2 cDNA expression on the capacity of the knockdown cells to grow again in spheroid assays (**Figure 33B**). Expectedly, re-expression of eIF4E2 was sufficient to shift cancer cell growth past the 100-200 micron diameter supporting that this gene alone was required for normoxic to hypoxic cancer growth (**Figure 33B**). Taken together, these experiments suggested that tumour cells grown from multicellular masses displayed an association

between the DNMT3a-mediated epigenetic program and the hypoxic protein translation machinery at the crux of the normoxic to hypoxic growth transition.

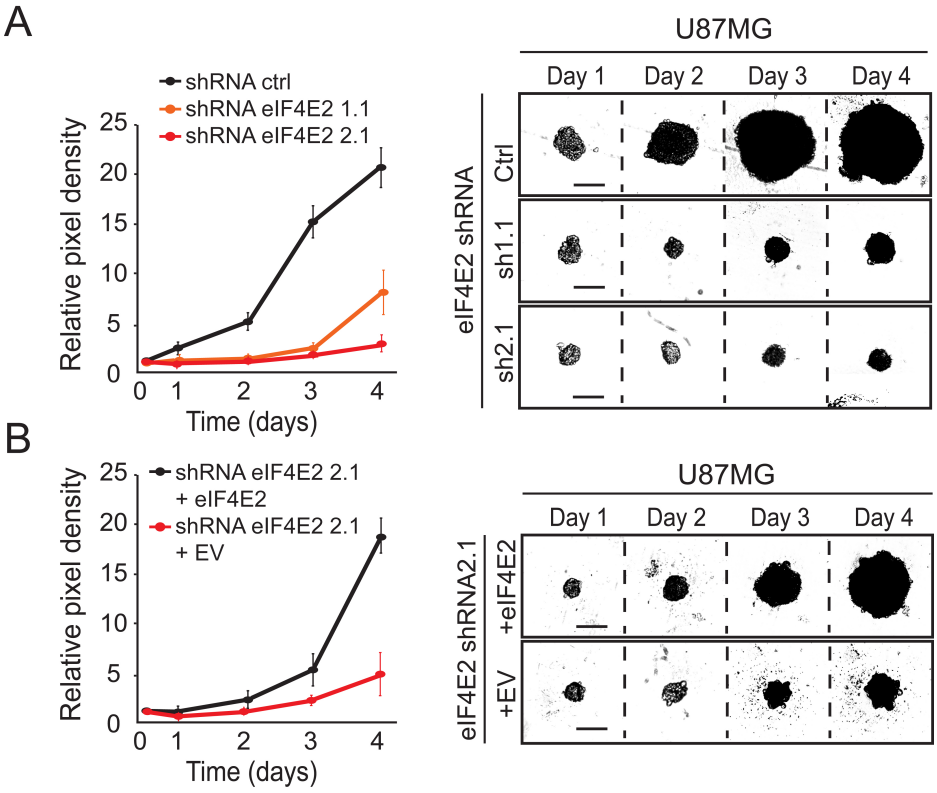

Figure 33

Figure 33. eIF4E2 regulates the hypoxic barrier for tumour cell growth: (A) 50 U87MG single cells with stable knockdown of eIF4E2 (shRNA eIF4E2 1.1 and shRNA eIF4E2 2.1) or control (shRNA ctrl.) were plated and agglomerated to form multicellular spheroid masses. At various days post-plating spheroids were visualized and their relative size measured by counting total pixel (left). Representative images of the experiment are shown (right). (B) 50 single U87MG shRNA eIF4E2 2.1 cells stably re-expressing eIF4E2 or a control cDNA were plated and measured as in A). Scale bars represent 100 microns.

5.3. Normal cells utilise HIF-2α translation activation pathway to survive in hypoxia.

Numerous evidence suggested that RBM4 and eIF4E2, the cofactors known experimentally to be required for HIF-2α-mediated hypoxic protein synthesis, were expressed and functional in normal renal cells. First, we found no change in RBM4 expression between normal and renal tumour cells when we interrogated the protein atlas data bank (**Figure 34A**). In addition, direct siRNA silencing of RBM4 blocked hypoxia-mediated induction of EGFR protein levels in RECs (**Figure 34B**), supporting that RBM4 was functioning normally in normal renal epithelial cells. Also, western blot analysis of eIF4E2 protein levels in primary RCC and matching normal adjacent renal tissue showed little change in eIF4E2 expression (**Figure 35A**).

More importantly, eIF4E2 was expressed in all normal kidney samples and also in cultures of renal epithelial cells (**Figure 35A, B**), suggesting that eIF4E2 could have functions in both normal and cancer cells in low oxygen. Backing this, silencing of eIF4E2 abrogated the capacity of the primary renal cells to upregulate EGFR protein levels under hypoxic conditions (**Figure 35B**) and to grow normally in low oxygen (**Figure 36A, B**). This proliferation defect correlated with a significant loss of viability in hypoxia as shown by the marked trypan blue positive staining in shRNA silenced eIF4E2 cells (**Figure 36C**). It thus appeared form these preliminary evidences that the core hypoxic protein translation machinery of normal renal epithelial cells worked to some extent.

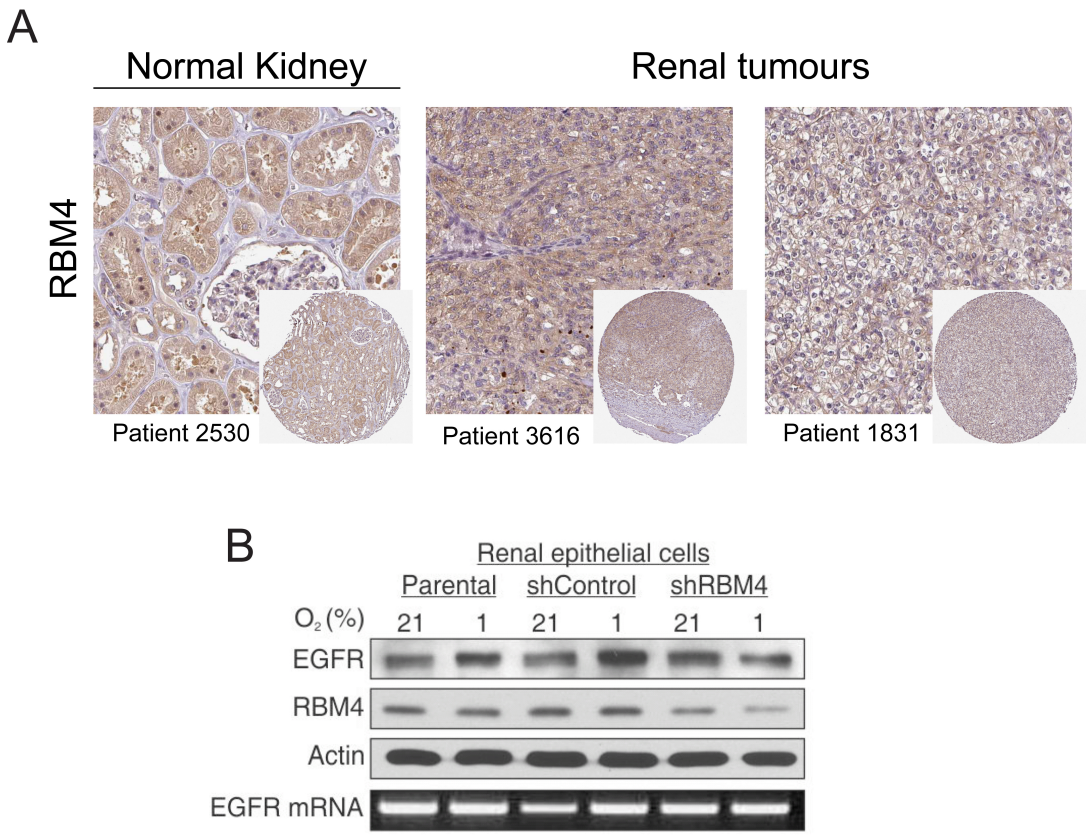

Figure 34

Figure 34. RBM4 is functional in normal renal cells: (A) The protein atlas databank was interrogated for the expression of RBM4 in normal kidney and RCC tumour samples stained with RBM4 antibody and representative samples are shown. (B) Normal human renal epithelial cells were transfected with a control and an siRNA targeting endogenously expressed RBM4. EGFR protein and mRNA expression was used as a readout for increased RBM4-dependent translation in hypoxic cells.

