From single cell to single molecule: 
Elucidation of the intracellular dynamics of 
the Hypoxia Inducible Factor (HIF) 

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By 
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Abstract

Hypoxia signalling pathway acts in all mammalian cells to try and avert damage occurring during periods of limited oxygen availability (hypoxia). The cellular response to a low oxygen environment is mediated by the transcription factor hypoxia-inducible factor (HIF), which activates the transcription of genes that aid cell survival and act to restore oxygen homeostasis. The heterodimeric transcription factor consists of a constitutively expressed beta subunit (HIF-1β) & an alpha subunit (HIF-1α / HIF-2α). The alpha subunits are regulated in an oxygen-dependent manner.

The main aim of this thesis was to elucidate if the spatial localisation and/or the temporal fluctuations of HIF-1α and HIF-2α are altered in response to hypoxia and participate to the regulation of HIF activity. The experimental strategy focused on studying HIF at several scales ranging from single cell to single molecule level. I first investigated the temporal and spatial dynamics of the HIF-α subunits in living cells using time-lapse imaging. This was part of a larger study, which was initiated before the start of this thesis. It was published in January 2014 in the Journal of Biological Chemistry. We demonstrated that HIF-α accumulates transiently in the nucleus in response to hypoxia and this was necessary to ensure cell survival. We further demonstrated using a combination of mathematical modelling and knock-down experiments that the negative feedback loop involving PHD2 has an essential role in these dynamics. Secondly, we observed that HIF-2α exhibits a non-homogenous sub-nuclear localisation, whereas HIF-1α is distributed homogenously within the nucleus. Since protein localisation is commonly linked to function we sought to elucidate the purpose of this heterogeneous distribution using a combination of live cell imaging, co-localisation and photo-bleaching experiments. Lastly, to avoid molecule averaging errors, we planned to employ single molecule tracking to elucidate sub-cellular dynamics of HIF-2α and gain further insight into the movement of HIF-2α between the speckles and the rest of the nucleus. Our aim was to image and monitor gold nanoparticles (GNPs) conjugated to HIF-2α in real time in living cells, using photo-thermal microscopy. In collaboration with Prof. D. G. Fernig and Dr. R. Levy’s research groups, we developed a protocol using the Halotag (an engineered enzyme that reacts with a substrate (Halo-ligand) to form a covalent bond) to label a protein of interest with gold nanoparticles. This was achieved by: immobilising the Halotag ligand on the surface of GNPs and producing purified recombinant Halotag-fusion protein. We have obtained positive results by using
the fibroblast growth factor 2 as proof of principle. Due to difficulties in expression and purification of recombinant Halotag-HIF-2α single molecule tracking of HIF-2α has not been achieved yet, but this work is still on-going.

Overall my results show that: (1) the temporal dynamics of HIF-1α elicited by hypoxia have a critical role in HIF activity and function, (2) the non-homogenous speckle localisation of HIF-2α is associated with a slow diffusion time within the nucleus, 7-10X (depending on condition) slower than the homogeneous HIF-1α. Finally, using single molecule measurements, we did not observe any significant difference in speckle organisation or HIF-2α mobility, so single molecular tracking will be required to provide a non-average quantitative measurement of HIF-2α movement between the speckles and inter-speckle space.
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M6 FRAP - Tracking individual HIF-2α speckle in bleached region 1
M7 FRAP - Tracking individual HIF-2α speckle in bleached region 2
List of Abbreviations

aa  Amino acid
ADP  Adenosine diphosphate
aka  Also known as
APS  Ammonium persulphate
ARNT  Aryl receptor nuclear transporter
ATP  Adenosine triphosphate
BAC  Bacterial artificial chromosome
bHLH  Beta helix loop helix
bp  nucleotide basepairs
BSA  Bovine serum albumin
CBP  CREB binding protein
CCI  Centre for cell imaging
CM  Carboxymethyl
CMV  Cytomegalovirus
CREB  cAMP response element-binding protein
Da  Daltons
DAEA  Diethylaminoethanol
ddH2O  Ultrapure water / double distilleds water
DM  Double mutant
DMEM  Dulbecco’s modified medium
DMOG  Dimethyloaloxyglycine
DMSO  Dimethyl sulphoxide
DNA  Deoxyribonucleic acid
dTOM  dTomato fluorescent protein
DTT  Dithiothreitol
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>e-</td>
<td>Electron</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EPAS</td>
<td>Endothelial PAS domain protein 1</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>FADH</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine serum (cell culture)</td>
</tr>
<tr>
<td>FCS</td>
<td>Fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FIH</td>
<td>Factor inhibiting HIF</td>
</tr>
<tr>
<td>FLIP</td>
<td>Fluorescence loss in photobleaching</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster / fluorescence resonance transfer</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GNP</td>
<td>Gold nanoparticles</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HAF</td>
<td>Hypoxia associated factor</td>
</tr>
<tr>
<td>HDAC5</td>
<td>Histone de-acetylase 5</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>His</td>
<td>Histadine</td>
</tr>
<tr>
<td>HisTag</td>
<td>Poly-Histadine tag</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HL</td>
<td>Halotag ligand</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response element</td>
</tr>
<tr>
<td>HT</td>
<td>Halotag</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>Lab</td>
<td>Laboratory</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth aka Luria-Bertani medium</td>
</tr>
<tr>
<td>LSM</td>
<td>Laser scanning microscope</td>
</tr>
<tr>
<td>MEM</td>
<td>Modified eagle medium</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSD</td>
<td>Mean squared displacement</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NINTA</td>
<td>Nickel nitrilotriacetic acid</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation signal</td>
</tr>
<tr>
<td>OD600</td>
<td>Optical density measured at a wavelength of 600</td>
</tr>
<tr>
<td>ODD</td>
<td>Oxygen dependent degradation</td>
</tr>
<tr>
<td>PAS</td>
<td>PER-ARNT-SIM domain</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethyleneimine)</td>
</tr>
<tr>
<td>PER</td>
<td>Period circadian protein</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain protein</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic leukemia protein</td>
</tr>
<tr>
<td>POI</td>
<td>Protein of interest</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
</tbody>
</table>
RNA  Ribonucleic acid
RNAPII  RNA polymerase II
ROI  Region of interest
RPM  Revolutions per minute
RT-qPCR  Real time quantitative polymerase chain reaction
SD  Standard deviation
SDS PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec  Seconds
SFC  Splicing factor compartments
sh  Short hairpin RNA
SIM  Single minded protein
SMSS  Slope of the moment scaling factor
SMT  Single molecule tracking
SR  Super-resolution
SUMO  Small ubiquitin-like protein
TAD  Trans-activation domain
TEMED  Tetramethylethylenediamine
TEV  Tobacco etch virus
TF  Transcription factor
TMR  Tetramethylrhodamine
Tris  Tris(hydroxymethyl)aminomethane
UV  Ultraviolet light
VEGF  Vascular endothelial growth factor
VHL  von Hippel Lindau protein
vs  Versus
WT  Wild type
YFP  Yellow fluorescent protein
Chapter 1: Introduction
1.1 Cell signalling

Cell to cell communication is vital in multi-cellular organisms for coordination of cellular events such as metabolism and growth. Cell signalling is orchestrated by several factors such as signalling molecules (e.g. hormones and cytokines), that are detected via receptors that trigger a chain of events whereby proteins are sequentially activated / deactivated leading to the up / down regulation of genes and a change in protein expression.

As well as signalling molecules, cells can sense changes in their environment. They relentlessly monitor and respond to environmental cues causing the activation / deactivation of signalling pathways. For example extreme temperatures, changes in pH and exposure to toxins trigger stress response pathways that orchestrate a change in gene expression facilitating cellular protection.

In addition to this, cells monitor the levels of oxygen, a vital component for life, and it is the signalling pathway that facilitates cells to sense and adapt to low oxygen (hypoxia) that is the focus of this study.

1.2 Oxygen and metabolism

As obligate aerobes, humans cannot survive without oxygen. Homo sapiens (and many other living organisms) require oxygen to generate chemical energy in the form of Adenosine triphosphate (ATP) to fuel energetically unfavourable processes and biochemical reactions at the cellular level. It is generated via a number of mechanisms in eukaryotic cells, but the greatest yield of ATP comes from aerobic respiration, a process involving the oxidation of organic compounds such as sugars (Figure 1.1).
**Figure 1.1 | Cellular metabolism.** A simplified schematic of the three stages of cellular metabolism. Glucose is broken down into pyruvic acid in the cytosol. This product is transported into the mitochondria where it is converted into acetyl CoA. Acetyl CoA enters the Krebs cycle (aka citric acid cycle) which produces high energy electrons that are passed onto the electron transport chain (ETC), situated in the inner membrane. As the electrons travel down the ETC, it creates a proton gradient which drives ATP synthase to create ATP from ADP and Pi. Oxygen acts as the electron acceptor at the end of ETC and is split to create two molecules of water. *Net gain is 32 or 34 ATP molecules depending on which electron shuttle transports the electrons from the cytosol into the mitochondria.*

Glycolysis literally means “lysis of sugar”. One molecule of glucose is converted to two pyruvate molecules in a ten step process to yield a net gain of two ATP molecules. It is a primitive form of ATP production and is present in nearly all organisms including anaerobes as it does not require molecular oxygen.

The pyruvate molecules produced are transported in the mitochondrial matrix where they are converted to acetyl CoA, the substrate of the Krebs cycle (citric acid cycle). Although the Krebs cycle is part of “aerobic metabolism”, this process does not itself require molecular oxygen. It does however produce high energy electrons, transported in the form of NADH and FADH, which are required for the subsequent oxygen-dependent step in aerobic respiration. These electrons pass down the electron transport chain and eventually combine with oxygen. The proton gradient created during this process drives ATP synthase which produces a net value of 32 (or 34) molecules of ATP. Meaning that in presence of sufficient oxygen, a cell can produce 36 (or 38) molecules of ATP per one molecules of glucose in total.
1.3 Hypoxia

Hypoxia occurs when oxygen levels fall below the threshold of a particular tissue. Figure 1.2 highlights the partial pressures of a selection of organs and tissues. In simple terms, organs, tissues and cells require delivery of sufficient oxygen to meet their particular metabolic needs and when the demand for oxygen becomes greater than the supply, they become hypoxic.

**Figure 1.2 | Examples of the partial pressure of oxygen in different organs and tissues.** Values taken from Wiener et al. (1976) and Carreau et al. (2011). Image source http://media.tumblr.com/tumblr_m5z390bppP1qmovzi.jpg

Hypoxia results from pathological conditions where the oxygen supply is reduced or impeded such as anaemia, sleep apnoea and ischemic events. Hypoxia has been shown to play a role in physiological and developmental processes, such as wound healing (Brahimi-Horn & Pouyssegur, 2009), embryogenesis (Dunwoodie, 2009) and maintenance of pluripotent stem cells (Forristal et al., 2010).
1.3.1 Maintaining oxygen homeostasis

Hypoxic events trigger a highly conserved canonical signalling pathway that results in the up-regulation of genes that aid cell survival by restoring oxygen levels, improving tissue oxygenation and/or increasing anaerobic respiration (Table 1.1).

For example, hypoxia triggers an increase in the levels of the hormone erythropoietin (EPO) that controls the maturation of red blood cells. This results in an increase in the number of red blood cells and therefore increases the oxygen carrying capacity of the blood. There is also an increase in vascular endothelial growth factor (VEGF). This protein stimulates angiogenesis (development of new blood vessels), which restores or increases blood supply to tissues. Together, these responses contribute towards re-establishing sufficient tissue oxygenation.

Table 1.1 | Examples of cellular processes that are up-regulated during hypoxia.

<table>
<thead>
<tr>
<th>Process</th>
<th>HIF target gene(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythropoiesis</td>
<td>EPO</td>
<td>Wang &amp; Semenza (1993)</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>VEGF</td>
<td>Forsythe et al. (1996)</td>
</tr>
<tr>
<td>Switching cellular metabolism</td>
<td>GLUT1, GLUT3, GAPDH</td>
<td>Wolfe and Voelkel (1983), Chen et al. (2001)</td>
</tr>
<tr>
<td>Iron metabolism</td>
<td>Transferrin, Transferrin receptor, Ferroxidase</td>
<td>Rolfs et al. (1997), Tecchini et al. (1999), Lok &amp; Ponka (1999), Mukhopadhyay et al. (2000)</td>
</tr>
</tbody>
</table>

1.4 Hypoxia-inducible factor (HIF)

In 1995 it was determined that the protein responsible for the hypoxic induction of erythropoietin (EPO) was hypoxia-inducible factor (HIF) (Wang et al., 1995). Subsequent studies found that HIF is a heterodimeric transcription factor that is composed of an alpha subunit, which is tightly regulated in an oxygen-dependent manner, and a constitutively expressed beta subunit (HIF-1β) (Wang & Semenza, 1995; Reyes et al., 1992; Huang et al., 1996).
1.4.1.1 HIF-α

There are three isoforms of the alpha subunit (HIF-1α, -2α and -3α). HIF-1α was discovered by Gregg Semenza in 1992 (Semenza & Wang, 1992). It was determined that this basic helix-loop-helix (bHLH) protein was made up of the following domains: PER/ARNT/SIM (PAS) domains, the oxygen-dependent degradation (ODD) domain and two transactivation domain (TAD) (Figure 1.3) (Depping et al., 2008; Jiang et al., 1996; Wang et al., 1995; Lee et al., 2004).

Five years after the discovery of HIF-1α, several laboratories published reports on an HIF-1α isoform, HIF-2α. The simultaneous discovery led to its publication under several names: endothelial PAS domain protein-1 (EPAS1), HIF-1α-like factor (HLF) or members of PAS superfamily 2 (MOP2) (Wenger & Gassmann, 1997; Tian et al., 1997; Ema et al., 1997; Hogenesch et al., 1997). This, like HIF-1α, is a beta-helix-loop-helix protein that heterodimerises with HIF-1β, recognises the HRE DNA consensus sequence and activates transcription of hypoxia inducible genes (Tian et al., 1997).

The third isoform, HIF-3α, has numerous splice variants. The resulting proteins range from 667 to 7 amino acids, with some splice variants having an ODD and an N-terminal TAD like HIF-1α and HIF-2α (Maynard et al., 2003; Gu et al., 1998). Although the role of HIF-1α and -2α in the cellular adaptation to low oxygen is well established, the function of this third isoform is complex, with some splice variants have been shown to act as negative or positive regulators in the hypoxia response (Hara et al., 2001; Maynard et al., 2005; Maynard et al., 2003; Gu et al., 1998).