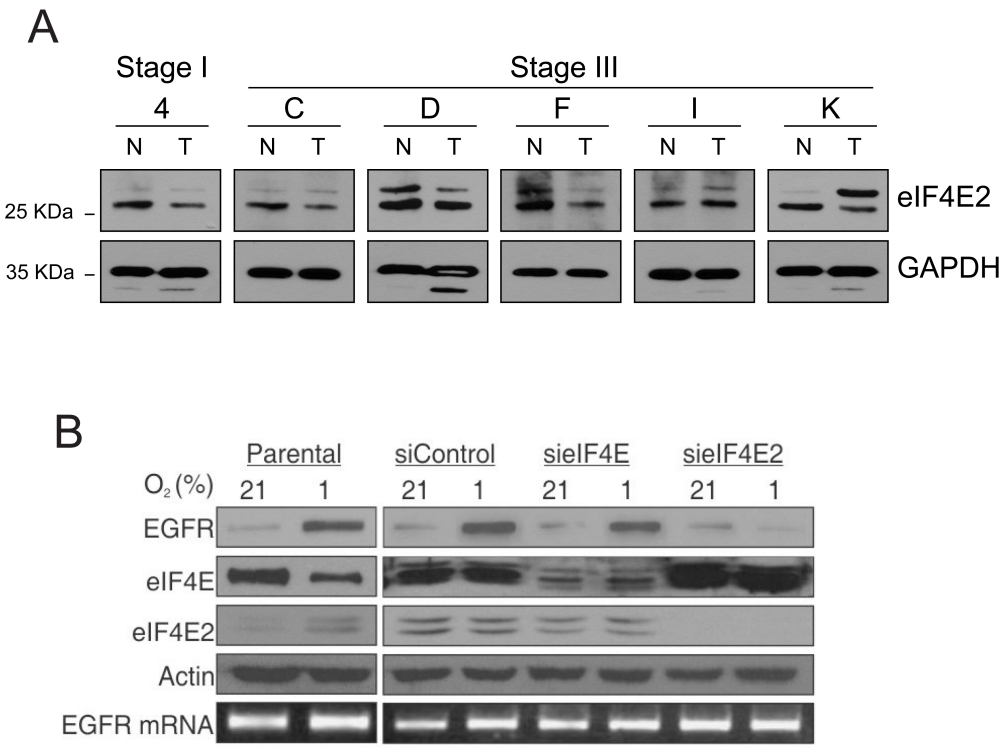

Figure 35

Figure 35. eIF4E2 is functional in normal renal cells: (A) Expression of eIF4E2 protein in a panel of primary RCC tumours samples obtained from the Ontario Tumour Bank and measured by western blot. Tumour samples were compared to their normal adjacent tissue counterparts. (B) Normal human renal epithelial cells were transfected with a control siRNA, siRNA targeting endogenously expressed eIF4E and eIF4E2. EGFR protein and mRNA expression was used as a readout for increased eIF4E2-dependent translation in hypoxic cells.

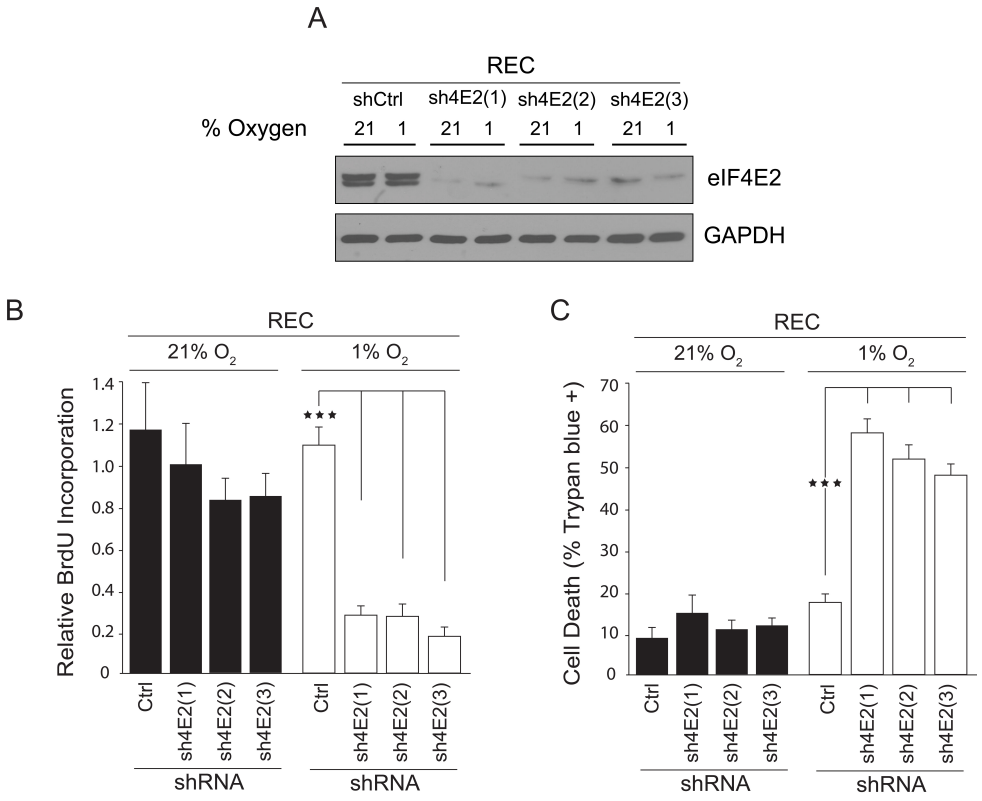

Figure 36

Figure 36. eIF4E2 is required for normal growth and survival of renal epithelial cells: (A) Western blot analysis of eIF4E2 protein levels in primary renal epithelial cells (REC) transiently expressing shRNAs targeting eIF4E2 (sh4E2(1), Sh4E2(2), Sh4E2(3)), or non-targeting control shRNA (Control). GAPDH was used as a loading control. (B) The ability of REC transiently expressing control shRNA (Ctrl) or shRNAs targeting eIF4E2 to proliferate in culture was measured by BrdU incorporation following incubation in normoxia or hypoxia for 24h. BrdU-positive cells were reported relative to cells stably expressing non-targeting shRNA. (C) % Cell death was measured for eIF4E2-depleted or control REC exposed to 48h of normoxia or hypoxia by dividing trypan blue-positive cells by the total number of cells. Columns, mean (n = 3); error bars, s.e.m. Significance measured by student's t test *** $p < 0.001$.

5.4. DNMT3a-depleted human renal epithelial cells require HIF-2α expression to grow and survive in hypoxia.

Since cofactors utilized for HIF-2α mediated hypoxic protein synthesis were active in normal epithelial cells, we expected to measure some effect of silencing DNMT3a and upregulating HIF-2α expression in primary cells. As mentioned previously we did not observe any oncogenic property activated following DNMT3a loss (**Figure 11**). In its place, we tested for a synergic effect of DNMT3a depletion on the normal growth and survival of RECs in low oxygen.

To test specifically for hypoxia-related phenotypes we measured the viability as well as the rate of proliferation of DNMT3a-depleted cells from different time points in low oxygen, growth factor supplemented primary cell cultures. Proliferation was measured via BrdU incorporation in parallel to viability assays using trypan blue viability dye (**Figure 37A, B**). Results showed that BrdU incorporation was impaired in unaltered and control primary cells after 24h of hypoxia but resumed after 96h suggesting that RECs could adapt to a prolonged period of low oxygen (**Figure 37A**). We then tested the effect of silencing DNMT3a for the growth and survival of primary renal epithelial cells after a prolonged hypoxia treatment. DNMT3a-depleted RECs displayed little reduction in BrdU incorporation after 24h of hypoxia compared to the significant drop of BrdU incorporation seen in control cells (**Figure 37A**).

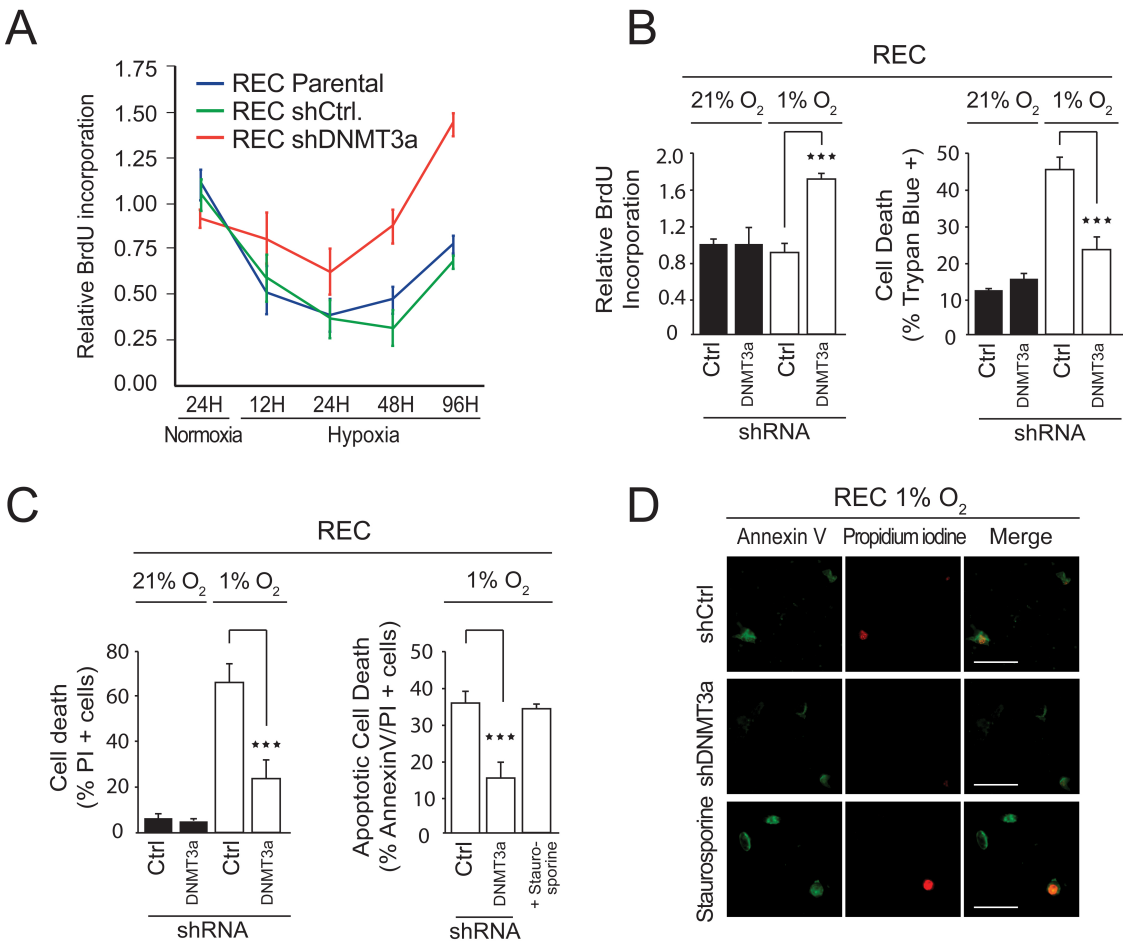
A
Relative BrdU incorporation
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
REC Parental
REC shCtrl.
REC shDNMT3a
24H
12H
24H
48H
96H
Normoxia
Hypoxia
B
REC
21% O2
1% O2
Relative BrdU Incorporation
2.0
1.6
1.2
0.8
0.4
0
***
Ctrl
DNMT3a
Ctrl
DNMT3a
shRNA
Cell Death (% Trypan Blue +)
50
40
30
20
10
0
***
Ctrl
DNMT3a
Ctrl
DNMT3a
shRNA
C
REC
21% O2
1% O2
Cell death (% PI + cells)
80
60
40
20
0
***
Ctrl
DNMT3a
Ctrl
DNMT3a
shRNA
1% O2
Apoptotic Cell Death (% AnnexinV/PI + cells)
50
40
30
20
10
0
***
Ctrl
DNMT3a
+ Stauro-sporine
shRNA
D
REC 1% O2
Annexin V
Propidium iodine
Merge
shCtrl
shDNMT3a
Staurosporine