1.4.1.2 HIF-β

Otherwise known as aryl hydrocarbon nuclear translocator (ARNT), this member of the bHLH-PAS superfamily was first described in 1991 as the binding partner of the aryl hydrocarbon receptor (AhR / dioxin receptor) (Hoffman et al., 1991; Reyes et al., 1992). When hetero-dimerised, this transcription factor is responsible for regulating xenobiotic (chemicals foreign to the body) metabolism. It was later discovered that ARNT also forms heterodimers with HIF-1α and HIF-2α, thus acquiring the name HIF-β (Wang et al., 1995; Wang & Semenza, 1995). There are two isoforms: HIF-1β and HIF-2β (HIF-1β2 / ARNT2). Both isoforms can mediate the hypoxic response, but HIF-2β has a more limited
tissue expression compared to the ubiquitous HIF-1β (Hirose et al., 1996; Drutel et al., 1996; Maltepe et al., 2000).

**Figure 1.3 | Domain structure of HIF-1α and HIF-1β.** PAS = PER/ARNT/SIM domains, ODD = oxygen-dependent degradation domain, TAD = transactivation domains.

### 1.4.2 Oxygen-Dependent regulation of HIF

The availability of oxygen regulates both the stability and transcriptional activity of HIF, via two independent processes. HIF-α subunits are continually synthesised but they are rapidly degraded in normoxia (Figure 1.4A). This is regulated by members of the Fe (II) and 2-oxoglutarate-dependent dioxygenase superfamily: the prolyl-4 hydroxylase domain (PHD1, 2 and 3) proteins (Bruick & McKnight, 2001; Kallio et al., 1999). These proteins use oxygen as a co-substrate, thus the availability of oxygen regulates their enzymatic activity and enables them to sense local or general hypoxia (Steinhoff et al., 2009; K et al., 2004; Epstein et al., 2001). Under normoxic conditions, the PHD proteins catalyse the hydroxylation of specific prolyl residues located within the oxygen dependent degradation (ODD) domain of the alpha subunit (Figure 1.4B) (Bruick & McKnight, 2001; Epstein et al., 2001).

Hydroxylation initiates the association of von Hippel-Lindau (VHL) tumour suppressor protein (an E3 ubiquitin ligase) with the alpha subunit and targets it for proteasomal degradation (Maxwell et al., 1999; Kallio et al., 1999; Ivan et al., 2001). During periods of hypoxia, the lack of oxygen leads to a marked reduction in the activity of the PHD proteins, resulting in fewer prolyl residues being hydroxylated and the stabilisation of the alpha subunit (Epstein et al., 2001).
Introduction
Figure 1.4 | Oxygen dependent regulation of HIF-α subunit. A) During periods of sufficient oxygen (normoxia) the HIF alpha subunits are degraded. Reduced oxygen (hypoxia) causes a decrease in PHD activity, allowing HIF-α subunits to accumulate and dimerise with HIF-1β to form active HIF in the nucleus. There it binds to a highly conserved consensus sequence, (A)CGTG, located within the enhancer domain. This sequence is termed the hypoxic response element (HRE) and leads to transcription of hypoxia-inducible genes (Luo & Shibuya, 2001). pVHL = Von Hippel-Lindau tumour suppressor protein. Negative feedback highlighted in orange. B) Schematic representation of the domain structures of HIF-1α highlighting the key protein interactions in normoxia and hypoxia. PER/ARNT/SIM (PAS-A & PAS-B) domains, oxygen-dependent degradation domain (ODD) and N-terminal / C-terminal transactivation domain (NTAD / CTAD) are shown. The proline residues highlighted in the oxygen degradation dependent domain are hydroxylated by Prolyl Hydroxylase Domain (PHD) enzymes and the asparagine residues at the C-terminus is hydroxylated by factor-inhibiting HIF (FIH). Adapted from Lee et al.(2004). Illustration of the key interactions that take place between the alpha subunit and other proteins, highlighting the domain through which they occur. HIF-1β = Hypoxia Inducible Factor Beta, PHD = Prolyl Hydroxylase Domain, FIH = Factor Inhibiting HIF, CBP = CREB Binding Protein, pVHL = Von Hippel-Lindau tumour suppressor protein.

Factor Inhibiting HIF (FIH) is an asparaginyl hydroxylase and regulates the transcriptional activity of HIF. During normoxia, factor inhibiting HIF (FIH) catalyses the hydroxylation of an asparagine residue which blocks the interaction of HIF with its co-activators CREB-binding protein (CBP)/p300 through the transactivation domain (TAD, Figure 1.4B) (Kallio et al., 1998; Zhang et al., 2010). In hypoxia, the asparagyl residues are not hydroxylated, allowing the interaction of HIF with CBP/p300 and therefore the transcription of HIF target genes. Therefore in hypoxia, the impaired activity of the PHD and FIH enzymes leads to the alpha subunit evading degradation allowing it to form transcriptionally active heterodimers with HIF-1β via the PAS domains in the nucleus.

1.4.3 Oxygen-independent regulation of HIF

HIF is not only activated in response to low oxygen. For example, HIF-1α levels increase in proliferating cells, because the resulting daughter cells require more oxygen than the single parent cell. It is likely that growth factors increase synthesis of HIF-1α in a pre-emptive move to maintain oxygen-homeostasis. This increase in HIF switches ATP production to glycolysis and increased VEGF expression to ensure the oxygen needs for the cells are met (Brand & Hermfisse, 1997; Seagroves et al., 2001; Jiang et al., 1997). It has been shown that HIF can be regulated by the PI3K/AKT/mTOR pathway, a regulator of the cell cycle. The activation of this signalling pathway leads to an increase in translation of a subset of mRNAs including HIF-1α and HIF-2α (Laughner et al., 2001; Hudson et al., 2002; Toschi et al., 2008; Hay & Sonenberg, 2004).
Non-hypoxic stimulation of HIF has also been observed in cells responsible for the immune response such as macrophages (Blouin et al., 2004). For example, cells exposed to the endotoxin lipopolysaccharide (LPS) have been shown to have increased levels of active HIF-1 and this regulation is believed to be at the transcriptional level as HIF-1α mRNA increase significantly in the presence of LPS (Blouin et al., 2004).

Several other components regulate the stability of the alpha subunit in an oxygen-independent manner. For example, all of the following lead to the alpha subunits evading proteasomal degradation in normoxia: inhibition of PHD proteins by intermediaries of the Kreb’s cycle, sequestration of pVHL in nucleoli due to increased acidity and overexpression of a deubiquitase (DUB) called pVHL protein-interacting deubiquitinating enzyme 2 (VDU2 aka USP20) (Fong & Takeda, 2008; Mekhail et al., 2004; Li et al., 2005). Stability of HIF-1α is also regulated by protein-protein interaction. For example, HSP90 and RACK1 actively compete to bind to HIF-1α and have antagonistic effects on protein stability (Hogenesch et al., 1997; Liu et al., 2007; Isaacs et al., 2002).

### 1.5 HIF-1α versus HIF-2α

This study focuses on the spatial and temporal dynamics of HIF-1α and -2α. Even though these two isoforms have been implicated in activating and regulating the hypoxic response, it has become clear that they do not have redundant roles and do have many differences. Here I discuss some of the key differences that have been described so far.

Firstly, HIF-1α and HIF-2α are encoded by different genes, HIF1A and EPAS1, which are located on chromosome 14 (14q23.2) and chromosome 2 (2p21-p16), respectively. The translated proteins differ slightly in length (826 and 870 amino acids, respectively) with an overall sequence similarity of 48%. They have a similar domain structure (Figure 1.5) with the greatest amount of similarity within the bHLH, PAS-A and PAS-B domains (Table 1.2). Both isoforms have an ODD domain where residues 549-572 and 517-534 are vital for the VHL-dependent degradation of HIF-1α and HIF-2α, respectively (Huang et al., 1998; O’Rourke et al., 1999; Ema et al., 1999; Ohh et al., 2000; Cockman et al., 2000; Tanimoto et al., 2000; Kamura et al., 2000). Additionally, both alpha subunits have nuclear localisation signals. Lys737, Arg738 and the surrounding amino acid sequence, function as the nuclear localisation for HIF-2α and residues 718-721 for HIF-1α (Hara et al., 1999).
### Figure 1.5 | Comparison of HIF-1α and HIF-2α domains.

A) Schematic representation of the domain structures of HIF-1α and HIF-2α. A) Highlighted are the key residues and their position that are hydroxylated in hypoxia. (p = proline, n = asparagine). B) The size and location of domains, regions and motifs found within the protein sequence of HIF-1α and HIF-2α. Sequence similarities were calculated by performing sequence alignment using BlastP. 

#### Table 1.5.1

<table>
<thead>
<tr>
<th>Domain</th>
<th>HIF-1α</th>
<th>HIF-2α</th>
<th>Sequence similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (aa)</td>
<td>Position (aa)</td>
<td>Length (aa)</td>
</tr>
<tr>
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<td>17-70&lt;sup&gt;§&lt;/sup&gt;</td>
<td>54&lt;sup&gt;§&lt;/sup&gt;</td>
</tr>
<tr>
<td>PAS-A</td>
<td>74&lt;sup&gt;§&lt;/sup&gt;</td>
<td>85-158&lt;sup&gt;§&lt;/sup&gt;</td>
<td>71&lt;sup&gt;§&lt;/sup&gt;</td>
</tr>
<tr>
<td>PAS-B</td>
<td>71&lt;sup&gt;§&lt;/sup&gt;</td>
<td>228-298&lt;sup&gt;§&lt;/sup&gt;</td>
<td>71&lt;sup&gt;§&lt;/sup&gt;</td>
</tr>
<tr>
<td>ODD</td>
<td>203&lt;sup&gt;§&lt;/sup&gt;</td>
<td>401-603&lt;sup&gt;§&lt;/sup&gt;</td>
<td>166&lt;sup&gt;§&lt;/sup&gt;</td>
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<td>NTAD</td>
<td>45&lt;sup&gt;§&lt;/sup&gt;</td>
<td>531-575&lt;sup&gt;§&lt;/sup&gt;</td>
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<tr>
<td>CTAD</td>
<td>41&lt;sup&gt;§&lt;/sup&gt;</td>
<td>786-826&lt;sup&gt;§&lt;/sup&gt;</td>
<td>41&lt;sup&gt;§&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

B) Figure 1.5 | Comparison of HIF-1α and HIF-2α domains. A) Schematic representation of the domain structures of HIF-1α and HIF-2α. A) Highlighted are the key residues and their position that are hydroxylated in hypoxia. (p = proline, n = asparagine). B) The size and location of domains, regions and motifs found within the protein sequence of HIF-1α and HIF-2α. Sequence similarities were calculated by performing sequence alignment using BlastP. 

#### 1.5.1 Differential regulation of the HIF-α isoforms

The two alpha subunits are regulated at all levels: transcription of the *HIF1A* and *EPAS1* genes, mRNA translation, protein stability and transcriptional activity of the isoforms.
themselves. Here are some examples of regulation that is unique to one isoform or an event that targets both isoforms but has a different effect.

**TRANSCRIPTION** – As previously discussed LPS has been shown to upregulate transcription of HIF-1α in an oxygen-independent manner. But cytokines have been shown to differentially regulate HIF-1α and HIF-2α mRNA expression. Takeda et al. (2010) demonstrated that interferon gamma (IFNγ) increases HIF-1α but significantly decreases HIF-2α mRNA expression. Conversely, interleukin-4 (IL-4) initiates prolonged increase in HIF-2α mRNA expression, but has no effect on HIF-1α (Takeda et al., 2010).

**TRANSLATION** – As mentioned previously, HIF-α can be regulated independently of oxygen levels by the PI3K/AKT/mTOR pathway (see chapter 1.3.2) however there is discrimination between the two HIF-α isoforms. Toschi et al. (2008) demonstrated that in renal cell carcinoma cell lines HIF1A translation is regulated by mTORC1 and mTORC2 but EPAS1 translation is regulated by mTORC2 only (Toschi et al., 2008). They also looked at Akt, a substrate of mTORC2, and found that the expression of HIF-1α and HIF-2α was dependent on different Akt isoforms (Toschi et al., 2008).

In addition to this, HIF-2α is regulated at the post-transcriptional level by iron-response element binding protein 1 (IREBP1). This protein specifically inhibits the translation of HIF-2α when bound to the iron response element within the EPAS1 transcript (Sanchez et al., 2007).

It has also been suggested that HIF-1α protein synthesis is regulated by antisense HIF-1α (αHIF1, a transcript that is complementary to the 3’ untranslated region (3’-UTR)). Upon hypoxic stimulation, in addition to an increase in HIF1A mRNA expression, there is an increase in the αHIF1 which associates with and destabilises the HIF1A mRNA transcript, thus preventing further translation (Uchida et al., 2004). It is unknown whether a similar mechanism exists for HIF-2α.

**PROTEIN STABILITY** – It is well established that the protein stability of both alpha subunits is regulated via the hydroxylation of specific prolyl residues (Ivan et al., 2001;Lando et al., 2002) and that this leads to degradation following VHL-dependent poly-ubiquitination (Maxwell et al., 1999;Kallio et al., 1999;Ivan et al., 2001). However, it has been shown that there are differences in the specificity of the PHD enzymes, for instance PHD3 preferentially hydroxylates HIF-2α (Appelhoff et al., 2004). Furthermore, it appears that there are
differences in the stabilisation of HIF-1α and HIF-2α in hypoxia. Firstly, it has been shown that, in HeLa and other cell lines, HIF-2α stabilises at oxygen levels of 2-5% but levels need to be lower for HIF-1α stabilisation (0-2% O2) (Nilsson et al., 2005; Holmquist-Mengelbier et al., 2006; Li et al., 2009). Secondly, a difference in the duration of protein stabilisation has been observed. Holmquist-Mengelbier et al. (2006) found that in prolonged hypoxia, high levels of HIF-2α levels were maintained whereas levels of HIF-1α decline after several hours in neuroblastoma cells (Holmquist-Mengelbier et al., 2006; Uchida et al., 2004). This will be discussed further in chapter 3.

There are a number of mechanisms that specifically regulate HIF-1α stability. For example, Heat shock protein 70 (HSP70) and carboxyl terminus of HSP70-interaction protein (CHIP) have been shown to bind HIF-1α in prolonged hypoxia and target the subunit for proteasomal degradation (Luo et al., 2010). Secondly, arrest defective 1 (ARD1) has been shown to specifically acetylate HIF-1α, which results in the destabilisation of the protein (Jeong et al., 2002; Yoo et al., 2006). And finally heat shock protein 90 (HSP90) and receptor for active kinase 1 (RACK1), previously mentioned in section 1.4.3 (Hogenesch et al., 1997; Liu et al., 2007; Isaacs et al., 2002).

**TRANSCRIPTIONAL ACTIVITY** – FIH regulates both HIF-1α and HIF-2α in an oxygen-dependent manner, however Bracken et al. (2006) demonstrated that FIH preferentially hydroxylates HIF-1α (Bracken et al., 2006). In addition to this, several kinases (CK1, MAPK, ATM) have been identified to phosphorylate HIF-1α and increase its activity (Kalousi et al., 2010; Mylonis et al., 2006; Cam et al., 2010). However, it is yet to be determined whether these kinases also modify HIF-2α. Sirtuin1 (SIRT1) has been shown to de-acetylate both HIF isoforms, but with differential effects. The removal of the acetyl group results in the transcriptional repression of HIF-1α and the transcriptional activation of HIF-2α (Dioum et al., 2009; Lim et al., 2010).

More interestingly, HIF associated factor (HAF aka SART1), fits into several categories. HAF promotes VHL-independent degradation of HIF-1α in acute hypoxia but upregulates HIF-2α transcriptional activation in chronic hypoxia, which has led to this molecule being put forward as a differential regulator that switches cells from HIF-1α to HIF-2α dependent signalling in prolonged hypoxia (Koh et al., 2008).
Table 1.2 | Examples of protein-protein interactions that regulate transcription and translation of HIF-1α and HIF-2α mRNA in an O₂-independent manner.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Isoform</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcription</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>HIF-1α</td>
<td>✓</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>HIF-2α</td>
<td>✓</td>
<td>↓</td>
</tr>
<tr>
<td>IL-4</td>
<td>HIF-1α</td>
<td>✗</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HIF-2α</td>
<td>✓</td>
<td>↑</td>
</tr>
<tr>
<td><strong>Translation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense HIF1A</td>
<td>HIF-1α</td>
<td>✓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>HIF-2α</td>
<td>✗</td>
<td>-</td>
</tr>
<tr>
<td>IREBP1</td>
<td>HIF-1α</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>HIF-2α</td>
<td>✓</td>
<td>↓</td>
</tr>
</tbody>
</table>

References:
- Takeda et al. (2010)
- Uchida et al. (2004)
- Sanchez et al. (2007)
Table 1.3 | Examples of post-translation modifications and protein-protein interactions that regulate protein stability and transcriptional activity of HIF-1α and HIF-2α in an O2-independent manner.

<table>
<thead>
<tr>
<th>Protein</th>
<th>PTM / interaction</th>
<th>Isoform</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARD1</td>
<td>PTM</td>
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<tr>
<td></td>
<td></td>
<td>HIF-2α</td>
<td>×</td>
<td>-</td>
</tr>
<tr>
<td>HSP70</td>
<td>PTM</td>
<td>HIF-1α</td>
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<td></td>
<td></td>
<td>HIF-2α</td>
<td>×</td>
<td>-</td>
</tr>
<tr>
<td>HAF</td>
<td>PTM</td>
<td>HIF-1α</td>
<td>✓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIF-2α</td>
<td>×</td>
<td>-</td>
</tr>
<tr>
<td>HSP90</td>
<td>Interaction</td>
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<td>✓</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIF-2α</td>
<td>×</td>
<td>-</td>
</tr>
<tr>
<td>Rack1</td>
<td>Interaction</td>
<td>HIF-1α</td>
<td>✓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIF-2α</td>
<td>×</td>
<td>-</td>
</tr>
<tr>
<td>HAF</td>
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<td>HIF-1α</td>
<td>×</td>
<td>-</td>
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<td>SUMO-1/2/3</td>
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<td></td>
<td></td>
<td>HIF-2α</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>SIRT1</td>
<td>PTM</td>
<td>HIF-1α</td>
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<td>↓</td>
</tr>
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<td></td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIF-2α</td>
<td>✓</td>
<td>↓</td>
</tr>
</tbody>
</table>

It should be noted that there is far less known about mechanisms that specifically regulate HIF-2α largely due to HIF-1α being the more popular focus of research in the field of hypoxia. But it is clear that the two isoforms are differentially regulated at all levels.
1.5.2 Differential roles of HIF-1α and HIF-2α in physiological processes

HIF-1α and HIF-2α exhibit some redundancy in function. However, there are some processes that are either differentially regulated or uniquely regulated by one of the two isoforms.

**HYPOXIA INDUCIBLE GENES** – Hundreds of HIF target genes have been identified so far (Mole et al., 2009) and there is increasing evidence that some genes are specifically regulated by either HIF-1α or HIF-2α (Table 1.4). Genomic studies have found that generally, but not exclusively, HIF-1α regulates genes involved in the metabolic response and HIF-2α target genes control the respiratory response to oxygen deprivation (Mole et al., 2009; Hu et al., 2003). There is substantial evidence that HIF-2α is solely responsible for regulating erythropoiesis (reviewed in Haase, 2013), iron homeostasis (Haase, 2013) and lipid metabolism (Schoenenberger et al., 2015).
### Table 1.4 | Examples of target genes unique to HIF-1α or HIF-2α. Adapted from Keith et al. (2012).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Process</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNIP3 (BNIP3)</td>
<td>Autophagy and apoptosis</td>
<td>Raval (2005)</td>
</tr>
<tr>
<td>HK1 (hexokinase 1)</td>
<td></td>
<td>Iyer (1998), Ryan et al. (1998)</td>
</tr>
<tr>
<td>HK2 (hexokinase 2)</td>
<td></td>
<td>Hu et al. (2003), Iyer (1998), Ryan et al. (1998)</td>
</tr>
<tr>
<td>PFK (phosphofructokinase)</td>
<td>Glycolysis</td>
<td>Hu et al. (2003), Iyer (1998), Ryan et al. (1998)</td>
</tr>
<tr>
<td>ALDOA (ALDA)</td>
<td></td>
<td>Hu et al. (2003), Iyer (1998), Ryan et al. (1998)</td>
</tr>
<tr>
<td>PGK1 (PGK1)</td>
<td></td>
<td>Hu et al. (2003), Ryan et al. (1998)</td>
</tr>
<tr>
<td>LDHA (LDHA)</td>
<td></td>
<td>Hu et al. (2003), Ryan et al. (1998)</td>
</tr>
<tr>
<td>EPO (erythropoietin)</td>
<td>Erythropoiesis</td>
<td>Scortegagna et al. (2003), Gruber (2007), Rankin et al. (2007), Kapitsinou (2010)</td>
</tr>
<tr>
<td>ANGPT2 (angiopoietin 2)</td>
<td>Blood vessel remodelling</td>
<td>Skuli (2009)</td>
</tr>
<tr>
<td>POU5F1 (OCT4)</td>
<td>Stem cell identity</td>
<td>Covello et al. (2006)</td>
</tr>
<tr>
<td>CCND1 (cyclin D1)</td>
<td>Cell cycle progression</td>
<td>Raval (2005)</td>
</tr>
</tbody>
</table>

**ACUTE VS CHRONIC HYPOXIA** – It has also been hypothesised that the response to different types of hypoxia is differentially controlled by the two isoforms i.e. HIF-1α is the mediator of the response to acute hypoxia, but during periods of prolonged hypoxia control switches to HIF-2α (Holmquist-Mengelbier et al., 2006; Koh et al., 2011).
CELL CYCLE – The HIF-α isoforms regulate components of other signalling pathways. For example, HIF-1α has been shown to up-regulate p53 and down-regulate c-Myc, mTOR and β-catenin, whereas induction of HIF-2α has the converse effect on these signalling molecules, suggesting that HIF-1α suppresses cell proliferation and HIF-2α promotes cell cycle progression (Koshiji et al., 2004; Koshiji et al., 2005; Gordan et al., 2007; Florczyk et al., 2011).

NO PRODUCTION – The two alpha subunits are regulated by the levels of interferon gamma (IFNγ) resulting opposing effects on nitrous oxide (NO) production. Low levels of IFNγ result in high levels of HIF-2α and reduced NO production due to an increase in arginase, the enzyme that catalyses the conversion of arginine (the substrate required for NO production) to ornithine and urea (Figure 1.6). Whereas high levels of IFNγ leads to HIF-1α domination and increased NO productions (Figure 1.6)(Takeda et al., 2010).

A. arginine + 3/2 NADPH + H+ + 2 O2 ↔ citrulline + nitric oxide + 3/2 NADP⁺
B. arginine + H₂O → ornithine + urea

C. 

IFNγ

*LOW*  

↑ HIF-2α → ↑ Arginase → ↓ iNOS substrate → ↓ NO production

*HIGH*  

↓ HIF-2α  

↑ HIF-1α  

↑ Arginase  

↑ iNOS  

↑ NO production

Figure 1.6 | Differential effect of HIF-α levels on nitrous oxide production. A) Reaction catalysed by nitrous oxide synthase (iNOS). B) Reaction catalysed by arginase. C) The antagonistic effect of interferon gamma (IFNγ) levels on NO production.

EMBRYONIC DEVELOPMENT – Knockout mice experiments have shown that the loss of the HIF-1α or HIF-2α gene is embryonic lethal. However, lethality is due to defects in the heart and impaired lung formation, respectively (Loboda et al., 2010). Thus, indicating that both play vital yet different roles in embryonic development (Peng et al., 2000; Tian et al., 1998; Iyer et al., 1998; Ryan et al., 1998).
1.5.3 Differential role of HIF-1α and HIF-2α in disease

The involvement of HIF in a range of disease makes it an interesting and promising therapeutic target. However, depending on the disease, HIF can have a protective role or can contribute to the pathogenesis (Figure 1.7).

![Diagram](image)

**Figure 1.7 | Differential role of HIF in disease.**

There is particular focus on the relationship between HIF and cancer, as many HIF target genes are related to tumour progression and metastasis (Gilkes & Semenza, 2013). Furthermore, it has been shown that a hypoxic tumour microenvironment is associated with aggressiveness and resistance to treatment (Liu et al., 2008; Nardinocchi et al., 2009). However, the role of HIF in cancer is complex. Both HIF-1α and HIF-2α can be over-expressed in many different cancers with HIF-2α being generally seen as a tumour promoter and HIF-1α a tumour suppressor; a classic example being clear cell renal cell carcinoma (ccRCC). However, this characterisation is not always true and in some cancers HIF-2α has a tumour suppressive role. For example, Mazumdar et al. (2010) demonstrated that knocking out HIF-2α in the KRAS mouse model led to increased tumour growth due to the down regulation of the tumour suppressor gene Scgb3al (Mazumdar et al., 2010).
To complicate matters further, not only does the prognostic role of HIF-1α and HIF-2α vary between cancers, they even vary within the same types of cancer e.g. hepatocellular cancer (Table 1.5).

Table 1.5 | Differential roles of HIF-1α and HIF-2α in cancer.

<table>
<thead>
<tr>
<th>Cancer / Model</th>
<th>HIF isoform</th>
<th>Up/down regulated</th>
<th>Prognosis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast Cancer</td>
<td>HIF-1α</td>
<td>↑</td>
<td>Poor</td>
<td>Gilkes and Semenza (2013)</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>HIF-1α</td>
<td>↑</td>
<td>Good</td>
<td>Keith et al. (2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑</td>
<td>Poor</td>
<td>Dungwas et al. (2012)</td>
</tr>
<tr>
<td>Renal Cell Carcinoma</td>
<td>HIF-1α</td>
<td>↑</td>
<td>Good</td>
<td>Qing and Simon (2009)</td>
</tr>
<tr>
<td>Soft Tissue Sarcoma</td>
<td>HIF-1α</td>
<td>↑</td>
<td>Poor</td>
<td>Eisinger-Mathason et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>HIF-2α</td>
<td>↓</td>
<td>Good</td>
<td></td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>HIF-2α</td>
<td>↑</td>
<td>Poor</td>
<td>Wang et al. (2010), Yoon (2014)</td>
</tr>
<tr>
<td>Hepatocellular cancer (HCC)</td>
<td>HIF-2α</td>
<td>↑</td>
<td>Poor</td>
<td>Talks et al. (2000), Bangoura et al. (2007), Yang et al. (2014), Zhao et al. (2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓</td>
<td>Poor</td>
<td>Sun et al. (2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑</td>
<td>Good</td>
<td>Menrad et al. (2010)</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>HIF-2α</td>
<td>↑</td>
<td>Poor</td>
<td>Talks et al. (2000), Criscimanna et al. (2013)</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>HIF-2α</td>
<td>↑</td>
<td>Poor</td>
<td>Talks et al. (2000), Hui et al. (2002), Franovic et al. (2009), Cleven et al. (2008), Koukourakis et al. (2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓</td>
<td>Poor</td>
<td>Imamura et al. (2009)</td>
</tr>
</tbody>
</table>

Although HIF is of great interest as a potential therapeutic target and it is evident that there is a link between deregulation of HIF and therapeutic outcome, there is not a clear cut relationship.
1.5.4 Tissue distribution

As with cancer, the expression of the alpha subunits differs in tissues. HIF-1α is ubiquitously expressed throughout the body in all cell types; however HIF-2α is limited to distinct populations on cells in certain tissues or organs (Wiesener et al., 2003). For example: in the liver, HIF-2α accumulates in the nuclei of hepatocytes, i.e. parenchymal distribution; in the kidneys, pancreas and brain, HIF-2α nuclear accumulation is predominantly in non-parenchymal cells; and, in myocardial tissue equal distribution is observed (Wiesener et al., 2003).

1.5.5 Cellular localisation

Within our research group, single cell imaging of HIF-1α and HIF-2α has revealed that the two proteins exhibit different nuclear localisation patterns. HIF-1α is only observed in cells that have been subjected to hypoxia or treated with an inhibitor of the PHD enzymes, Dimethyloxalylglycine (DMOG). Approximately 2-4 hours after oxygen levels fall, homogenous extranucleolar accumulation of HIF-1α is observed in the nucleus. Whereas, in cells expressing EGFP-HIF-2α, punctate foci are observed (see Figure 1.8). Moreover, some HIF-2α stabilisation is observed without subjecting cells to hypoxia or DMOG treatment, therefore hinting that HIF-2α may evade oxygen-dependent degradation.

Figure 1.8 | Nuclear localisation of HIF-1α and HIF-2α in HeLa cells. HeLa cells transiently transfected with HIF-1α-EGFP (right) and HIF-2α-EGFP (left) showing different nuclear sub-localisation patterns. HIF-1α shows a homogenous nuclear localisation during hypoxia, whereas HIF-2α localises in sub-nuclear foci or “speckles”.
These observations strongly correlate with previous findings:

1) Hypoxic accumulation of HIF-1α in the nucleus was first described by Huang et al (1996). They also observed the translocation of HIF-1α from the cytoplasm into the nucleus when in hypoxia. However, they too observed that HIF-2α tagged with GFP was stabilised in the nucleus regardless of oxygen levels (Hara et al., 1999).