Figure 37

Figure 37. Silencing DNMT3a enhances growth and survival of renal epithelial cells in low oxygen: (A) Parental, DNMT3a-depleted or control-expressing primary human renal epithelial cells (REC) were tested for proliferation in prolonged hypoxia using a BrdU incorporation assay. Cells were incubated in normoxia or hypoxia for 24h and up to 96h for the latter. BrdU was labelled for 2h and detected by immunohistochemistry using Hoescht for total cell count and expressed as a relative ratio of BrdU:Hoescht incorporation. (B) RECs transfected with control (Ctrl) or DNMT3a-specific shRNA were exposed to normoxic (21% O_2) or hypoxic (1% O_2) conditions for 96h before a 2h BrdU labeling (left) or trypan blue staining (right). (C) RECs depleted of DNMT3a via lentiviral-based shRNA silencing were grown for 96h in normoxic (21%) or hypoxic (1%) environments in parallel with control shRNA-expressing cells prior to cell death analysis with propidium iodide incorporation detected by flow cytometry (Left). Annexin V in combination with PI was used to label apoptotic cells in hypoxic cultures of DNMT3a-silenced or control RECs. Staurosporine-treated (1 μM for 2h) cells were used as a positive control (Right). (D) Representative images from A are shown. (Scale bars, 30 μm). Columns, mean (n=3). Error bars represent SEM. Significance was measured by Student t test, *** $p < 0.001$.

Overall DNMT3a-depleted RECs had a consistently faster rate of BrdU incorporation that persisted throughout the prolonged hypoxia treatment (**Figure 37A**). Normal cells survived surprisingly well up to 48h after which viability started to be an issue and data was collected after 96h of hypoxia treatment when cell death was significantly higher than normoxic cells (**Figure 37B, right panel**). shRNA-directed DNMT3a loss caused the RECs to survive significatively better after prolonged hypoxia treatment (**Figure 37B, right panel**). For every instance, the growth rates measured by BrdU incorporation were inversely proportional to the amount of dead cells stained with trypan blue (**Figure 37B**). We also used the annexinV/Propidium iodine (PI) staining technique to test for apoptosis in control and DNMT3a-silenced RECs (**Figure 37C, D**). As expected, the silencing of DNMT3a reduced the extent of apoptotic cell death in hypoxic RECs (**Figure 37C, D**). Altogether the trypan blue viability dye and the AnnexinV/PI double positive cell assay both showed a reduced rate of cell death in DNMT3a-depleted cells compared to those expressing control shRNA, further supporting the survival advantage of DNMT3a-depletion for primary renal epithelial cells growing in low oxygen.

To test the possibility that HIF-2α expression could be the limiting factor regulating hypoxic growth and survival of DNMT3a-depleted primary renal epithelial cells, double silencing experiments of both DNMT3a and HIF-2α were sequentially performed in RECs. QPCR and Western blot were used in parallel to detect the expression level of DNMT3a and HIF-2α in hypoxic cells with double gene silencing (**Figure 38A, B**). DNA methylation assays of DNMT3a-silenced and DNMT3a/HIF-2α-silenced cells confirmed that DNMT3a epigenetic silencing was not changed following the double shRNA silencing,

supporting that the observed phenotypes were a result of HIF-2α knockdown specifically (**Figure 39**).

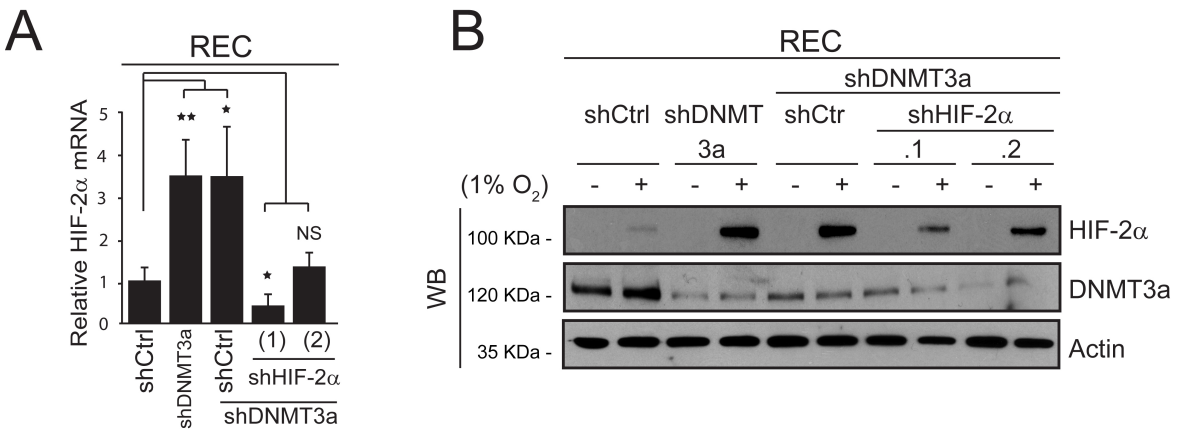

Figure 38

Figure 38. Restoration of normal HIF-2α expression in DNMT3a-depleted REC by double shRNA silencing: (A) Primary renal epithelial cells (REC) expressing lentiviral shRNAs targeting DNMT3a and HIF-2α and appropriate lentiviral shRNA controls were included where indicated and samples were tested by qPCR for the expression of HIF-2α mRNA in DNMT3a/HIF-2α double-silenced RECs. (B) Western blot analysis of HIF-2α protein levels in DNMT3a/ HIF-2α double-silenced RECs incubated in normal (-) or hypoxic (+) environments. DNMT3a and actin protein levels were tested in parallel as controls.

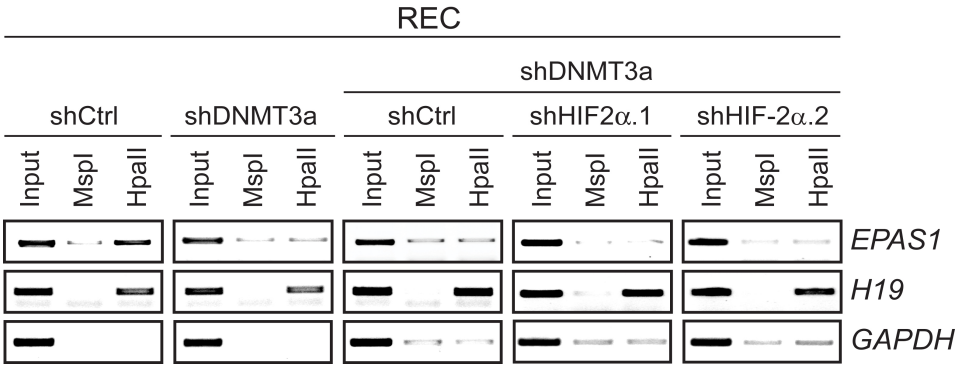

Figure 39

Figure 39. Double silencing of DNMT3a/HIF-2α is not changing the epigenetic status of endogenous *EPAS1* locus: (A) DNMT3a and HIF-2α double silencing in primary renal epithelial cells (REC). MSRPCR analysis of *EPAS1*, *H19* (positive DNA methylation control), and *GAPDH* (negative DNA methylation control) genes in RECs expressing lentiviral shRNAs targeting DNMT3a and HIF-2α mRNA. Appropriate lentiviral shRNA controls were included where indicated.

Lentiviral-mediated shRNA knockdown of HIF-2α abrogated the growth and survival advantage granted by shRNA silencing of DNMT3a in REC cells exposed to prolonged hypoxia (**Figure 40A**). Importantly, the specific knockdown of HIF-2α returned growth and survival of DNMT3a inactivated cells back to that of unaltered controls (**Figure 40A**). Trypan blue viability assay and annexinV/PI apoptosis analysis further confirmed that silencing HIF-2α fully abrogated the survival advantage granted by silencing DNMT3a in primary cells (**Figure 40A, right panel, B, C**). Altogether these functional assays confirmed that the growth and survival phenotypes activated by DNMT3a loss in hypoxic primary cells were imputable to HIF-2α expression.

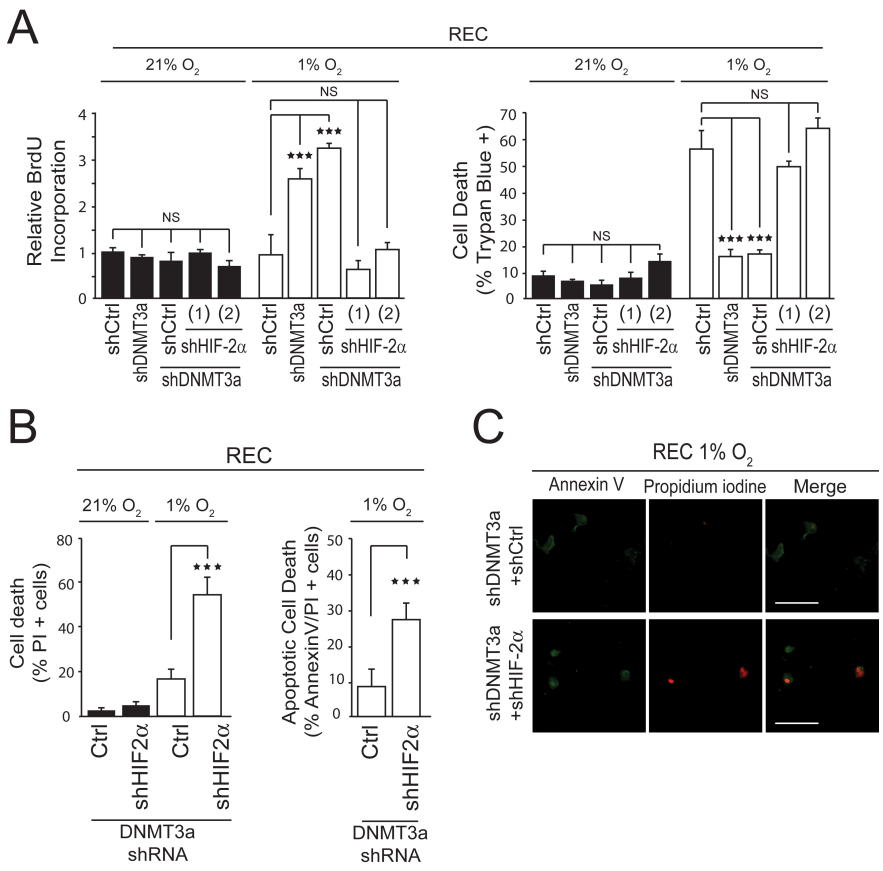

Figure 40

Figure 40. Silencing HIF-2α in DNMT3a-depleted RECs suppresses adaptation to hypoxia: (A) Primary renal epithelial cells (REC) were transduced with lentiviruses expressing shRNAs against DNMT3a and HIF-2α mRNA or control shRNA. Infected cells were grown under prolonged normoxic or hypoxic conditions (96 h) before proliferation analysis by BrdU incorporation (left panel) and trypan blue viability analysis (right panel). (B) Viability of RECs expressing DNMT3a shRNA was assayed in a low-oxygen environment using flow cytometry to count PI incorporated (left panel) or Annexin V/PI staining (right panel). (C) Representative images are shown. Columns, mean (n=3). Error bars represent SEM. Significance was measured by Student t test, *** $p < 0.001$. Scale bars represent 100μm.

DISCUSSION

1. *EPAS1* is methylated

After first observing the structure of the *EPAS1* DNA locus we tested for the possibility that an epigenetic silencing mechanism directed by CpG DNA methylation was responsible for restricting HIF-2α expression in normal renal epithelial cells. Various techniques were combined to correlate *EPAS1* DNA methylation with the low expression of HIF-2α mRNA in non-malignant tissues and cells. We first utilized the MSRPCR technique since it is highly sensitive and requires little starting DNA material for analysis. Despite this, HpaII digestions are limited by the coverage of CCGG sequence, the presence of sequence variants (SNPs) or DNA structure prohibiting access to the DNA (Halder et al., 2010; De and Michor, 2011). For these reasons we included a second isoschizomer constitutively active restriction enzyme and thus controlled for the access and the presence of the recognition sequence (Waalwijk and Flavell, 1978). We found this assay was highly reproducible and sensitive but still limited by the representation of 5'-CCGG-3' on the DNA tested. The MeDIP technique circumvented this limitation and we were able to detect several sites of DNA methylation on *EPAS1* that were also detected by MSRPCR. We further assessed *EPAS1* DNA methylation with bisulfite sequencing. Utilizing multiple assays, we concluded that at steady state *EPAS1* was methylated in normal human kidney, primary renal epithelial cells and normal human astrocytes.

Considering repressed HIF-2α mRNA expression in methylated cells, the presence of DNA methylation suggested two main gene silencing mechanisms. DNA methylation could be regulating the interaction of transcription activating/repressing factors or signalling the recruitment of additional epigenetic regulators to mark the gene for heterochromatin formation. Our bioinformatics analyses using Genomatix MatInspector software matched the sites of DNA methylation with putative sites of regulation by transcription factor binding including Fork Head Domain factor (FKHD), Homeobox Factor (HOXF), E2FF, SMAD, Transcription Factor IIB (TF2B), Early Growth Response (EGR), Signal Transducer and Activator of Transcription (STAT) and CCCTC-Binding factor (CTCF). Recognition sequences for CTCF and CTCF-binding factor (zinc finger protein)-like (CTCFL) are regulated by DNA methylation (de la Rosa-Velazquez et al., 2007). More importantly, *EPAS1* is differentially methylated in CTCF haploinsuficient and CTCF mutated human endometrial cancers (Kemp et al., 2014), suggesting that CTCF could be an important DNA methylation target at the *EPAS1* locus.

The fact that we found *EPAS1* to be equally methylated in renal epithelial cells and astrocytes suggests that a common progenitor cell in the development of these two lineages transmitted this epigenetic silencing mark. Both renal epithelial cells and astrocytes are part of the epiblast lineage and a genome-wide epigenetic study has shown that epiblast and mouse embryonic stem cells have different patterns of DNA methylation with increased overall gene methylation in the epiblast cells (Veillard et al., 2014). Interestingly, embryonic development is known to occur in a period of oxygen limitation (Dunwoodie SL, 2009). Also, the *in vitro* conversion of mouse epiblast stem cells to embryonic stem

cells causes a significant enrichment in differential expression of HIF-2α target genes (Ware et al., 2014). Human and mouse ES cells do not have a methylated *EPAS1* gene (Varley et al., 2013; Brunner et al., 2009), altogether suggesting that *EPAS1* expression was possibly repressed at the epiblast stage or before. Understanding these initial DNA methylation events leading to *EPAS1* silencing during embryonic cell lineage commitment could inform us on the mechanisms targeting DNMTase activity at this locus.

1.1 Cancers lose *EPAS1* DNA methylation.

We were able to correlate HIF-2α mRNA levels in cancers with the simultaneous loss of DNA methylation at the CpG island. Using MSRPCR assays, we compared both normal renal cell/RCC and normal astrocytes/astrocytomas and found consistent hypomethylation of *EPAS1* at two different loci targeting both sides of *EPAS1* TSS. Since 786-0 cells displayed residual DNA methylation as measured with the bisulfite assay (**Figure 2**) we asked whether this activity could be attributed to some residual DNMTase activity on *EPAS1*. We reported that 5-aza treatments had no effect on the expression level of HIF-2α in renal cancer cells, suggesting that RCC cells fully reprogrammed the epigenetic silencing of *EPAS1* (**Figure 4**). This suggested that loss of DNA methylation observed in these cancer cells accounted for most of the *EPAS1* oncogenic expression.

We found the same correlation between *EPAS1* DNA methylation and HIF-2α mRNA levels in primary tumour and normal adjacent kidney DNA. We limited our MSRPCR analyses to a 200bp section of *EPAS1* CpG in primary tumours. Although we found no

cancer samples with high *EPAS1* DNA methylation, it could be interesting to further map the sites of DNA methylation on *EPAS1* in these samples since we might have underestimated the hypomethylation rate of *EPAS1* in cancers. In parallel, ChIP analysis of DNMT3a binding would complement these assays and could allow deciphering of which site of DNA methylation is key for the regulation of *EPAS1* differential methylation in cancer.

An important question that was raised by the loss of *EPAS1* methylation in cancers was what is the leading cause of this hypomethylation? Our results suggest that DNMT3a is required for maintaining the methylation of *EPAS1*, since the acute silencing of DNMT3a in renal epithelial cells causes spontaneous demethylation of *EPAS1*. A number of research groups also reported that deletion of DNMT3a in somatic cells causes the acute loss of DNA methylation, supporting the notion that this *de novo* DNMTase can maintain the epigenetic landscape of normal cells. These experiments do not exclude the possibility that *EPAS1* could be demethylated via active DNA demethylation and that the loss of DNMT3a-mediated epigenetic silencing would cause the balance to shift towards greater DNA demethylation of *EPAS1*.

Demethylation of ^{m}C can be catalyzed by enzymes *in vitro* but the relevance of enzymes for direct DNA demethylation *in vivo* is controversial (Wu and Zang, 2010). Active routes for DNA hypomethylation encompass glycosylation of the ^{m}C and subsequent modification for a cytidine by the DNA repair machinery (Wu and Zang, 2010). The limiting step for the conversion of ^{m}C to C is thought to be the formation of 5-hydroxymethylcytosine from 5-methylcytosine by the Ten-eleven translocation (Tet) enzymes. In T-cell lymphomas and

acute myeoloid leukemia, DNMT3a mutations are often concurrent with Tet2 mutations (Couronné et al., 2012). Tet hydroxylation of ^{m}C was recently proposed as an intellectually satisfactory explanation for genome-wide DNA hypomethylation observed in early embryogenesis (Kinney and Pradhan, 2013). There are several genome-wide analyses of the hydroxymethylated genome publicly available but *EPAS1* is not differentially expressed in Tet KO cells (Dawlaty et al., 2014; Huang et al., 2014).