2) Takahashi et al also noticed that in Bovine aortic endothelial cells HIF-2α localised in the nucleus (excluded from the nucleoli) in “dots” and they too found that hypoxia or treatment with a hypoxia mimic (CoCl₂) did not affect the nuclear localisation of HIF-2α (Takahashi et al., 2004).

1.6 The Cell Nucleus

The nucleus was first described by Franz Bauer in 1802. It holds the majority of the cells genetic material and controls the activities of the cell by regulating gene expression. As with the cytoplasm, spatial organisation plays an important role within the nucleus to contain, regulate and streamline processes. However, unlike the cytoplasm, where processes are contained within organelles, nuclear functions are compartmentalised into domains. The different nuclear bodies can be distinguished by their size, shape, number and presence of particular proteins. Some examples follow.
NUCLEOLUS – The nucleolus is the largest and most well studied substructure in the nucleus. Many processes relating to ribosome biosynthesis takes place here such as transcription of ribosomal DNA and assembly of the ribosomes (Cmarko et al., 2008; Busch & Smetana, 1970). Although it is mainly seen as a “ribosome factory” it has recently come to light that it may also have a role in the regulation of cell cycle, proliferation and lifespan of a cell (Pederson, 1998; Carmo-Fonseca et al., 2000; Visintin & Amon, 2000; Takemura et al., 2002; Chestukhin et al., 2002).

CAJAL BODIES – Also known as coiled bodies (CBs) due to being composed of tangled fibrillar strands (Almeida et al., 1998; Pombo et al., 1999; Iborra et al., 2001), there are between 1-5 of these spherical compartments per nucleus but this can change depending on cell cycle stage and disease state (Brasch & Ochs, 1992; Boudonck et al., 1999; Gall, 2000). They are highly mobile and dynamic and have been observed: moving throughout the nucleoplasm; merging with and travelling through the nucleolus; and, going through joining and separation events, to make larger or smaller CBs (Platani et al., 2000; Lyon et al., 1997). Although reasonably well studies, the function of CBs has not been clearly defined as

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**Figure 1.9 | Structure of the nucleus.** Schematic diagram of the nucleus, highlighting key domains and sub-compartments. (Taken from http://www.fli-leibniz.de/www_imaging/structure_en.php)
yet, but there is some evidence to suggest that they are involved in the storage of small nuclear ribonucleic proteins (snRNPs) and may deliver these to the nucleolus (Lamond & Earnshaw, 1998; Matera, 1999; Spector, 2001; Zhou et al., 2002). It has also been suggested that the transcription machinery assembles within CBs before relocating to transcription factories (Platani et al., 2002).

**PML BODIES** – PML bodies get their name from the distinguishing presence of promyelocytic leukemia (PML) protein. They are donut shaped nuclear bodies, typically 0.25–0.5 μm in diameter and number around 10–30 per nucleus (Ascoli & Maul, 1991; Stuurman et al., 1992). As well as PML, many other proteins such as CBP, SP100, p53, NRF2 and elf-4E have been shown to localise at these nuclear bodies (LaMorte et al., 1998; Lai & Borden, 2000; Sternsdorf et al., 1999; Fogal et al., 2000; Ben-Dor et al., 2005). The function of the PML bodies is not clearly defined but based on the additional occupants it has been suggested that they may play a role in transcriptional regulation or translational regulation. They have also been linked to viral replication (Maul, 1998).

**TRANSCRIPTION FACTORIES** – It was previously thought that RNA polymerase II (RNAPII) is recruited to the promoters of genes that are to be transcribed, however it is now believed that active loci migrate to domains where the transcriptional machinery is located (Iborra et al., 1996; Jackson et al., 1998). Different genes, even genes that are separated by great distances, in the genome have been shown to concurrently occupy the same transcription factories (Osborne et al., 2004; Jackson et al., 1993; Jackson et al., 1998).

**SPlicing FACTOR COMPARTMENTS** – Pre-mRNA splicing occurs co-transcriptionally. Splicing factors continuously exchange between splicing factor compartments (SFCs) and sites of transcription. SFCs are irregular in shape but following inhibition of transcription these nuclear speckles increase in size and change shape, indicating further these are storage sites of inactive splicing factors (Sinclair and Brasch, 1978).

There is little known about how nuclear bodies are formed as they are not delineated by a lipid bilayer (like the nucleus itself). The lack of intra-nuclear membranes has led to suggestions that they form via “self-organisation”, whereby the architecture and function of the structure is determined via the physical interaction of its components (Misteli, 2001).

There is some evidence that there is a sub-nuclear framework, similar to the cytoskeleton, which is composed of a mixture of proteins such as lamin B (Stuurman et al., 1990; Berezney
& Coffey, 1977). It has been suggested that this nuclear matrix may play a role in the orchestrating nuclear processes and is likely to act as a “substratum” for nuclear bodies (Mitchell & Fraser, 2008; Ascoli & Maul, 1991; Stuurman et al., 1992).

The discovery and study of the heterogeneous localisation of nuclear proteins and nuclear domains has been facilitated by the advancements in microscopy and protein labelling techniques made over the last century.
1.7 Microscopy

1.7.1 A brief history of fluorescent confocal microscopy

1595 saw the invention of the first instrument that facilitated the visualisation of objects that could not be seen by the naked eye but it wasn’t until 1911 that the first successful fluorescent microscope was constructed by the physicist, Oskar Heimstädt (Heimstadt, 1911). This design was reconfigured by Ellinger and Hirt (1929) so that the light source was on the same side of the specimen as the objective, resulting in both the emission and excitation light passing through the objective (Figure 1.10), creating the epi-fluorescence microscope (Ellinger & Hirt, 1929).

![Figure 1.10](image)

**Figure 1.10** | **Lightpath of an epi-fluorescence microscope.** *Schematic representation of the basic setup of an inverted epifluorescence microscope.*

The problem of “out of focus light” associated with epi-fluorescence microscopes was circumvented by Minsky’s confocal microscope, which incorporated pinhole apertures to block this non-specific fluorescence from reaching the detector (Minsky, 1961). He also introduced “raster scanning”, whereby an image is created by systematically illuminating
the field of interest line by line, to reduce the amount of the specimen that is illuminated at any one time. Minsky’s design was further improved by the addition of dichroic mirrors (Ploem, 1967) and upgrading the light source to a laser beam (White et al., 1987; Van Meer et al., 1987) resulting in the basis of the modern laser scanning confocal microscope.

Table 1.6 | Timeline of key events in the history of light microscopy.

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1595</td>
<td>Invention of microscope</td>
</tr>
<tr>
<td>1911</td>
<td>First fluorescent microscope</td>
</tr>
<tr>
<td>1929</td>
<td>First epi-fluorescence microscope</td>
</tr>
<tr>
<td>1942</td>
<td>First example of immunofluorescence</td>
</tr>
<tr>
<td>1961</td>
<td>Minsky’s patent of confocal microscope accepted</td>
</tr>
<tr>
<td>1972</td>
<td>Development of Fluorescence correlation spectroscopy (FCS)</td>
</tr>
</tbody>
</table>
| 1976 | Development of Fluorescence recovery after photobleaching (FRAP)  
Försters / fluorescence resonance transfer (FRET) experimentally proven |
| 1987 | Laser scanning confocal microscope |
| 1993 | Light sheet microscopy  
Single molecule microscopy |
| 1992-1996 | Cloning and development of GFP |
| 1999 | Cloning of red fluorescent proteins |
| 2000 | Development of super-resolution method - STED & SIM |
| 2002 | Photoactivatable fluorescent proteins |
| 2006 | Development of Super-resolution methods - PALM / fPALM / STORM |
| 2008 | Nobel prize for chemistry awarded to researchers involved in the cloning and development of GFP |
| 2014 | Nobel prize for chemistry awarded to developers of super-resolution imaging |
1.7.1.1 Fluorescence microscopy and labelling methods

Fluorescence refers to the ability of a substance to absorb light at a certain wavelength and emit light at a higher wavelength (Figure 1.11) and was first described in 1852 by Sir George Stokes. However, the earliest recorded use of fluorescence in biological investigations wasn’t until almost a century later. However, when the fluorescent microscope was first invented, scientists relied upon autofluorescence (the natural emission of light from a specimen) until Ellinger and Hirt pioneered the use of “secondary fluorescence”, whereby they introduced an exogenous fluorescent substance (fluorescein) to improve contrast of the sample (Ellinger & Hirt, 1929).

Figure 1.11 | Overview of fluorescence. A) Schematic representation of the principle of how fluorescence occurs. Briefly, an orbital electron of a molecule or atom is electronically excited by absorbing photons from higher energy light (e.g. blue). As the electron relaxes through energy levels (orange lines) to its ground state, photons of a lower energy wavelength (e.g. green) are emitted. B) Example excitation - emission spectra of enhanced green fluorescent protein (EGFP).
It was only in the 1940’s that immunofluorescence, a technique that permits specific labelling of cells, structures and proteins with fluorescent probe, emerged. Coons et al took antibodies raised against strains of pneumococcus and attached a fluorescent molecule (Coons et al., 1941). They labelled tissue from patients positive and negative for this bacteria and observed a clear difference in the signal between the two samples (Coons et al., 1942). However, the biggest breakthrough for fluorescence microscopy came in 1994 when Chalfie et al. demonstrated how the green fluorescent protein (GFP), from the jellyfish Aequorea victoria, could be used to visualise proteins in living organisms (Chalfie et al., 1994). The cloning of GFP opened the door for the creation of genetically labelled proteins of interest facilitating the study of intracellular localisation and dynamic behaviour of proteins of interest in real time in vivo.

Further developments were made when Roger Tsien and colleagues conducted exploratory mutagenesis of GFP creating enhanced GFP (EGFP), a derivative that is 30x brighter than the wildtype, and derivatives with a shift in the excitation / emission spectra: BFP (blue), CFP (cyan) and YFP (yellow) (Heim & Tsien, 1996). To add to these tools, the orange-red fluorescent protein, dsRED, that emits at 583 nm following excitation at 558 nm, was cloned from a reef coral Discosoma sp in 1999 (Matz et al., 1999). The availability of these different fluorescent proteins (only a subset mentioned here) has meant that genetically-encoded spectrally-discrete reporters can be created, thus facilitating the simultaneous study of several proteins of interest in situ. Furthermore, it has permitted the non-invasive study of protein kinetics and interactions in living cells. The invaluableness of GFP (and its variants) and the genetic tagging of biomolecules to scientific research was recognised by the award of the Nobel Prize in chemistry in 2008.

1.7.2 Probing the dynamics of proteins further

In the 1970s, it was proposed that fluorescence microscopes could be utilised for more than just imaging, but could also be used to study reaction kinetics, diffusion and intermolecular interactions. Some examples include: fluorescence correlation spectroscopy (FCS), Förster / fluorescence resonance electron transfer (FRET) and fluorescence recovery after photobleaching (FRAP).
1.7.2.1 FCS

This technique measures fluctuations in fluorescent signal coming from labelled molecules moving in and out of a defined region. These measurements can be used to determine the concentration and diffusion rate of the molecule being studied. Advancements in detection methods led to the quantification of the dynamics of single molecules, with Rigler and Widengren being the first to demonstrate this in 1990.

In principle, FCS can be used to measure intermolecular interactions of a fluorescently labelled protein as the diffusion rate would slow upon binding to a larger molecule. However, the development of two-colour FCS (fluorescence cross-correlation spectroscopy / FCCS) has meant the interaction of any two molecules, regardless of size, can be studied (Schwille et al., 1997).

1.7.2.2 FRET

This technique is based on the principle, proposed by Theodor Förster in the 1940s (Förster, 1946) and confirmed experimentally in 1967 (Stryer & Haugland, 1967), that the energy generated from electronic excitation can be passed from one fluorophore (donor) to another (acceptor) when the two chromophores are within close enough proximity (10 nm). However, it wasn’t until the development of GFP variants CFP and YFP, that FRET was utilised to study protein-protein interactions in vivo (Bacskai et al., 1993). Shortly after, FRET-sensors were developed that act as biological indicators. Examples include calcium (Miyawaki et al., 1997; Romoser et al., 1997; Emmanouilidou et al., 1999), cyclic AMP (Zaccolo et al., 2000), apoptosis (Xu et al., 1998) and synaptic activity (Vanderklish et al., 2000).
Table 1.7 | Key landmarks of FRET

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1993</td>
<td>First application of FRET to study molecular interactions in situ.</td>
<td>Bacskai et al. (1993)</td>
</tr>
<tr>
<td>1997</td>
<td>Development of FRET-sensors</td>
<td>See text</td>
</tr>
</tbody>
</table>

1.7.2.3 FRAP

This fluorescence perturbation technique involves irreversible bleaching of fluorescently labelled proteins. The redistribution of non-bleached molecules (or “recovery”) into the bleached region is monitored (Figure 1.12) and subsequent analysis of the recovery of fluorescent molecules provides information on mobility.

![Figure 1.12](image)

**Figure 1.12 | Fluorescent recovery after photobleaching.** A) Schematic of time-lapse imaging to capture recovery of fluorescence. B) Schematic of molecular redistribution following photobleaching. Black broken circle = bleached region, black circles = bleached molecules, green circles = non-bleach molecules.

Although FCS can be used to measure diffusion of single molecules, for answering certain biological questions FRAP is more advantageous as the whole cell can be monitored and it can detect the presence of immobile populations of molecules.
Due to ease of labelling, FRAP was first utilised to study the movement of protein within the cell membrane (Peters et al., 1974) as these could be easily labelled. However, today, genetic labelling enables the study of protein trafficking anywhere within the cell.

It may seem surprising that these aforementioned techniques were all invented before the realisation of the laser scanning confocal microscope and the cloning of GFP (Table 1.7). However, these later developments only broadened the applications of these techniques by facilitating the non-invasive study of real-time diffusion and interactions of fluorescently labelled protein in living cells.

1.7.3 Super-resolution microscopy

The diffraction limit theory was conceived in 1873 and states that the smallest resolvable distance between two points is half the wavelength of the light used for illumination (Abbe, 1873), which essentially means that objects less than 150-200 nm apart cannot be resolved with conventional light microscopy. Now, over 100 years later, with the arrival of super-resolution we are able to see beyond the diffraction limit, with distances down to tens of nanometres being resolved. Up until recently, to resolve structures that were closer than 200 nm, either electron microscopy (Ruska, 1987) or total internal reflection (TIRF) microscopy (Axelrod, 1981) were used. Although modern electron microscopes are capable of achieving more than 1 nm resolution (Erni et al., 2009), they cannot be used to image living cells and there is an intensive sample preparation protocol. TIRF is also limited to certain applications as only structures that are no more than 100 nm away from the coverslip can be studied. However, the last twenty years has seen the emergence of new super-resolution (SR) imaging techniques which can be divided into two categories: illumination-based such as stimulation emission depletion (STED) (Hell & Wichmann, 1994) or structured illumination (SIM) microscopy (Gustafsson, 2000); and, single-molecule / probe-based e.g. photo-activated localisation (PALM) (Betzig et al., 2006;Hess et al., 2006) or stochastic optical reconstruction (STORM) microscopy (Rust et al., 2006). These methodologies mean that structures can be visualised in living cell at the molecular level and have already been utilised in discerning the three-dimensional organisation of molecules in the nuclear pore complex (Schermelleh et al., 2008) and the distribution of proteins in structures such as microtubules (Huang et al., 2008) and lysosomes (Betzig et al., 2006). SR has also been utilised to study dynamic processes such as the formation of
macromolecular complexes e.g. focal adhesions (Shroff et al., 2008). The recent ground-breaking developments to surpass the diffraction limit of conventional fluorescence microscopy resulted in E. Betzig, S. Hell and W. Moerner being awarded the Nobel Prize in chemistry in 2014.