No matter how *EPAS1* methylation is lost, cells with hypomethylated *EPAS1* could potentially have a significant advantage in conditions of low oxygen. If a DNA demethylation pathway acted on *EPAS1* it would potentially be active in hypoxia, however we do not know if *EPAS1* DNA methylation is reversible. For this reason, we analysed *EPAS1* DNA methylation in primary renal epithelial cells treated for 96h in hypoxia and observed no change at any time point at the locus tested (**Appendix 2**, see **Figure 2** for primer set 4). In support for a role of hypoxia in regulating the epigenetic silencing of *EPAS1*, a research group recently showed that *EPAS1* DNA methylation was lost in early-onset preeclampsia human placenta samples (Preeclampsia is a syndrome associated with high-blood pressure during pregnancy and is likely caused by abnormal placental development. The most severe consequence of preeclampsia is the onset of hypoxic conditions during foetus development). This abnormal *EPAS1* hypomethylation in preeclampsia inversely correlated with *EPAS1* gene (Blair et al., 2013) and protein expression (Rajakumar et al., 2001), suggesting an adaptive epigenetic response to oxygen deficit.

1.2 Functional analysis of *EPAS1* DNA methylation.

We tested the relevance of DNA methylation for *EPAS1* silencing by treating primary human renal epithelial cells with a general inhibitor of DNA methylation, 5-azacytidine (5-aza). 5-aza traps cellular DNMTases thereby causing progressive DNA demethylation in replicating cells via a passive effect. Accordingly, primary cells were incubated for 72h with 5-aza to ensure minimal replication and we observed that upregulation of HIF-2α mRNA levels inversely correlated with *EPAS1* hypomethylation. As expected, this caused HIF-2α protein to be stabilized in hypoxia. Interestingly, HIF-2α mRNA was also shown to be activated by 5aza in ES cells when triggered for differentiation by removing leukemia inhibitory factor (LIF) (Banerjee and Bacanamwo, 2010). Notably, 5-aza caused the cells to differentiate into endothelial cells, the cell type with the most abundant HIF-2α expression. This leads to the interrogation of why suppressing HIF-2α expression would require two independent silencing mechanisms in normal epithelial cells: DNA methylation and oxygen-dependent degradation of the protein? Our results suggest that renal epithelial cells epigenetically silence the HIF-2α isoform to limit the growth and survival properties of hypoxic cells. In support for this, animals with HIF-2α Knock-in in place of HIF-1α have several developmental defects including abnormal vasculature and expanded epiblast tissue. Those phenotypes were different from HIF-1α-/- suggesting a gain-of-function effect of abnormal HIF-2α isoform expression during the normal hypoxic response (Covello et al., 2006).

2. DNMT3a is lost in genetically different cancer

ChIP experiments revealed DNMT3a interacts with *EPAS1* CpG in primary renal epithelial cells. By taking advantage of the protein atlas online databanks, we found several tumour cases with alterations in DNMT3a expression and localization (**Figure 15, 16**). Consistent with our independent qPCR analysis of DNMT3a mRNA levels in RCC, we found DNMT3a protein was down in several renal tumours and renal cancer cell lines (**Figure 6**). We were able to reconcile DNMT3a loss with the epigenetic status of *EPAS1* in two independent cellular systems of normal renal epithelial cells/RCC and normal human astrocytes/astrocytomas. In renal and brain tumourigenic cells, both DNMT3a mRNA and protein were depleted. Interestingly, another research group also observed that DNMT3a was not expressed (both mRNA and protein) in gliomas and brain tumour stem cells compared to normal human astrocytes and normal neural progenitor cells (Fanelli et al., 2007).

The fact that DNMT3a mRNA and protein levels are equally lost in renal and brain cancer cell lines suggests that mutational events directly target the stability or the expression of DNMT3a in cancers. In AML, an internal promoter of the DNMT3a gene is responsible for the expression of an alternative DNMT3a2 transcript (Chen et al., 2002). A CpG site within that alternative promoter was found to be methylated in 40% of the total AML samples tested suggesting that expression of DNMT3a can be targeted for epigenetic silencing. This CpG epimutation was not associated with coding sequence mutants of DNMT3a, such as R882H somatic mutations providing an alternative explanation for the loss of DNMT3a expression in cancers (Jost et al., 2013). The specific loss of DNMT3a mRNA in cancer

was also observed in lung and colorectal cancer tissues (Kim et al., 2013; Li et al., 2014). Interestingly, HIFα-activated pathways are required for the *in vivo* tumour growth of lung (Franovic et al., 2009; Kim et al., 2009), colorectal (Franovic et al., 2009; Imamura et al., 2009; Xue et al., 2012) and possibly other carcinomas (Omar T, 2012). In addition to the loss of DNMT3a mRNA expression, we found several cases of colorectal, prostate and ovary tumours that displayed atypical cytoplasmic expression of DNMT3a that contrasted with the strong nuclear expression seen in all normal tissues (**Figure 15, 16**). Independent immunohistochemistry analyses of lung cancers also reported a similar pattern for DNMT3a localization whereby the protein was found in the cytoplasm of some tumours or absent from cancer tissues compared to adjacent normal bronchial epithelial tissue (Kim et al., 2012). Overall several lines of evidence suggest alternative routes to target DNMT3a in human cancers. Future experiments need to address whether DNMT3a-mediated epigenetic silencing is altered in cancer cells from lung, colon, prostate or ovary and contribute to their hypoxic growth.

Our data also raise concerns about the widespread use of 5-aza in cancer therapy. Several groups reported encouraging results for leukemia chemotherapy and prompted its therapeutic usage for solid cancers (Mantalban-Bravo and Garcia-Manero, 2014; Li et al., 2014). Gene knockout experiments demonstrated that 5-aza cytotoxicity was mediated by DNMT3a and DNMT3b (Oka et al., 2005). We have shown that a number of renal cancers lose DNMT3a expression (**Figure 6**) and therefore it could be important to screen patients based on DNMT3a expression/activity before challenging tumours with 5-aza since the cytotoxic effect of 5-aza might not be achievable in DNMT3a-inactivated tumour cells.

2.1 Our result suggest DNMT3a is constitutively targeted in renal cancers

Since HIF-2α expression is mainly required in hypoxic conditions, it is unclear why DNMT3a would need to be constitutively low in RCC and brain cancer cell lines and not targeted specifically in hypoxia? This is particularly puzzling in the case of non-VHL cancers since there are no advantage *a priori* to lose *EPAS1* DNA methylation and express HIF-2α mRNA in normoxia because VHL constitutively degrades HIF-2α protein. This would suggest that loss of DNMT3a-mediated epigenetic silencing confers a positive advantage to renal and brain cancer cells in normoxia. Indeed, DNMT3a loss was well tolerated in RECs, suggesting the overall negative effects were counterbalanced by gain of function events. Furthermore, our DNMT3a knockdown experiments revealed a role for DNMT3a in normal cellular growth of primary renal epithelial cells since DNMT3a-depletion augmented the number of passages in normal growth conditions (**Figure 11**). In support for this, it was recently found that a rare overgrowth syndrome was linked to mutations in the DNMT3a gene (Tatton-Brown et al., 2014). These novel DNMT3a mutations were in functional domains, predicted dysfunctional activity and were thought to cause facial protuberance and intellectual disabilities. In addition, the overexpression of DNMT3a R882H mutant found in leukemic cells causes a dominant increase in cellular proliferation when expressed in DNMT3a wild-type cells (Yan et al., 2011). As for the case of many new DNMT3a mutations, the downstream-activated pathways are not known yet. In our experiments, the only non-hypoxic DNMT3a-activated gene we studied was *EYA4* and this gene is not directly related to proliferation, longevity or telomere maintenance. EYA4 is a transcription coactivator required for normal hearing and mutations in EYA4 are

linked to deafness (Wayne et al., 2001). *EYA4* is also differentially methylated in hepatocarcinoma, melanoma and lung cancer tissues although its direct participation in tumour growth is not clear (Miller et al., 2010; Hou et al., 2013; Ecsedi et al., 2014; Wilson et al., 2014). Alternatively, several mutations in tumour suppressor genes, VHL, FH, SDH and IDH are known to cause a pseudohypoxic phenotype by causing the abnormal expression of HIF-2α in normoxic renal and brain cancer cells. In the eventuality that these mutations would precede the loss of DNMT3a-mediated epigenetic silencing, HIF-2α expression could be attained in these pseudohypoxic conditions. The co-occurrence of IDH and DNMT3a mutation in AML suggest that this is plausible (Lin et al., 2013; Im et al., 2014). Gene expression microarray analysis of DNMT3a-silenced REC cells would help clarify the contribution of both normoxic and hypoxic DNMT3a-epigenetic programs to malignant tumour growth.

3. DNMT3a regulates HIF-2α expression

We gathered significant evidence to implicate DNMT3a activity in the normal epigenetic silencing of *EPAS1*. In order to confirm its direct involvement in regulating HIF-2α expression we tested the effect of reintroducing DNMT3a in cancer cells and silencing DNMT3a in normal epithelial cells. By reintroducing DNMT3a in renal and brain cancer cells, we were able to restore normal expression and epigenetic silencing of *EPAS1* (**Figure 9, 21**). This suggested that the mechanism targeting DNMT3a to the DNA, at least to *EPAS1* and *EYA4* (**Figure 9**) was intact in these cancer cells. We were not able to stably express DNMT3a in KTCL and RCC4 cells suggesting that either the normal DNMT3a-

associated machinery was not functional or that active silencing mechanisms were targeting DNMT3a in these cells. The fact that we were able to repress HIF-2α expression by transiently expressing DNMT3a in RCC4 cells suggests that the latter is probably true (**Figure 9**). We restored the expression of DNMT3a in several other cancer types (**Figure 17, 20**). The next logical step would be to obtain normal corresponding cells and compare the epigenetic status of *EPAS1* to those of DNMT3a-reintroduced lung and colorectal tumourigenic cells.

In normal human primary cells, our shRNA-mediated silencing experiments showed that DNMT3a was required for targeting *EPAS1* for DNA methylation (**Figure 10**). Several genome-wide DNA methylation studies showed the extent of genes regulated by DNMT3a. Knockout animals revealed that DNMT3a prevented cancer-driven hypomethylation (Raddatz et al., 2012) and that DNMT3a was required to maintain DNA methylation boundaries in normal mouse heamatopoietic stem cells (Jeong et al., 2014). A close examination of some of these studies confirmed that several sections of the *EPAS1* CpG island were either hypomethylated or strongly hypomethylated in DNMT3a-/- cells. (chr17:8715068-87155080, 87130613-87132289, 87180675-87181650, 87168821-87169659, 87211179-87211638, 87185381-87185793) (Appendix 3). In these cells, other DNMT3a targets such as *EYA4* were also hypomethylated (chr10:23212589-23213839). Reckoning the similarity between the hypomethylated genes in DNMT3a-/- cells could predict the CpG site preferentially methylated by DNMT3a or a frequent transactivator binding site targeted by DNMT3a-mediated DNA methylation.

Importantly, these targeting experiments, including our shRNA experiments showed that loss of DNMT3a alone was sufficient to abolish the epigenetic silencing of several genes including *EPAS1*. This goes against the theory that *de novo* DNA methylation is only required for early genome reprogramming and embryonic development. Several groups including us provided evidence that *de novo* DNA methylation function is required for normal gene regulation in somatic cells and tissues (Wu et al., 2010; Oliveira et al., 2012; Colquitt et al., 2014; Morris et al., 2014). In addition these findings suggest that DNMT3a and DNMT3b have non-overlapping roles in somatic cells. This notion was supported by our experiments in which silencing DNMT3b did not change the epigenetic status of *EPAS1*, and furthermore was predicted based on our non-exhaustive ChIP analysis that revealed DNMT3b did not interact with *EPAS1* while DNMT3a did.

3.1 DNMT3b cannot compensate for DNMT3a loss

The catalytic domains of DNMT3a and DNMT3b share 84% homology. With the exception of a short palindromic sequence (2-6bp) including the CpG base pair, no DNA primary sequence can reliably predict the site of DNMT3a or DNMT3b activity *in vivo* (Yokochi and Robertson, 2002; Bethany et al., 2010). *In vitro* however, DNMT3a methylates DNA in a distributive manner while DNMT3b displays processive DNA methylation activity of the same DNA template (Takeshima et al., 2006). Interestingly, similar to DNMT3a mutation in AML, a R823X mutation is found in the DNMT3b gene, causing Immunodeficiency, Centromeric region instability, Facial anomalies (ICF) syndrome, a rare recessive disorder characterized by hypomethylation of satellite DNA in juxtacentromeric heterochromatin. More specifically, DNMT3b mutation causes

hypomethylation of chromosomes 1 and 16, predisposing lymphoid cells to chromosomal rearrangements that lead to impaired immunity (Ehrlich M., 2002). ICF patients do not have increased susceptibility to develop cancer, however the longevity of ICF patients is significantly reduced (Ehrlich M, 2003). This evidence could explain why DNMT3a cannot fully compensate for DNMT3b-dependent DNA methylation loss in ICF and the converse for DNMT3a-mutated cells.

4. DNMT3a epigenetic silencing of *EPAS1* is critical for the normoxic to hypoxic growth transition

4.1 DNMT3a regulates HIF-2α-dependent autonomous growth of RCC

Silencing DNMT3a in normal renal cells triggered a premalignant cellular state of improved growth factor-dependent proliferation and survival in the hypoxic tumour microenvironment but was not sufficient to impart autonomous growth properties to renal epithelial cells. In fully malignant cells, we observed that reintroduction of DNMT3a phenocopied the effect of directly silencing HIF-2α via shRNA or indirectly silencing HIF-2α by reintroducing VHL expression in RCC. Serum growth was unaffected in DNMT3a-reintroduced cells or cells with shRNA-mediated HIF-2α knockdown suggesting that neither DNMT3a nor HIF-2α were implicated in core cell cycling pathways. HIF-2α is required for the autonomous growth and tumour formation of RCC, brain, lung and colorectal human cancers cells (Franovic et al., 2009). Accordingly, reintroduction of DNMT3a repressed the autonomous growth properties of brain, colorectal and lung cancer

cells in hypoxia but had no effect on their normoxic growth. The similarity between re-introducing DNMT3a and directly silencing HIF-2α suggests that these are part of the same biological pathway responsible for conferring autonomous growth properties to cancer cells. We confirmed the importance of HIF-2α expression in DNMT3a-reintroduced RCC cells by transiently rescuing HIF-2α levels. Ectopic expression of HIF-2α fully restored the autonomous growth properties of DNMT3a-reintroduced 786-0 cells, supporting that the loss of DNMT3a-mediated epigenetic silencing of *EPAS1* is an important event for conferring the abnormal growth of renal cancer cells. We thus conclude that the expression of HIF-2α alone, via loss of DNMT3a, is not sufficient to cause abnormal growth but is instead required for growth of cancer cells in low oxygen. This is supported by the observations that a panel of tumourigenic cell lines, including renal cancers, require HIF-2α for *in vivo* growth (Franovic et al., 2009; Imamura et al., 2009; Kim et al., 2009; Xue et al., 2012) but the forced expression of HIF-2α in normal renal epithelial cells does not cause cancer (Schietke et al., 2012).

5. DNMT3a suppresses tumour formation

Overall, our experiments suggested that DNMT3a functions like a tumour suppressor in renal and brain cancers and to a lesser extent in colorectal and lung cancer cell lines (**Figure 18**). Several other groups also reported evidence for a tumour suppressive function of DNMT3a in other human cancers. For example, deletion of DNMT3a caused the cellular transformation of hematopoietic cells and over a long period of time DNMT3a-/- cells developed a form of lymphocyte neoplasm morphologically similar to human chronic

lymphocytic leukemia (Peters et al., 2013). Interestingly, knockout of DNMT3b was shown to accelerate formation of tumours in DNMT3a-/- animals (Peters et al., 2013). Genetic alteration of DNMT3a in combination with five other genes is implicated in leukemia (Tet2, Runx1, Nf1, Ezh2 and Smc3) using small guide RNA editing mouse hematopoietic stem cell leads to myeloid malignancy and recapitulated the human AML disease (Heckl et al., 2014). Finally some evidence suggests that DNMT3a gene could be targeted in human cancers since leukemia, lymphomas and several other solid cancers including lung and colorectal tumours display loss of heterogenecity (LOH), a classical feature of tumour suppressor gene inactivation. (Ciriello et al., 2013; Kim et al., 2013; Zack et al, 2013). Altogether, a growing number of studies highlight the tumour suppressive function of DNMT3a but at this stage no oncogenic pathways have been clearly established and therefore we only partially understand the role of DNMT3a in restricting tumour growth.

5.1 DNMT3a is a gatekeeper of the hypoxic barrier

Our results are in agreement with a prominent role for DNMT3a-mediated silencing in cancer cell growth and a role in supporting normal cell proliferation in hypoxia. For example, shRNA silencing DNMT3a caused normal renal epithelial cells to express both HIF-1α and HIF-2α isoforms while control cells would chiefly express HIF-1α. Consequently, we observed a significant improvement in both proliferation and viability of DNMT3a-depleted renal cells in prolonged hypoxia. The opposite was also true; DNMT3a reintroduction in brain cancer cells significantly abrogated their capacity to grow and survive in low oxygen for a prolonged period of time. Altogether, this suggests that

silencing DNMT3a is beneficial for both normal and cancerous growth in hypoxia. However, untransformed cells are unlikely to grow once oxygen becomes limiting since hypoxia was shown to have a cytoprotective effect in normal cells only if proliferation was abrogated (Wang et al., 2006). On the contrary, once cancer cells have out paced the normal capacity of oxygen blood delivery, malignant cells are likely to sustain growth past the hypoxic barrier in order to form larger tumours and metastasize.

5.2 Targeting the hypoxic barrier

Hypoxia has been identified as a major source of therapeutic resistance in most common cancer treatments including radiotherapy and chemotherapy (Vaupel and Mayer, 2014). A hypoxia gene expression signature was shown to be useful to predict response to therapy (Eustace et al., 2013). Hypoxia directly impacts tumour recurrence after surgery (Govaert et al., 2014). Irradiated tumour cells post-therapy utilise HIF to translocate into blood vessels (Harada et al., 2013). Hypoxia-resistant profiles implie vulnerability of cancer stem cells to physiological agents, which suggests new therapeutic targets (Cipolleschi et al., 2014; Sun and li, 2014). However, the precise stage where cancer cells first experience oxygen limitation is unclear. Our results support the idea that cancer cells have regions of low oxygen when forming microscopic masses and by consequence cancer cells activate different pathways when growing in regions below or past the oxygen diffusion limit. To simulate the passage of cancer cells from a normoxic to a hypoxic growth *in vitro*, we established a spheroid assay using U87MG multicellular masses of a size bellow the oxygen diffusion limit. We were able to plate as little as 50 cells to form oxygenated tumour cell spheres *in vitro*. Overtime, these masses grew past the point of the oxygen

diffusion limit and showed features of hypoxia, accordingly. Reintroduction of DNMT3a stalled the growth of U87MG multicellular spheres before the point of acute hypoxia. For this reason we expected the DNMT3a-reintroduced cells would fail to grow in the tumour microenvironment. Indeed, U87MG cells form tumours in xenograft experiments but DNMT3a-reintroduced U87MG cells did not.