1.7.4 Single molecule tracking (SMT)

Although perturbation (e.g. FRAP) and correlation (e.g. FCS) techniques can provide information on diffusion, this information is based on the average behaviour of the protein of interest meaning that, as with any population technique, any heterogeneity within the population will be masked (Brameshuber & Schuetz, 2008). Single molecule tracking (SMT) is a technique that allows the elucidation of the spatial and temporal dynamics of individual molecules by direct monitoring of said molecules in real time.

Several techniques have been developed for SMT of fluorescently labelled proteins (reviewed in Meuller et al., 2013) and have been implemented in tracking RNA polymerase II in three dimensions (Abrahamsson et al., 2013) and deciphering how transcription factors search target binding sites (Chen et al., 2014).

One alternative to fluorescence microscopy, which can be utilised for SMT in living cells, is photothermal imaging of protein labelled with gold nanoparticles, which is discussed in Chapter 5.
1.8 Project aims

The aim of my project is to study the spatial and temporal regulation of the main intracellular effectors of the hypoxia signalling pathway.

The specific aims of my project were:

- Improving molecular and cellular tools for imaging HIF-1/2α.
- Investigating the temporal dynamics of HIF-1/2α at the single cell level
- Investigating the sub-nuclear spatial localisation of HIF-2α at the single cell and single molecule levels

I have employed live cell imaging techniques to elucidate the spatial and temporal dynamics of the key mediators of the hypoxia signalling pathway at the cellular level. I have also utilised confocal microscopy to study sub-cellular localisation and perturbation techniques to elucidate molecular dynamics. All of this was done with a view to contributing to the understanding of the mechanisms of cellular responses and adaptation to environmental stress but also to potentially provide insight into improving therapeutic strategies developed to either inhibit (e.g. cancer) or enhance (e.g. stroke) the hypoxic response.
Chapter 2: Materials and Methods
In this chapter, the general materials and methods used during this project are described. For some areas, such as recombinant protein expression and purification, specific details can be found in the respective chapters.

2.1 Chemicals and reagents

All chemicals and reagents were from Sigma Aldrich (MO, USA) unless stated otherwise. With the exception of cell culture regents which were from Life Technologies (CA, USA) and all plastic ware was Corning® (Corning Inc., NY, USA), unless stated otherwise.

2.2 Molecular Biology

2.2.1 Plasmids

Plasmids encoding fluorescent HIF-1α and HIF-2α fusion proteins were as described in Bagnall et al. (2014). pLNT-Ubc-HIF-2α-Venus was created by replacing the region encoding HIF-1α in pLNT-Ubc-HIF-1α-Venus (a generous gift from J. Bagnall, University of Manchester, UK) with a fragment encoding HIF-2α using Infusion HD cloning protocol (see Plasmid Cloning). pCMX-PL1-YFP-mHDAC5 was a generous gift from R. M. Evans Laboratory, (The Salk Institute, CA, USA). CMV-Halotag-HIF-2α (pFN21AB4384) was purchased from Kazusa DNA Research Institute, Japan. pET-M11-His-Halotag-HIF-2α was created by inserting the Halotag-HIF-2α coding region (from CMV-Halotag-HIF-2α) and inserting this into the pETM-11-His plasmid (obtained from G. Stier, EMBL. See Plasmid Cloning).

2.2.2 Propagation of Expression Plasmid DNA

2.2.2.1 Transformation

50 µL commercial competent DH5α E. coli cells (Invitrogen, CA, USA) were thawed on ice and transferred to pre-chilled 14 mL BD Falcon 17x100 mm round-bottom polypropylene tube (Becton Dickinson, NJ, USA). 5 ng of plasmid DNA was added to the competent cells and were incubated on ice for 30 min. The cells were then heat-shocked at 42 °C for 45 sec in a water bath, followed by a second incubation on ice for 2 min. 950 µL pre-warmed SOC medium (Invitrogen, CA, USA) was added and the culture was incubated for 1 hour at 37 °C in an orbital shaker (225 rpm). The cells were diluted 1:10-1:100 in pre-warmed SOC
medium and 100 μL spread onto LB agar (Merck KGaA, Germany; prepared according to manufacturer’s instructions) plates containing the appropriate antibiotic (at a final concentration as stated in Table 2.1) and incubated overnight at 37 °C.

Table 2.1 | Antibiotic concentrations used

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Final Concentration (μg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>34</td>
</tr>
</tbody>
</table>

2.2.2.2 Small scale Plasmid DNA purification (Mini-prep)

A single colony was selected from the agar plate for mini-culture using aseptic techniques and added to 5 mL LB broth (Merck KGaA, Germany; prepared according to manufacturer’s instructions) containing the appropriate antibiotics. Following incubation for 6 h-8 h at 37 °C in an orbital shaker (225 rpm), 2 mL of the bacterial culture was processed and the plasmid DNA extracted using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Ma, USA) following the manufacturers guidelines.

2.2.2.3 Large Scale Plasmid DNA purification (Maxi-Prep)

Using aseptic technique, 2 mL of bacterial mini-culture was transferred to a 1 L flask containing 200 mL LB broth & selective antibiotic(s). This was then incubated overnight at 37 °C in the orbital shaker (225 rpm). Cells were harvested using a Sorvall centrifuge, they were spun at 4 °C, 6800 x g for 15 min. DNA was isolated from bacterial cells using Purelink TM HiPure Plasmid Filter Purification Kit (Invitrogen, CA, USA), following the protocol provided by manufacturer. The purified DNA was diluted to 1 μg μL⁻¹.
2.2.3 Polymerase Chain Reaction (PCR)

PCR was used to amplify gene inserts for In-Fusion cloning (see below). KOD Hot Start DNA polymerase (Novagen, Germany) was used according to Table 2.2. PCR was carried out in a Px2 Thermal Cycler (Thermo Scientific, MA, USA). The standard PCR conditions used are stated in Table 2.3.

Table 2.2 | Standard set up for PCR reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume / amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO4</td>
<td>3 μl</td>
</tr>
<tr>
<td>2 mM dNTPs</td>
<td>5 μl</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>5 μl</td>
</tr>
<tr>
<td>10 μM Forward-primer</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>10 μM Reverse-primer</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>20 ng</td>
</tr>
<tr>
<td>KOD hot start polymerase</td>
<td>1 μl</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Up to final volume of 50 μl</td>
</tr>
</tbody>
</table>

Table 2.3 | Standard conditions used for PCR reaction

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>20 seconds</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>50-70</td>
<td>1 min / 1kb $T_{hyb}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>3 min</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
2.2.4 Restriction Digests

Digests were set up according to Table 2.4. The 10x buffer used was dependent on the restriction enzyme used and chosen based upon manufacturers guidelines. Total reaction volume made up to 20 μL with ddH₂O and incubated at 37 °C (or the temperature recommended by the manufacturer) for a minimum of 2 h followed by enzymes inactivation (if required) by heating to 65 °C for 15 min. Digests were validated via gel electrophoresis.

Table 2.4 | Standard set up for restriction digest

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume / amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single digest</td>
<td></td>
</tr>
<tr>
<td>Enzyme 1</td>
<td>1 μl</td>
</tr>
<tr>
<td>10x Buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>DNA</td>
<td>1μg</td>
</tr>
<tr>
<td>Double digest</td>
<td></td>
</tr>
<tr>
<td>Enzyme 1</td>
<td>1 μl</td>
</tr>
<tr>
<td>Enzyme 2</td>
<td>1 μl</td>
</tr>
<tr>
<td>10x Buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>DNA</td>
<td>1 μg</td>
</tr>
</tbody>
</table>

2.2.5 Plasmid Cloning

The constructs listed in Table 2.5 were created using the Clontech In-Fusion® HD cloning protocol. Inserts were amplified using specifically designed primers that have 20 bp insert specific regions and 15 bp that are homologous to the destination vector (Table 2.6). The destination vectors were linearised using the protocol in Table 2.4 using the enzymes listed in Table 2.5 Both the PCR product and linearised vector were run on a 1% agarose gel at 100 V for 1 h and excised. These DNA fragments were purified using E.Z.N.A. gel extraction kit (Omega Biotek, GA, USA) according to manufacturer’s instruction.

The In-Fusion® (Clontech, Canada) reaction was carried out according to the manufacturer’s instructions. The volume of linearised vector and insert were calculated based on the mass
of each required, as determined by the In-Fusion® Molar Ratio Calculator (http://bioinfo.clontech.com/infusion/molarRatio.do; insert:vector ratio = 2). Briefly, 2 μL of 5X In-Fusion HD Enzyme was added to make a total reaction volume of 10 μL. The reaction was incubated in a heat block at 50 °C for 15 min. In order to reduce toxicity and improve the number of transformants the In-fusion enzyme was digested via incubation with 1 μL Proteinase K (Invitrogen, CA, USA) for 10 min at 37 °C prior to transformation into Stellar™ competent cells (Clontech, Canada). 50 μL of cells were thawed on ice and transferred to pre-chilled 14 mL BD Falcon 17x100 mm round-bottom polypropylene tube (Becton Dickinson, NJ, USA). Approximately 5 ng of DNA was added. The cells were incubated on ice for 30 min, heat shocked at 42 °C for 45 sec and returned to ice for 2 min. 450 μL pre-warmed SOC medium was added and the culture was incubated in an orbital shaker for 1 h at 37 °C (225 rpm). 200 μL was then spread onto selective LB agar plates and incubated for 16 h at 37 °C. DNA from isolated colonies was purified (see mini-prep) and screened using restriction digest and imaging of transiently transfected cells. The best candidate was sent away for sequencing (GATC, Germany) before large scale DNA purification (maxi-prep).
### Table 2.5 | Components used to create plasmids via Infusion HD cloning method

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Destination Vector</th>
<th>Restriction Enzymes</th>
<th>Insert</th>
<th>Insert amplified from</th>
</tr>
</thead>
<tbody>
<tr>
<td>pETM-11-HIS-HaloTag-HIF-2α</td>
<td>pETM-11-HIS*</td>
<td>NcoI &amp; EcoRI (Roche, UK)</td>
<td>Halotag-HIF-2α</td>
<td>CMV-Halotag-HIF-2α</td>
</tr>
<tr>
<td>CMV-HaloTag-HIF-2α-IRES-dTomato</td>
<td>CMV-Halotag-HIF-2α</td>
<td>NotI &amp; Xbali (Roche, UK)</td>
<td>IRES-dTomato</td>
<td>pHIV-EGFP-HIF-2α-IRES-dTOM</td>
</tr>
<tr>
<td>pLNT-Ubc-HIF-2α-Venus</td>
<td>pLNT-Ubc-HIF-1α-Venus #</td>
<td>Xbali &amp; BamiHi (New England Biolabs, MA, USA)</td>
<td>HIF-2α</td>
<td>pG-EGFP-HIF-2α</td>
</tr>
</tbody>
</table>

[*G. Stier, EMBL; #J. Bagnall, University of Manchester*]

### Table 2.6 | Primers used for infusion cloning

20 bp are specific for the insert and 15 bp (red) are specific to the destination vector, this creates overhangs allows the amplified insert to be directionally ligated into the linearised destination vector.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pETM-11-HIS-HaloTag-HIF-2α</td>
<td>Forward</td>
<td>GACGGAGCTCGAATTCAACGGTGCGCTGGTCCAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTTCAAGGGCCATGCGAAGAAATCGGTACTGCG</td>
</tr>
<tr>
<td>CMV-HaloTag-HIF-2α-IRES-dTomato</td>
<td>Forward</td>
<td>TACCCGGGGATCCTTAGAAGTACCCGGGCTAGGATCG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCATGCTGCTCGAGCGGCCGCATTTAATTCTGACAGCTCGT</td>
</tr>
<tr>
<td>pLNT-Ubc-HIF-2α-Venus</td>
<td>Forward</td>
<td>GGGCTGCAGGTCGACTCTAGAATGACAGCTGACAAAGGAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGTGGGCACCGGTGGATCCGCAGTGCGCCCTGGTCCAGG</td>
</tr>
</tbody>
</table>

### Table 2.7 | Sequencing primers

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>pETM-11-HaloTag-HIF-2α</td>
<td>TCAAGACCGTTTAGAGG; ATACATGGACTGGCTGGC; CATGCAAGTTCGTAGG; TCACCTCTCGAGTTCCG; CTCTCAAAAGGTGAGGC</td>
<td>Covered full Halotag-HIF-2α insert</td>
</tr>
<tr>
<td>CMV-HaloTag-HIF-2α-IRES-dTomato</td>
<td>Same as those used for cloning (Table 2.6)</td>
<td>Covered IRES and dTOM insert</td>
</tr>
<tr>
<td>pLNT-Ubc-HIF-2α-Venus</td>
<td>CATGAAGTTCACCTACCTG</td>
<td>Binds in the middle of EPAS1 and sequences towards 3’ end</td>
</tr>
</tbody>
</table>

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2.3 Cell Culture

2.3.1 Cell line propagation

All cell lines were maintained in humidified air at 37 °C in a Sanyo CO₂ (CO₂ set at 5%) incubator (SANYO Electric Biomedical Co., Japan).

Table 2.8 | Cell lines and culturing conditions

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Growth Conditions</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HeLa</strong></td>
<td>MEM 10% v/v FCS, 1% v/v NEAA</td>
<td>ECACC no: 93021013</td>
</tr>
<tr>
<td><strong>shPHD2 HeLa</strong></td>
<td>MEM 10% v/v FCS, 1% v/v NEAA, 10 µg mL⁻¹ Puromycin (Invitrogen, CA, USA).</td>
<td>generous gift from D Hoogewijs, D Stiehl and R Wenger, University of Zürich, Switzerland</td>
</tr>
<tr>
<td><strong>shPHD3 HeLa</strong></td>
<td>MEM 10% v/v FCS, 1% v/v NEAA, 10 µg mL⁻¹ Puromycin (Invitrogen, CA, USA).</td>
<td>HeLa cells transduced with shPHD3 lentivirus. shPHD3 plasmid from D Stiehl and R Wenger, University of Zürich, Switzerland</td>
</tr>
<tr>
<td><strong>PML-YFP HeLa</strong></td>
<td>MEM 10% v/v FCS, 1% v/v NEAA, 1 µg mL⁻¹ Blasticidin, 1% v/v Penicillin/Streptomycin</td>
<td>generous gift from E. G. Jaffrey, University of Dundee</td>
</tr>
<tr>
<td><strong>HEK 293TN</strong></td>
<td>DMEM 10% v/v FCS.</td>
<td>#LV900A-1; System Biosciences, Inc., CA, USA</td>
</tr>
<tr>
<td><strong>HIF-1α-GFP BAC HeLa</strong></td>
<td>MEM 10% v/v FCS, 1% v/v NEAA, 400 µg mL⁻¹ geneticin</td>
<td>Stably cell line created by transfection of HIF-1α-GFP BAC (generous gift from Prof R van de Water, Leiden University)</td>
</tr>
<tr>
<td><strong>C2C12</strong></td>
<td>–</td>
<td>Provided by Dr J Ankers, University of Liverpool, UK</td>
</tr>
</tbody>
</table>
2.3.2 Cell treatments

Hypoxic incubation was carried out either directly on the microscope stage or incubated in a H35 Hypoxysation (Don Whitley, UK). DMOG was used as a hypoxia mimic at a final concentration of 0.5 mM used (Enzo Life Sciences; NY, USA).

Table 2.9 | Definitions of different cell treatments

<table>
<thead>
<tr>
<th>Treatment Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>19% O₂, 5% CO₂, 37 °C</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>1% O₂, 5% CO₂, 37 °C</td>
</tr>
<tr>
<td>Dimethylloxalylglycine (DMOG)</td>
<td>2OG-hydroxylase (PHD) inhibitor</td>
</tr>
</tbody>
</table>

2.3.3 Transient Transfection

The transfection reagent, FuGene®6 (Roche, Switzerland) was used for delivery of plasmids into cells. Transfection of cell was carried out 24 h before imaging or other analysis, following the manufacturer’s protocol and using a ratio of 2:1 (transfection reagent: plasmid DNA mass).

Table 2.10 | Volumes of reagents used for different vessels for transient transfection of HeLa cells.

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Volume of Serum Free Medium (µL)</th>
<th>Volume of Fugene (µL)</th>
<th>Total Mass of DNA (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 well plate</td>
<td>50</td>
<td>1.5</td>
<td>0.75</td>
</tr>
<tr>
<td>35 mm glass bottom dish</td>
<td>100</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>60 mm culture dish</td>
<td>200</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>10 cm culture dish</td>
<td>1700</td>
<td>34</td>
<td>17</td>
</tr>
</tbody>
</table>
2.4 Stable cell line production

2.4.1 Lentivirus

The shPHD3 and ODD-EGFP stable cell lines were made via lenti-viral transduction. The pLKO.1.shPHD3 construct was obtained from D Hoogewijs and R Wenger (University of Zürich). The pHIV-ODD-EGFP-IRES-dTomato lentiviral construct was cloned by Amelie Schober (Erasmus Masters student). This was done by amplifying the ODD-EGFP coding region from pG-ODD-EGFP (Bagnall, 2011) and ligating this into a pHIV-IRES-dTomato plasmid (plasmid 21374; Addgene, MA, USA).

2.4.1.1 Viral particle production

HEK 293TN cells were plated in 10 cm at a density of $1.5 \times 10^6$. The lentiviral construct was transfected into HEK 293TN cells using FuGene®6 (Roche Applied Science, UK) along with packaging plasmids psPAX2 (plasmid 12259; Addgene, MA, USA) and pMD2.G (plasmid 12260; Addgene, MA, USA). The three plasmids were mixed in the ratio of 4:2:1 respectively and transfected using a ratio of 2:1 (Fugene®6 volume: total DNA mass). 16 h post transfection, the media was aspirated and replaced with fresh media. The media was then collected 72 h after transfection and the viral particles concentrated by ultracentrifugation, following the protocol in Kutner et al. (2009). In brief, the 20 ml of viral-particle containing medium was placed in sterilized Ultra-clear SW28 centrifuge tubes with a 4 mL 20% sucrose solution (20 g of ultrapure sucrose dissolved in 100 mM NaCl, 20 mM Hepes pH 7.5 and 1 mM EDTA) at the bottom. Ultracentrifugation was carried out as stated in protocol. Viral particles were resuspended in 100 μL PBS and were combined to make a total of 200 μL.

2.4.1.2 Transduction of HeLa cells

Wt HeLa cells were seeded in a 24 well plate and grown to approximately 60% confluency. The concentrated virus was added to the cells along with polybrene (8 μg mL$^{-1}$; Sigma, MO, USA). The media was changed after 16 h. When confluent, the transduced cells were transferred to a 25 cm$^2$ flask and eventually maintained in 75 cm$^3$ flask. The ODD-EGFP and
shPHD3 HeLa cell lines were grown in MEM (10% FCS, 1% NEAA). The shPHD3 medium was supplemented with 10 μg mL⁻¹ puromycin (Invitrogen, CA, USA).

### 2.4.2 Bacterial artificial Chromosome (BAC)

Bacteria containing the HIF-1α-GFP BAC (generous gift from Dr S Le Dévédec, Leiden University; RP11-867-L15, http://www.mitocheck.org/cgi-bin/BACfinder) were streaked onto an LB agar plate (containing 31.25 μg mL⁻¹ Kanamycin and 12.5 μg mL⁻¹ Chloramphenicol) and incubated at 37 °C overnight. Isolated colonies were picked and grown in 4 mL of LB broth (containing 31.25 μg mL⁻¹ Kanamycin and 12.5 μg mL⁻¹ Chloramphenicol) at 37 °C in an orbital shaker (225 rpm) for 8 h. Five hundred microlitres of this culture was transferred to 500 mL LB broth (containing 31.25 μg mL⁻¹ Kanamycin and 12.5 μg mL⁻¹ Chloramphenicol) and incubated at 37 °C in an orbital shaker (225 rpm) for 16 h. The bacteria were harvested via centrifugation (Sorvall RC-5B Refrigerated Superspeed Centrifuge; GSA rotor) at 6000 rpm, 4 °C for 10 min. The supernatant was discarded and the BAC was extracted and purified from the pellet using a NucleoBond Bac 100 kit (Macherey-Nagel GmbH, Germany) following the manufacturers protocol (BAC 100 Maxi).

One million HeLa cells were seeded in 10 cm dishes 24 h hours before transfection. HIF-1a-GFP BAC was transfected into HeLa cells using polyethylenimine (PEI) “Max” (Polysciences Inc., PA, USA) in a ratio of 2:1 (PEI:BAC) following the same protocol as used for Fugene6® (see section 2.3.3). A second plate was left non-transfected as a selection control. After 72 h, the medium was replaced with medium containing 400 μg mL⁻¹ geneticin (Sigma Aldrich, MO, USA). The medium was changed every 48 h until all cells on control plate had died and colonies had started to form on the transfected plate.

Isolated colonies were picked and transferred to a 96 well plate (one colony per well). Cells were kept under selection until confluent enough to be transferred into a larger vessel. This process was repeated until there were enough cells to create frozen stocks. Expression of HIF-1α-GFP was verified by western blot and microscope experiments.
2.5 Microscopy

2.5.1 Overview of Microscopes used

Table 2.11 | Microscopes used and the application used for

<table>
<thead>
<tr>
<th>Microscope</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeiss, LSM 510</td>
<td>EGFP-HIF-2α FRAP</td>
</tr>
<tr>
<td></td>
<td>HIF-1α-EGFP FRAP</td>
</tr>
<tr>
<td>Zeiss, LSM 710</td>
<td>Co-localisation of HIF-2α</td>
</tr>
<tr>
<td></td>
<td>TMR-Halotag-FGF2</td>
</tr>
<tr>
<td>Zeiss, LSM 780</td>
<td>EGFP-HIF-2α FLIP</td>
</tr>
<tr>
<td></td>
<td>HIF-1α-EGFP BAC imaging</td>
</tr>
<tr>
<td>Epifluorescence</td>
<td>Speckle characterisation</td>
</tr>
<tr>
<td></td>
<td>Microinjection</td>
</tr>
</tbody>
</table>
Table 2.12 | Details of settings used for different fluorophores on each microscope

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Laser</th>
<th>Filter set up</th>
<th>Objective</th>
<th>Image capture software</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LSM 510</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFP</td>
<td>488 nm laser</td>
<td>Emitted light reflected by a 540 nm dichroic mirror through a 505-550 nm bandpass filter and detected through a 530 nm longpass filter.</td>
<td>Plan-apochromat 63× 1.4 oil</td>
<td>LSM510 version 3 software</td>
</tr>
<tr>
<td>dTomato</td>
<td>543 nm laser</td>
<td>Emitted light was detected through a 560 nm longpass filter</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LSM 710</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AlexaFluor488 / YFP</td>
<td>488 nm laser</td>
<td>MBS 488/561/633</td>
<td></td>
<td>Zen 2010 software</td>
</tr>
<tr>
<td>AlexaFluor555</td>
<td>561 nm laser</td>
<td>MBS 458/561</td>
<td>Plan-Fluar 100× / 1.40 oil</td>
<td></td>
</tr>
<tr>
<td>Topro-3-Iodide</td>
<td>633 nm laser</td>
<td>MBS 488/561/633</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LSM 780</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFP</td>
<td>Argon ion laser at 488 nm</td>
<td>488 MBS, detection range 493-598 nm.</td>
<td>Plan-apochromat 63× 1.4 oil DIC</td>
<td>Zen 2012 software</td>
</tr>
</tbody>
</table>

MBS = main beam splitter
2.5.2 Immunofluorescence (IF)

Cells were seeded at a density of $1 \times 10^5$ / glass cover slip, 24 h before treatment. Following treatment, cells were rinsed three times with PBS and subsequently fixed for 15 min with 4% paraformaldehyde at room temperature, followed by three 10 min PBS washes. To reduce auto-fluorescence, 50 mM NH$_4$CL was added for 20 min, removed and the cells were blocked for 20 min (blocking buffer: 1% w/v BSA, 0.1% v/v Triton X-100, and 0.4% v/v Tween 20 in PBS). Cells were then incubated for 1 h with primary antibody (see table). Cells were washed three times in blocking buffer and then incubated for 30 min with the secondary antibody. All steps were carried out at room temperature and antibodies were diluted in blocking buffer according to table. Cover slips were mounted onto glass microscope slides with Dako Fluorescent Mounting Medium (Dako UK Ltd., UK). Precautions were taken throughout to ensure minimal exposure of samples to light.

Table 2.13 | Antibodies used for immunocytochemistry

<table>
<thead>
<tr>
<th>Reactive against</th>
<th>Species</th>
<th>Source</th>
<th>Reference</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-HIF-2α</td>
<td>Rabbit</td>
<td>Abcam, UK</td>
<td>ab20654</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-HIF-2α</td>
<td>Rabbit</td>
<td>Abcam, UK</td>
<td>ab179825</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-HIF-1α</td>
<td>Mouse</td>
<td>BD Biosciences, NJ, USA</td>
<td>610959</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-HIF-1β</td>
<td>Mouse</td>
<td>Novus Biologicals, CO, USA</td>
<td>NB100-124</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-RNAPII (phospho ser5)</td>
<td>Mouse</td>
<td>Abcam, UK</td>
<td>ab24759</td>
<td>1:50</td>
</tr>
<tr>
<td>Anti-SC35</td>
<td>Mouse</td>
<td>Abcam, UK</td>
<td>ab11826</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-Sart1</td>
<td>Mouse</td>
<td>Abcam, UK</td>
<td>ab88583</td>
<td>10 μg / mL</td>
</tr>
<tr>
<td><strong>Secondary</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-rabbit-AlexaFluor555</td>
<td>Goat</td>
<td>Invitrogen, CA, USA</td>
<td>A-21428</td>
<td>1:500 - 1:1000</td>
</tr>
<tr>
<td>Anti-mouse-AlexaFluor488</td>
<td>Goat</td>
<td>Invitrogen, CA, USA</td>
<td>A-11008</td>
<td>1:500 - 1:1000</td>
</tr>
<tr>
<td><strong>Nanobodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-GFP-Atto594</td>
<td>Alpaca</td>
<td>ChromoTek GmbH, Germany</td>
<td>gba594</td>
<td>1:200</td>
</tr>
</tbody>
</table>
2.5.3 Imaging Fixed samples

Fixed samples were imaged with a Plan-Fluar 100× / 1.30 oil immersion objective on a LSM 710 confocal microscope (Zeiss, Germany). Z-stacks were taken through the nucleus of single cells, with a distance of 0.5 μm between each slice. 1024x1024 pixel images were acquired using a 100x oil-immersion objective with a numerical aperture >1.40, zoom x2 and the pinhole set at 70 μm. Images were captured using the Zen 2010 software (Zeiss, Germany).

2.5.4 Live cell imaging

2.5.4.1 LSM 510

Twenty four hours before each experiment, 1.5 × 10⁵ cells were seeded in a 35 mm glass bottom dish (Greiner Bio One, UK). Cells were transiently transfected at the time of seeding. Imaging dishes were placed in a PeCon O₂ controller incubator (PeCon GmbH, Germany) mounted on the confocal microscope stage. Conditions were as described in Table 2.9.

2.5.4.2 Epifluorescent microscope

Imaging dishes were prepared as described above and mounted on the stage of an Axio Observer Z.1 Epifluorescent microscope (Zeiss, Germany) fitted with an incubation system. Conditions were as described in Table 2.9. Cells expressing EGFP-HIF-2α were imaged for 1000 frames with an Andor iXon 879 (16 μm pixels 512×512) camera (Andor, UK). To achieve high magnification a 63× objective and 2.5× optovar lens were used.

2.5.5 Analysis

2.5.5.1 Co-localisation analysis

Post-acquisition processing and co-localisation analysis was carried out using ImageJ (Schneider et al., 2012). The middle slice from each z-stack was analysed. The background was subtracted for both the red and green channel (pixel size of 5 for HIF-2α, 5 for RNAPII,
10 for HDAC5-YFP). Analysis was performed with an ImageJ plugin for co-localisation analysis (http://fiji.sc/Colocalization_Threshold).