In support for specifically targeting the hypoxic cells as a therapeutic approach, targeting vasculature and normalizing oxygenation of tumours was shown to increase survival (Jones B, 2014). Also, hypoxia modification is useful to treat invasive bladder cancer (Hunter et al., 2014). Hypoxia and HIFα are known to upregulate many components in the EGFR pathway (de Paulsen et al., 2001; Smith et al., 2005; Franovic et al., 2007; Wang et al., 2009; Zhao et al., 2014). In addition, conventional receptor tyrosine kinase inhibitors were shown to preferentially target the hypoxic cancer cell fraction (Ahmadi et al., 2014). For example, Sunitinib therapy was shown to transiently reverse the state of tumour hypoxia in *vivo* (Chapman et al., 2014). Unfortunately there are very little active compounds known to target the hypoxic population of tumour cells, which warrants the development of tools to identify hypoxia-activated pathways. Interestingly, the spheroid model is compatible with high content analysis and thus could be utilised to screen for drugs that restrict hypoxic growth (Wenzel et al., 2014).

Recent work established that the selective inhibition of HIF-2α is possible *in vitro* (Scheuermann et al., 2013). However before systematically targeting HIF-2α for cancer therapy it is important to address the contribution of HIF-regulation to the normal hypoxic

response. Interestingly, a number of epidemiologic studies concluded that populations adapted to life at high altitude either had no change or lower cancer incidence and death rates (Amsel et al., 1982; Hart J., 2010; Youk et al., 2012; Ezzati et al., 2012). Since thinness of air at high altitude increases amount of UV radiation (Mason and Miler, 1974; Hart J, 2011) and the cancer incidence rates are lower or not higher in this environment, it suggests that people adapted to high altitude have some kind of protection against malignant growth.

6. HIF-2α is a central modulator of the DNMT3a epigenetic program in hypoxia

Several independent investigations reported a genetic link between the HIF-2α gene and adaptation to life at high altitude in various mammalian species including pigs (Dong et al., 2013), horses (Hendrickson et al., 2013), dogs (Gou et al., 2014; Li et al., 2014), grey wolves (Zhang et al., 2014) and humans (Simonson et al., 2010; Beall et al., 2010), supporting a predominant role for HIF-2α in the hypoxia response. To test the importance of *EPAS1* for the DNMT3a-mediated epigenetic gene expression, especially in hypoxia, we silenced DNMT3a and HIF-2α together in primary renal epithelial cells. The hypoxic improvement in viability and growth made by silencing DNMT3a was completely abolished when HIF-2α was also silenced (**Figure 40**). In addition ectopically expressed HIF-2α rescued all the DNMT3a-related phenotypes in hypoxic U87MG. Growth and viability defects were restored in DNMT3a-reintroduced U87MG cells expressing HIF-2α cDNA. In addition to the allowance of spheroid growth past the hypoxic barrier HIF-2α reinstated tumour growth of DNMT3a-reintroduced U87MG cells completely.

7. DNMT3a and hypoxic protein synthesis

It is well documented that hypoxia results in dephosphorylation and activation of eIF4EBP, an inhibitory protein of the eIF4F complex that represses the translation by directly binding and blocking the recruitment of eIF4E to the cap which is a rate-limiting step of protein synthesis (van den Beucken et al., 2006; Koritzinsky and Wouters, 2007). This repression of translation is thought to be a systemic response aimed at preserving cellular ATP levels in low oxygen (Liu et al., 2006). The proteome of various tumour specimens was shown to have a much faster rate of decay (Carmichael et al., 1980; Norton et al., 1981; Richards et al., 1993), suggesting that cancer cells were unlikely to sustain the attenuation of protein synthesis for a prolonged period of time. Polysomal fractions extracted from cancer cells exposed to normoxic and hypoxic conditions showed that some mRNAs were continuously associated with polysomes supporting the existence of an alternative protein synthesis mechanism functioning in hypoxic conditions (Thomas and Johannes, 2007; Stein et al., 1998; Gorlach et al., 2000; Lang et al., 2002). Work in our laboratory suggested that HIF-2α was uniquely involved in the systemic response to low oxygen by regulating the hypoxic protein synthesis machinery (Uniacke et al., 2012). Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) experiments showed that HIF-2α was associated with hundreds of mRNA transcripts and ^{35}S incorporation in HIF-2α-depleted cells validated that the bulk of protein synthesis in hypoxia required HIF-2α expression (Uniacke et al., 2012).

In depth analysis of the proteins associating with HIF-2α in the hypoxic protein fraction revealed specific interactions with RBM4 and eIF4E2. shRNA-mediated silencing experiments and ^{35}S incorporation assays confirmed that both RBM4 and eIF4E2 were equally required for protein synthesis in low oxygen. More precisely, RBM4 was important for targeting HIF-2α to a specific sequence, the rHRE, found on the mRNA transcripts targeted for translation in hypoxia. eIF4E2 replaces the function of eIF4E specifically in hypoxia and was required to recruit HIF-2α/RBM4 bound transcripts to the polysomes for Cap-dependent translation. Using our multicellular spheroid assay, we found that silencing eIF4E2 blocked the capacity of brain tumourigenic cell lines to traverse the hypoxic barrier (**Figure 33**). This effect was similar to what we observed with DNMT3A reintroduction in U87MG cells, suggesting that HIF-2α-directed hypoxic protein translation is important for the fitness of tumour cells in low oxygen. It would be interesting to address the contribution of both HIFα-driven gene expression and HIF-2α-dependant hypoxic translation for the cellular adaptation to hypoxia.

Surprisingly, we found that both RBM4 and eIF4E2 were required to drive the induction of EGFR protein levels in hypoxic renal epithelial cells (Uniacke et al., 2012; **Figure 34**). RBM4 was shown to bind EGFR in both normoxic and hypoxic conditions, in addition eIF4E2 was also seen constitutively expressed in normal and cancer renal tissues (Uniacke et al., 2012; **Figure 35**). eIF4E2 is a weak competitor of eIF4E2 for binding to the Cap structure of mRNAs (Tee et al., 2004). Therefore it suggests that a pool of mRNAs can be directed to the hypoxic protein synthesis machinery in normal cells when the normoxic machinery fails. We also found that eIF4E2 was important for the growth and survival of

normal renal epithelial cells in hypoxia, altogether suggesting that either: HIF-2α protein level is sufficient to drive protein synthesis or that additional hypoxia-regulated factors can associate with the hypoxic translation machinery in normal cells. HIF-2α mRNA transcript was found in the high-throughput analysis of mRNA sequences bound by HIF-2α/RBM4 complex in hypoxic U87MG cells. This would suggest that HIF-2α drives its own translation in hypoxia and would explain why renal epithelial cells expressing very low levels of HIF-2α protein could still utilise eIF4E2-dependent hypoxic protein synthesis during a period of prolonged hypoxia. To test this further, ^{35}S incorporation assays were performed to measure the rate of protein synthesis and assess the extent of hypoxic protein translation occurring in normal human primary renal epithelial cells. shRNA depleted DNMT3a primary cells and controls were tested in parallel with tumourigenic renal cell line 786-0. ^{35}S experiments showed that primary cells had a much lower protein synthesis activity in hypoxia than the 786-0 matched RCC cells. The rate of protein synthesis in normoxic primary cells was lower than the hypoxic rate of 786-0. shRNA knockdown of DNMT3a did not seem to increase the overall ^{35}S incorporation in primary cells but could nevertheless induce synthesis of some polypeptides. eIF4E2 is definitely required for normal cell survival in low oxygen. This data agreed with the observations that forced expression of HIF-2α in renal epithelial cells does not cause cancer (Schietke et al., 2012; Fu et al., 2013).

First, transcripts available for HIF-2α-mediated translation could be different between normal and cancer cells. The rHRE transcriptome could be different from the genes upregulated via the canonical pathway. To test this one would have to build a PAR-CLIP

analysis of eIF4E2-bound transcripts in primary epithelial cells and compare conditions where HIF-2α is expressed, such as in DNMT3a-depeleted hypoxic primary cells. Secondly, other factors could be required to drive HIF-2α-dependent protein synthesis. This includes the processing of mRNA transcripts prior to their translation in hypoxia. RBM4 is primarily a splicing factor (Markus and Morris, 2009) and HIF and non-HIF target genes were shown to be alternatively spliced in hypoxia (Sena et al., 2014). The fact that HIF-2α is not expressed in all cells suggests that the rHRE may not be used by all cells when exposed to low oxygen or other that factors can replace HIF-2α activity. Third, repressive factors may be required to be inactivated for HIF-2α to mediate protein translation. For example, VHL loss in renal epithelial cells was shown to induce a growth-arrested senescent phenotype that was reversed by hypoxia (Welford et al., 2010). In support for this, silencing of eIF4E2 in cells constitutively expressing HIF-2α (786-0) blocked the normoxic expression of EGFR but failed to inhibit total protein synthesis in normoxia (Uniacke et al., 2014). ^{35}S experiments showed that 786-0 cells depleted of eIF4E2 required an additional hypoxia treatment to exhibit defects in the total rate of protein synthesis, supporting the idea that additional yet unknown additional mechanisms restrict the hypoxic translation machinery event in the presence of constitutively expressed HIF-2α and a functional hypoxia translation unit.