### 2.5.5.2 Cell Tracker

For each time lapse the fluorescence intensities, for a number of cells, was determined using Cell Tracker version 0.6 software (software website: www.dbkgroup.org/celltracker/) (Shen et al., 2006). Using this software, EGFP positive cells were manually tracked by drawing a circle in the cell and adjusting it accordingly to ensure it remained within the cell boundaries through each frame. Cells were tracked from the start of imaging up to point of cell death or until the end of the experiment. For any cell division, the parent cell was tracked up to point of division and after division tracking continued following one of the daughter cells. Any cells that started outside of the frame or moved out of the frame during the time course were not tracked. All data was exported as the mean intensity of fluorescence.

### 2.5.5.3 Characterisation of EGFP-HIF-2α Speckles

Analysis was performed in Fiji (Schindelin et al., 2012) on calibrated image stacks (1000 frames). The image stack was duplicated: one was processed as follows. Background was subtracted using a 5 pixel filter and a mask applied (threshold type Li, dark background). To separate touching objects a watershed was applied and any holes were filled (using Fill Holes option). The masked images was analysed (size of pixel = 10-infinity) and measurements were redirected to the original (unprocessed) image. The results output gave the number, size, total area etc.

### 2.5.5.4 Analysis of EGFP-HIF-2α Speckle Dynamics

Image stacks (1000 frames) were analysed with the plugin Particle Tracker 2D/3D (Sbalzarini & Koumoutsakos, 2005; parameters using radius = 5, cut-off = 1, percent = 5, link = 1, displacement = 5) in ImageJ. The trajectory coordinates were exported to Excel (Microsoft Corporation, WA, USA). Analysis of trajectories of individual speckles was performed using a macro written by Dr D Mason (Image Analyst, Centre for Cell Imaging, University of Liverpool, UK). The velocity and slope of the moment scaling spectrum
(corresponds to diffusion mode) were calculated based on formulas in Ewers et al. (2005), Sbalzarini & Koumoutsakos (2005) and Scheweizer (2007).

2.5.6 Advanced Microscopy Techniques

2.5.6.1 Fluorescence Recovery After Photobleaching (FRAP)

FRAP was performed on an Axiovert 200M LSM510 (Zeiss, Germany) confocal microscope. Ten pre-bleach and 290 post-bleach images were acquired every 300 ms, using a 63× oil immersion objective. Bleaching of a region of interest (ROI) was performed with an Argon ion laser (488 nm) at 100% output power for 50 iterations for EGFP-HIF-2α. The pinhole was set at ~3 airy units. Data was captured by LSM510 version 3 software (Zeiss, Germany).

Figure 2.1 | Overview of FRAP EGFP-HIF-2α analysis. A) Example of the three regions acquired for analysis Region of interest (ROI) 1 = bleached region, ROI2 = whole fluorescent region / acquisition bleach control, ROI3 = background fluorescence. B) Schematic of the quantitative data obtained from the three regions in B plotted against time. C) Schematic of a normalised recovery curve and the different information that can be extrapolated. D) Example of final recovery curve after background subtraction and normalisation from a single bleached cell. Half time and mobile fraction calculated from the fitted curve. All performed in Matlab (see methods).
For each FRAP experiment, fluorescent intensities for the bleached region (ROI1), non-bleached region (acquisition control; ROI2) and background (ROI3) were extracted using the LSM510 software (Figure 2.1). ROI1 and ROI2 were background subtracted (average) and normalised to their respective pre-bleach values (average). The resulting ROI1 values were normalised to the ROI2 values (ROI1 / ROI2). This ratio was then corrected for any non-specific bleaching during acquisition using the following equation:

\[
\frac{(R_t - R_p)}{(1 - R_p)}
\]

Where \( R_t \) is the bleach value at a given time point and \( R_p \) is the first post-bleach value. These values were plotted against time and the curves were fitted using the following equation:

\[
(a - b) \times e^{-cx} + b
\]

Where \( a \) = Value of Y intercept, \( b \) = Value of Y at infinity, \( c \) = rate constant for graph.

Both the normalisation and curve fitting was performed in Matlab (Mathworks, UK) using a code written by SiSeet Chan (Honours project student, Violaine See Group, University of Liverpool, UK). Using the curves the mobile fraction and half time for each FRAP experiment was calculated.

Due to the rapid recovery exhibited by HIF-1α-EGFP, the experimental set-up was altered slightly whereby a strip across the nucleus was bleached and was performed at 100% output power for 50 iterations.

### 2.5.6.2 Fluorescence Loss in Photobleaching (FLIP)

FLIP was performed on a LSM780 (Zeiss, Germany) confocal microscope. Following the acquisition of 10 images, cells expressing EGFP-HIF-2α were bleached within a region of interest with an Argon ion laser (488 nm) for 100 iterations at 100% output power. Twenty post-bleach images were captured before bleaching was repeated. Cycles of imaging and bleaching were carried out until fluorescence in the non-bleached region was lost. For acquisition of images, Images were captured by Zen 2012 software (Zeiss, Germany).
For each FLIP experiment, the fluorescent intensities for the bleached region (ROI1), non-bleached region (ROI2) and background (ROI3) were extracted using Fiji. The average of the background fluorescence over the course of the time-lapse was subtracted from each ROI2 value. These background corrected values were then normalised to average of the pre-bleach fluorescence values. This data was plotted against time. The half time for each experiment was calculated by fitting each FLIP curves with a one component exponential decay curve in Matlab.

### 2.6 Bulk cell analysis

#### 2.6.1 Western Blot

Cells were plated at a density of $4 \times 10^5$ – $5 \times 10^5$ per 60 mm culture dish (Corning, NY, USA) 24 h before the experiment. Following the respective treatment, samples were washed with PBS and 350 µL lysis buffer (50 mM Tris-HCl pH 7.5, EDTA 1 mM, EGTA 1mM, Na$_3$VO$_4$ 1 mM, 1% v/v Triton X-100, 50 mM Sodium Fluoride, 5 mM Sodium pyrophosphate, 19 mM sodium B-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µl / mL Phosphatase Inhibitor Cocktail) added. Cells were detached with a cell scraper and collected in 1.5 mL eppendorf tubes. Samples were rotated for 45 min at 4 °C and then centrifuged at 14000 g (≈10,000 rpm) for 15 min at 4 °C and the supernatant was collected. The protein concentration of the supernatant was measured using the BCA Protein Assay Kit (Thermo Scientific Pierce) according to manufacturer’s instruction. Protein samples were mixed in a 1:1 ratio with Laemmli (2x) buffer (25% v/v 0.5 M Tris base pH 6.8, 20% v/v glycerol, 10% w/v SDS, 5% v/v β-mercaptoethanol and 0.1% w/v bromophenol blue) and boiled for 5 min prior to loading. Samples were resolved on 10% SDS-PAGE resolving gel (0.4 M Tris base pH 8.8, 0.1% w/v SDS, 10% acrylamide, 0.25 ng/µl APS and 0.125% v/v Temed) and 4% stacking gels (0.4 M Tris base pH 6.8, 4% acrylamide, 0.65 ng/µl APS and 0.325% v/v Temed). 30 µg of protein was loaded per well and 8 µL of protein ladder (Precision Plus Protein All Blue Standards; Biorad, CA, USA) was loaded in one well. Gels were run at 130 V in running buffer 1x (25 mM Tris, 192 mM glycine and 3.4 mM SDS) until adequate separation achieved. Protein was transferred for 1.5 h (4 °C, 300 mA) onto a nitrocellulose membrane (Biorad, CA, USA) in transfer buffer (20% v/v ethanol, 25 mM Tris, 192 mM glycine). Membranes were blocked with 5% w/v milk (in TBST; 0.2 M Tris pH 7.6, 0.14 M NaCl, 0.1% v/v Tween 20) for 1 h and incubated with primary antibody (prepared in
5 % w/v BSA in TBST) for 16 h at 4 °C. Following three TBST washes, membranes were incubated with the secondary antibody (prepared in 5% w/v skimmed milk powder in TBST) for 1 h. Protein bands were visualised with Amersham ECL western blotting detection reagent (GE Healthcare, UK) and membranes imaged on the Syngene G-box (Geneflow, UK).

Table 2.14 | Antibodies used for immunoblotting.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Source</th>
<th>Reference</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>Mouse</td>
<td>BD Biosciences, NJ, USA</td>
<td>610959</td>
<td>1:1000</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>Rabbit</td>
<td>Abcam, UK</td>
<td>ab20654</td>
<td>1:1000</td>
</tr>
<tr>
<td>PHD3</td>
<td>Rabbit</td>
<td>Novus Biologicals, CO, USA</td>
<td>NB100-139</td>
<td>1:1000</td>
</tr>
<tr>
<td>GFP</td>
<td>Rabbit</td>
<td>Abcam, UK</td>
<td>ab290</td>
<td>1:1000</td>
</tr>
<tr>
<td>Beta-Actin</td>
<td>Mouse</td>
<td>Abcam, UK</td>
<td>ab8226</td>
<td>1:1000</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>Rabbit</td>
<td>Abcam, UK</td>
<td>ab3563</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-mouse HRP conjugated</td>
<td>Horse</td>
<td>Cell Signalling, MA, USA</td>
<td>7073S</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-rabbit HRP conjugated</td>
<td>Goat</td>
<td>Cell Signalling, MA, USA</td>
<td>7074S</td>
<td>1:3000</td>
</tr>
</tbody>
</table>

2.6.2 Real-time qPCR (RT-qPCR)

Cells were plated 24 hours before treatment at a density of 4-5 x 10⁶ cells in 60 mm culture dishes. Following treatment cells were rinsed with PBS and lysed with 350 µL of RLT buffer (provided in the RNeasy kit; QIAGEN, Netherlands) with 1% v/v β-mercaptoethanol, cells were scraped and collected in eppendorf tubes and stored at -80 °C. When all samples were collected, the lysates were homogenised using QIAshredder mini spin columns (QIAGEN, Netherlands). RNA extraction was carried out according to manufacturer’s instruction. RNA was eluted in 30 µL RNase free water and concentration was measured using a spectrophotometer (Nanodrop 2000; Thermo Scientific, MA, USA).1 µg of extracted RNA was converted to cDNA using SuperScript® VILO cDNA Synthesis Kit (Invitrogen, CA, USA) according to manufacturer’s instruction. RT-qPCR reactions were ran in triplicates and carried out in a 96 well plate. Each reaction consisted of 18 µL master mix (10 µL 1x SYBR Green PCR master mix, 1 µL 5 µM forward primer, 1 µL 5 µM reverse primer, 6 µL RNase
free H\textsubscript{2}O) and 2 µL cDNA sample. Temperature cycling was performed using a LightCycler® 480 (Roche, UK) using the parameters stated in Table 2.15. Results were analysed using the LightCycler® 480 software (version 1.5.0.39; Roche, UK). Target genes were normalised to the house-keeping gene cyclophillin A, with control sample used as the calibrator.

**Table 2.15 | Cycle parameters for RT-qPCR**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time</th>
<th>Ramp Rate (°C/s)</th>
<th>No. Of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Incubation</td>
<td>95 °C</td>
<td>5 min</td>
<td>4.4</td>
<td>1</td>
</tr>
<tr>
<td>Amplification</td>
<td>95 °C</td>
<td>10 s</td>
<td>4.4</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>60 °C</td>
<td>30 s</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Melt Curve</td>
<td>95 °C</td>
<td>5 s</td>
<td>4.4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>65 °C</td>
<td>61 s</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>97 °C</td>
<td>Continuous</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Cooling</td>
<td>40 °C</td>
<td>10 s</td>
<td>1.5</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 2.16 | Primers used for RT-qPCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophillin A (forward)</td>
<td>gctttgggtccaggaatgg</td>
</tr>
<tr>
<td>Cyclophillin A (reverse)</td>
<td>gttgtccacagtcaatggt</td>
</tr>
<tr>
<td>PHD2 (forward)</td>
<td>tgcagatgagagacacg</td>
</tr>
<tr>
<td>PHD2 (reverse)</td>
<td>ttagcgaaccaatctgaagg</td>
</tr>
<tr>
<td>PHD3 (forward)</td>
<td>agatcgtgtagaaccacacg</td>
</tr>
<tr>
<td>PHD3 (reverse)</td>
<td>ttctgcctctctcagcat</td>
</tr>
<tr>
<td>VEGF (forward)</td>
<td>ggcagaatcatcacaagact</td>
</tr>
<tr>
<td>VEGF (reverse)</td>
<td>cacacaggatgcttgaga</td>
</tr>
</tbody>
</table>
2.7 Recombinant protein and nanoparticle work

2.7.1 Bacterial transformation for recombinant protein expression

Fifty microlitres of C41 (DE3) E. coli, were thawed on ice and 5 ng of Halotag-FGF2 were added. The cells were incubated on ice for 20 min, heat shocked at 42 °C for 1 min and returned to ice for 2 min. 950 μL pre-warmed SOC medium was added and the cells incubated for 1 h at 37 °C in an orbital shaker (225 rpm). Following this, the cells were centrifuged at 8000 g for 1 min, the supernatant removed and the pellet re-suspended in 200 μL of pre-warmed SOC medium. 20-50 μL transformed cells were spread on selective LB agar plates (for antibiotics used see Table 2.1, note: BL21.pLysS also required 50 μg mL⁻¹ Chloramphenicol) and incubated at 37 °C overnight. See Table 2.17 for the variations in the transformation protocol for each strain of E. coli used.

Table 2.17 | Details of the variations in protocols for transforming pET-M11-Halotag-HIF-2α into different strains of competent cells

<table>
<thead>
<tr>
<th>Strain of E. coli</th>
<th>Mass of DNA</th>
<th>Time on ice</th>
<th>Heat shock</th>
<th>Return to ice</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21(DE3)</td>
<td>5 ng</td>
<td>30 min</td>
<td>10 sec at 42 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>C41 (DE3)</td>
<td>5 ng</td>
<td>30 min</td>
<td>45 sec at 42 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>BL21(DE3).pLysS</td>
<td>10 ng</td>
<td>10 min</td>
<td>45 sec at 42 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>Rosetta(DE3)</td>
<td>5 ng</td>
<td>5 min</td>
<td>30 sec at 42 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>Lemo21</td>
<td>5 ng</td>
<td>30 min</td>
<td>10 sec at 42 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>SoluBL21</td>
<td>5 ng</td>
<td>15 min</td>
<td>45 sec at 42 °C</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.18 | Antibiotics used for recombinant protein expression

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Antibiotic</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET-14b-Halotag-FGF2</td>
<td>Ampicillin</td>
<td>As in Table 2.1</td>
</tr>
<tr>
<td>pETM-11-Halotag-HIF-2α</td>
<td>Kanamycin</td>
<td></td>
</tr>
</tbody>
</table>
2.7.2 Assessing recombinant protein expression

Single colonies of transformed bacteria were picked using aseptic techniques and added to 10 mL LB broth (Merck KGaA, Germany; prepared according to manufacturer’s instructions) containing respective antibiotic. The 10 mL cultures were incubated at 37 °C in an orbital shaker (240 rpm). When the OD$_{600}$ (optical density / absorbance measured at a wavelength of 600 nm) reached 0.6-0.8 protein expression was induced by the addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG; Bioline, MA, USA). Cultures were then incubated at different temperatures for varying times in an orbital shaker (240 rpm). From this point all samples were kept on ice. Cells were pelleted via centrifugation at 4 °C, 8000 x g for 30 min and the supernatant discarded. The pellet was re-suspended in PBS and the centrifugation step repeated. The supernatant was discarded and samples were taken for analysis via SDS PAGE.