The capacity to detect early tumours continues to directly improve outcomes of anti-cancer therapies and makes a significant impact on patients' chances of survival. However, the tools to understand and test the early oncogenic events conferring the premalignant state of cancer cells are not numerous. The VHL cancer system uniquely revealed the multistep event causing HIF-2α oncogenic expression at the earliest stages of renal cancer development (Mandriota et al., 2002). By investigating the mechanism responsible for HIF-2α gene (*EPAS1*) expression in tumourigenic renal cancer, we were able to trace DNMT3a loss to a disruption in the normal epigenetic silencing of *EPAS1*. We believe our findings warrant further characterization of the molecular pathways allowing early cancers to traverse the hypoxic barrier. Targeting these enabling characteristics could restrict the development of malignant tumours and help manage therapy for resistant tumours.

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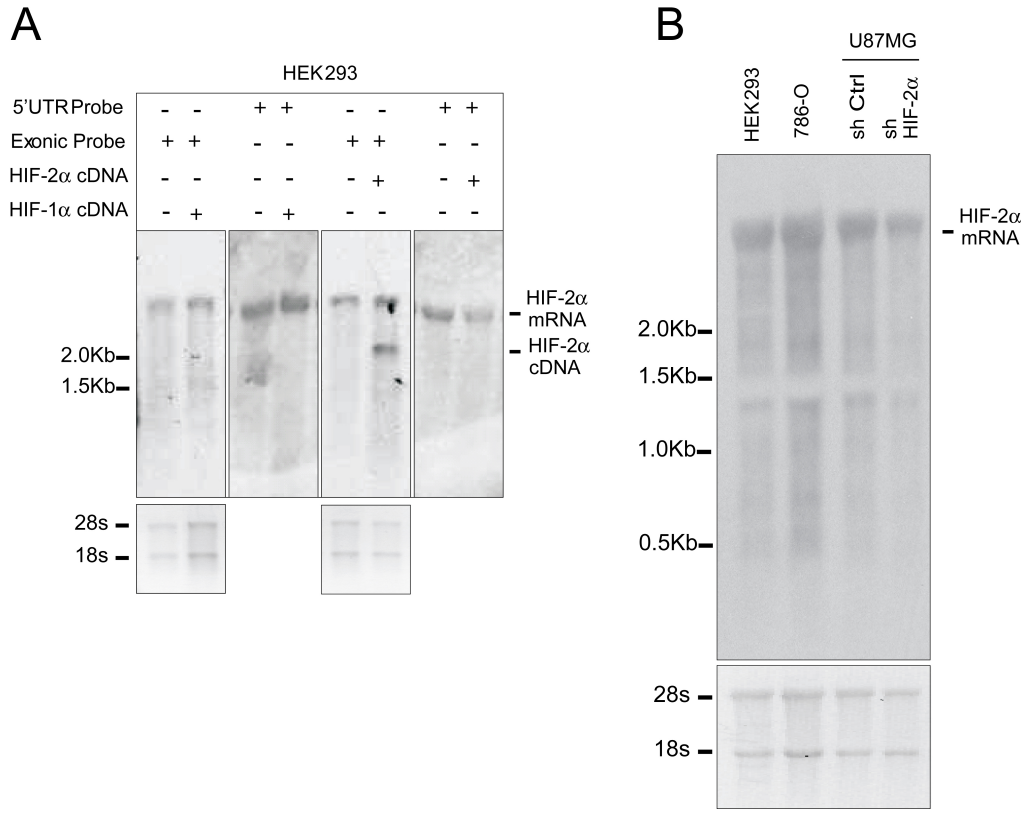
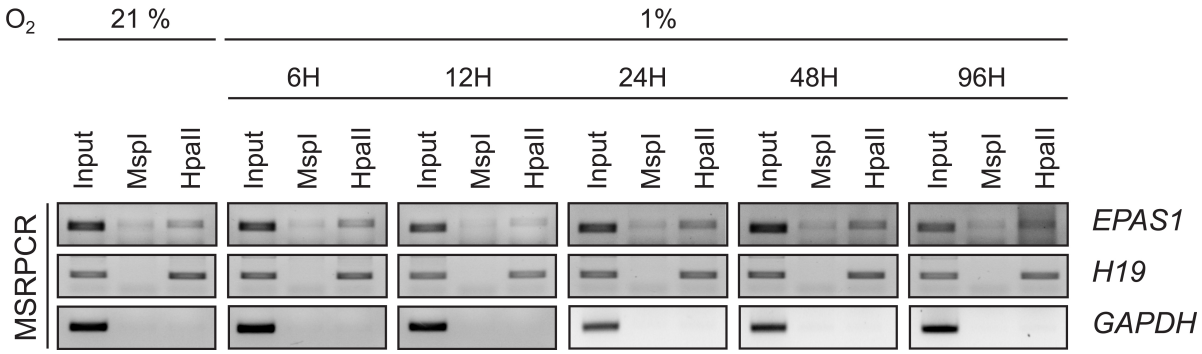


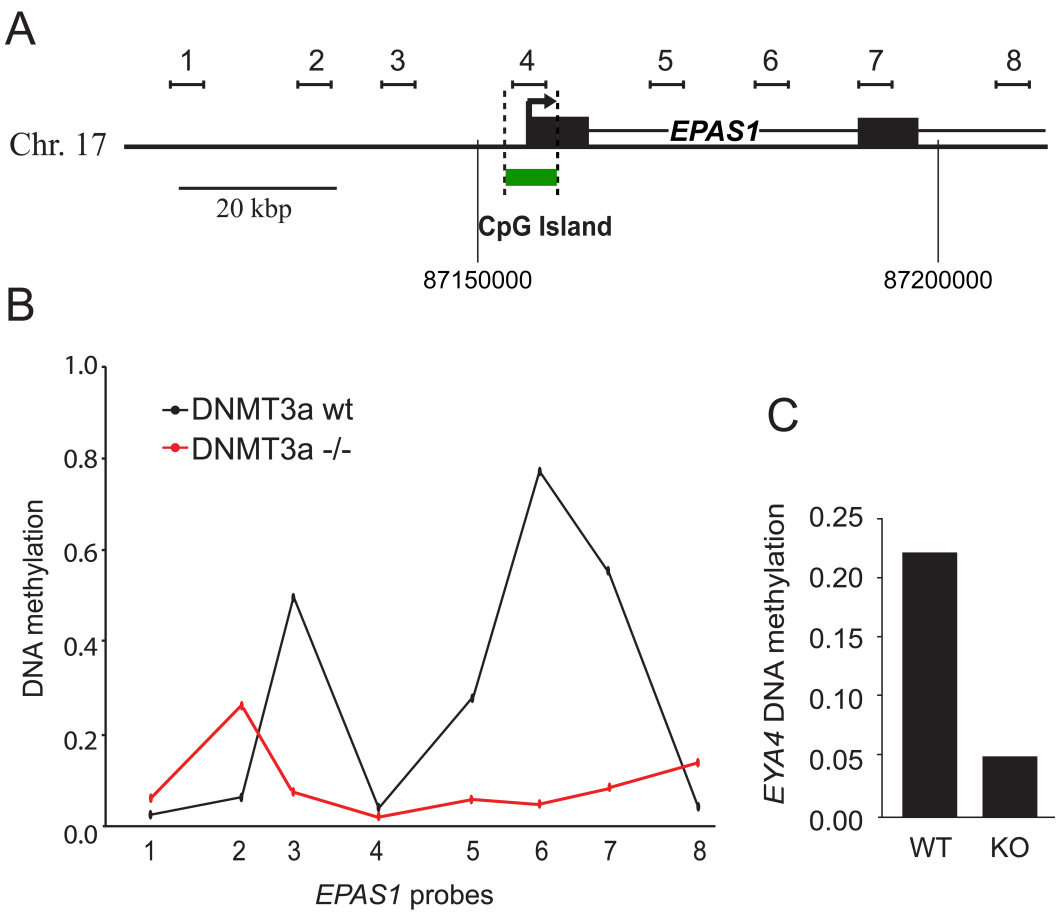
Appendix 1

Appendix 1. Northern blot validation of HIF-2α riboprobe used for *in situ* hybridization: (A) HEK293 cells were transfected with plasmids expressing HIF-2α or HIF-1α cDNA. Total RNA samples were run on a denaturing agarose gel, transferred onto a nitrocellulose membrane and hybridized with *in vitro* DIG-labelled RNA probes complementary to the 5'UTR (5'UTR Probe) or the coding region of HIF-2α mRNA (Exonic Probe). (B) Same procedure as in A) was done on total cell extracts of HEK293, 786-0 or U87MG cell with stable knockdown of HIF-2α (shHIF-2α) that were described before (Franovic et al., 2009). The riboprobe complementary to the 5'UTR region of HIF-2α mRNA was tested in B).



Appendix 2

Appendix 2 : *EPAS1* methylation in hypoxia-treated RECs. MSRPCR analysis of *EPAS1*, *H19* (positive methylation control), and *GAPDH* (negative methylation control) genes in REC incubated for several hours in hypoxia (1% O_2) or normoxia (21% O_2).



Appendix 3

Appendix 3 : Differential DNA methylation of *EPAS1* in DNMT3a-/- cells. (A) The mouse *EPAS1* locus on chromosome 17, the CpG island (green) and 8 regions differentially methylated between wildtype (wt) and DNMT3a knockout (KO) mouse hematopoietic stem cells. (B) Ratio of *EPAS1* methylated DNA measured by whole genome bisulfite sequencing in DNMT3a-/- and control DNMT3a wt cells. The probes are corresponding to the regions highlighted in (A). (C) DNMT3a target gene EYA4 is demethylated in DNMT3a-/- cells compared to wild type (wt). Adapted from Jeong et al., 2014.