2.7.3 Assessing recombinant protein solubility

10 mL culture was inoculated with a single colony and incubated overnight at 37 °C, 225 rpm. 5 mL of the overnight culture was transferred to 200 mL LB broth (100 µg mL$^{-1}$ Ampicillin) and incubated at 37 °C, 225 rpm. When the OD$_{600}$ reached 0.6-0.8, 1 mM IPTG added and incubated for a further 4 h. Bacteria was pelleted via centrifugation at 8000 rpm, 16 min, 4 °C (RC6+ Centrifuge). Pellets re-suspended in 20 mL PBS and centrifuged again.

Cells were lysed via sonication. Each pellet was thawed on ice, resuspended in 30 mL resuspension buffer (30 mM Tris, 0.6 mM EDTA, 0.5 M NaCl, 3 mM MgCl$_2$, 6% v/v Glycerol) and transferred to a pre-chilled 50 mL beaker. The cells were sonicated for 30 s (DAWE Ultrasonic Generator 7533A, pulsed sonication, 40-50% duty of cycles, output 10) followed by 60 s on ice. This was repeated five times. The sample was transferred to a 50 mL tube and centrifuged at 18000 rev / min for 30 min at 4 °C. The supernatant (soluble fraction) was transferred to a new tube and samples from the pellet and supernatant were analysed via SDS-PAGE and coomassie staining.

2.7.4 Purification of Recombinant protein

Preliminary purification described in Chapter 5. Final purification protocol as described in Sun et al. (2015).
2.7.5 Labelling bacterial cell lysates with Fluorescent Halotag Ligand for SDS PAGE

Purified protein: 10 µL of protein was incubated with 2 µL 50 µM Oregon Green Halotag Fluorescent Ligand for 15 min at room temperature. 4 x loading buffer was added and samples heated to 80 °C for 2 min. 15 µL of sample was analysed via SDS PAGE.

Bacterial lysate: Bacterial cell pellets were re-suspended in 1 mL 50 mM Hepes buffer (Life Technologies, CA, USA) and 1 mL lysis buffer (1x Fast Break Cell lysis reagent (Promega, WI, USA) diluted in PBS, 2 mg mL⁻¹ Lysozyme (Sigma Aldrich, MO, USA), 0.02 units RQ1 RNase Free DNase (Promega, WI, USA)). Samples were rotated for 30 mins at 4 °C, followed by centrifugation for 30 min. 100 µL of supernatant, 45 µL 50 mM Hepes buffer and 5 µL Oregon Green Halotag ligand (50 µM) were incubated for 20 min at room temperature in a 1.5 mL Eppendorf tube. Appropriate volumes of each sample were mixed with 5x loading buffer (250 mM TrisHCl pH6.8, 10% SDS, 30% Glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue) and heated at 80 °C for 2 min prior to analysis via SDS-PAGE with coomassie staining and by western blot.

Mammalian cell lysate: 20 µL of protein (whole cell lysate) was incubated with 4 µL Oregon Green Halotag ligand (50 µM) for 20 min at room temperature. 6 µL 5x loading buffer was added, and each sample heated to 80 °C for 2 min. Samples were analysed by SDS PAGE and western blot.

2.7.6 Fluorescent labelling of Halotag-Fusion protein for microscopy

Purified protein: Two microlitres of recombinant protein (30 µM) was mixed with 3 µL TMR Halotag Ligand (50 µM, diluted in DMSO) and made up to a total reaction volume of 20 µL with PBS. The reaction was allowed to proceed at room temperature for 30 minutes. The labelled protein was passed through a Heparin column. To remove any unbound ligand the column was washed with PBS and the TMR-Halotag-FGF2 was eluted with 2x 30 µL 1.5 M NaCl. The eluted fraction was diluted to 0.8 µM. Rama-27 cells seeded and fixed in 35 mm glass bottom microscope dishes (Greiner Bio-One, UK) were provided by Daniel Nieves (Prof. D. G. Fernig’s group, University of Liverpool). The cells were incubated for 10 minutes...
with Ringer BSA (10 mg / mL) buffer (stock Ringer solution: 10 mM Hepes pH 7.4, 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 11 mM Glucose. Diluted 1:1 with 20 mg / mL BSA prepared in PBS). The cells were incubated for 1 hour in 200 µL incubation buffer (TMR-Halotag-FGF2 diluted in Ringer and added in ratio of 9:1 to PBS supplemented with 10 mg / mL BSA to give final protein concentration of 2 nM). This was followed by 3x 1 mL Ringer BSA (10 mg / mL) washes.

Cells ectopically expressing Halotag-Fusion: HeLa cells were seeded in 35 mm glass bottom imaging dishes (Greiner Bio One, UK) and transiently transfected with the CMV-Halotag-HIF-2α construct as described earlier. Cells were labelled with the Oregon Green or TMR Fluorescent Halotag ligand following Promega’s guidelines for live cell imaging (rapid protocol). The cells were directly observed on a LSM 710 confocal microscope (For details see microscopy).

**2.7.7 in vitro Protein Expression**

Protein synthesis was carried out using the PURExpress® In Vitro Protein Synthesis kit (NEB, MA, USA) following the manufacturers guidelines. 250 ng of pET-M11-Halotag-HIF-2α was used per 25 µL reaction. 5 µL of protein was labelled with various concentrations (as stated in Figure x) of Oregon Green Halotag ligand for 30 minutes at room temperature. Samples were analysed via SDS PAGE and western blot.

**2.7.8 Expression of recombinant protein in Mammalian Cells**

HeLa cells: 5 x 10⁵ HeLa cells were seeded in 6 cm dishes in a total of 4 mL medium and were transiently transfected with CMV-Halotag-HIF-2α using FuGene®6 (as described in section X). 24 h post transfection, cells were incubated in normoxia or hypoxia or treated with 0.5 mM DMOG for 8 h. Cells were lysed with 150 µL western blot lysis buffer (see Western Blot). Samples were labelled with Halotag Oregon Green ligand (Promega, WI, USA) following the manufacturer’s guidelines.

HEK 293TN: cells were seeded in 10 cm dishes at a density of 1 x 10⁷ (total) in 10 mL medium. The CMV-Halotag-HIF-2α construct was transiently transfected following the protocol in section xx. 48 h post transfection the cells were harvested. The medium was carefully aspirated away and cells washed gently with PBS. 500 µL lysis buffer (50 mM Tris
HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1x Protease Inhibitor Cocktail (G6521; Promega, WI, USA) was used per 10 cm dish. A sterile cell scraper was used to detach the cells, which were collected in pre-chilled 1.5 mL Eppendorf tubes. All following procedures were done on ice or at 4 °C. The samples were incubator on a rotator for 1 h to aid cell lysis and then centrifuged for 1 h, 13000 rpm (Eppendorf MiniSpin). The supernatant was transferred to pre-chilled Eppendorf tubes. Total protein concentration was determined using a BCA assay (as described in Section 2.6.1)

2.7.9 Gold Nanoparticles

2.7.9.1 Synthesis and Functionalisation of Gold Nanoparticles

Gold nanoparticle synthesis and functionalisation was carried out in collaboration with Dr U Shaheen (Dr R Levy’s group, University of Liverpool). Nanoparticles with a gold core 10 nm in diameter and a ligand shell comprised of polyethylene glycol (PEG) only or PEG and a custom made Halotag ligand (Promega, WI, USA) were synthesised. Briefly, capped nanoparticles were made by mixing colloidal gold (Sigma Aldrich, MO, USA) with the ligand(s) in a ratio of 9:1, respectively (Duchesne et al., 2012). To make 1 mL of 1% Halotag ligand (HL) GNPs 2 μL of Halotag Ligand (1 mM) and 98 μL PEG (2 mM) was used. The ligands were added sequentially, the HL ligand first followed by the PEG. In between each step the nanoparticles were vortexed gently for 1 min. The nanoparticles were incubated at room temperature for 16 h on a rotator. Excess ligands were removed by washing with (PBS with 0.2% v/v Tween20), nanoparticles were centrifuged (13200 rpm for 1.5 h) and resuspended in PBST four times.

2.7.9.2 Gold nanoparticle conjugation

Nanoparticle (70 nM; PEG-GNPs or 1% HL-GNPs) were incubated with 750 μg protein (HaloTag-HIF2α or control HEK-293TN lysate) made up to a final volume of 100 μL with PBST. Protein degradation was prevented by inclusion of 1x EDTA-free protease inhibitor (Promega, WI, USA). Additional control reactions were set up with nanoparticles or lysate only. The nanoparticles were mixed with lysates (Halotag-HIF-2α) and for 18 h at 4 °C with mixing to allow conjugation to occur. To remove unbound protein, the nanoparticles were pelleted via centrifugation (13200 rpm at 4°C for 1 h) and the supernatant removed. The nanoparticles were then resuspended in 500 μL PBST. This was repeated four times and after each wash a small volume was taken for analysis by western blot or dot blot.
2.7.9.3 UV-visible spectroscopy

To monitor loss of nanoparticles following each step (of functionalisation or conjugation), samples were taken so the absorption spectrum could be measured. Measurements were made at room temperature using a Spectra Max Plus spectrophotometer (Molecular Devices, Wokingham, UK) between 450–700 nm.

2.7.9.4 Stripping Gold Nanoparticles

After washing, nanoparticles (PEG-GNPs or 1 % HL-GNPs) incubated with the various lysates were incubated in stripping buffer (60 mM TrisHCl, 10 % Glycerol, 50 mM DTT, 2 % SDS, 0.01 % Bromophenol Blue) with mixing for 36 h and then centrifuged (13200 rpm at 4 °C for 1 h) to remove the stripped gold nanoparticle cores. The supernatant was then boiled for 5 min prior to analysis via western blot.

2.7.9.5 Dot Blot

Four microlitres of nanoparticles was added to nitrocellulose membrane and allowed to dry. The dot blots were blocked for 1 h in 5 % milk (in TBST) and incubated overnight with primary antibody (prepared in 5 % BSA in TBST) at 4 °C. Following three TBST washes, membranes were incubated with the secondary antibody (prepared in 5 % milk powder) for 1 h. Protein was visualised with Amersham ECL Select western blotting detection reagent (GE Healthcare UK, UK) and membranes developed on a Syngene G-Box (Geneflow, UK).

2.7.9.6 Microinjection

Cells were plated sparsely (1.5 x 10^5 / mL, 2 mL total) in 35 mm gridded dishes (Grid500; Ibidi GmbH, Germany) 24 h prior to injection. Were required, transfection was carried out concurrently.

Microinjection was performed on a Zeiss Epifluorescent Axio Observer Z.1 (Zeiss, Germany) fitted with an incubation system that maintains humidity and allows control of temperature, CO₂ and O₂ levels. The microinjection system was set up in a manner that allows injection to occur whilst the imaging dish is mounted on the microscope stage. Microinjection was performed by Dr J Wnetrzak (Technician, Centre for Cell Imaging, University of Liverpool). Cells were injected into nucleus via microcapillaries (Eppendorf, Germany) with the injection time of 1 s, injection pressure of 150 KPa and compensation pressure of 30 kPa using the Eppendorf FemtoJet® Microinjector (Eppendorf, Germany).
Chapter 3: Investigating the temporal dynamics of HIF-α at the single cell level
This chapter starts with a brief introduction to single cell imaging and its advantages over bulk-cell analysis techniques. It is followed by the presentation of a paper published in January 2014 and then additional work that I conducted relating to this study.

3.1 Investigating Protein Dynamics

Live cell imaging of fluorescently labelled proteins allows the quantification of protein abundance over time as well as allowing the visualisation of intracellular localisation. Studies using live cell imaging have revealed that temporal and spatial dynamics play a role in regulating the activity of key signalling molecules. For example, Lahav et al. (2004) were the first to observe the pulsatile nuclear accumulation of p53 and showed that the frequency of pulses positively correlated with the amount of DNA damage. Continually, NFκB has been shown to transiently translocate from the cytoplasm into the nucleus upon stimulation with TNFα (Nelson et al., 2004). This group also determined that the timing of the nuclear-cytoplasmic shuttling directly affected gene expression, highlighting that temporal and spatial dynamics of transcription factors has a role in regulating their activity (Ashall et al., 2009).

The aforementioned discoveries were made by utilising live cell imaging, which has numerous advantages over other methods used for protein analysis. For example, with western blot analysis any spatial information such as sub-cellular localisation is lost and so spatial kinetics such as nuclear-cytoplasmic oscillations (such as those observed for NFκB) are missed. Also, heterogeneity within cell populations can be obscured when using bulk cell techniques such as western blot and quantitative PCR, due to “averaging effects” (Spiller et al., 2010). Observations at the single cell level have highlighted that the timing of cellular responses are not always synchronised (Nelson et al., 2004; Bagnall et al., 2014) and this cell to cell variation can only be detected by observing single cells in real-time.

Our hypothesis was that HIF-1/2α might have complex/oscillatory dynamics due to their regulation by negative feedback loops (Epstein et al., 2001; Stiehl et al., 2006). Our aim was to utilised single cell imaging techniques to capture the intracellular dynamics of the HIF-α subunits to determine which components are involved in this auto-regulatory mechanism.
3.2 Tight Control of HIF-α Transient Dynamics Is Essential for Cell Survival in Hypoxia

As previously mentioned, live cell imaging has revealed that temporal and spatial dynamics play a key role in the regulation of the activity of transcription factors such as p53 and NFκB (Lahav et al., 2004; Nelson et al., 2004). At the start of my PhD project, I had the opportunity to participate towards an on-going project (initiated by Dr James Bagnall, former PhD student in the laboratory) utilising live cell imaging to study the dynamics of HIF-1α/-2α at the single cell level. Our findings were published in an article entitled “Tight Control of Hypoxia-inducible Factor-α Transient Dynamics Is Essential for Cell Survival in Hypoxia” in the Journal of Biological Chemistry (Bagnall et al., 2014).

We found that in hypoxia HIF-1α accumulates transiently in the nucleus after approximately 4 hours with a peak in protein levels lasting 2-4 hours. In order to validate these data obtained from HeLa cells ectopically expressing HIF-1α-EGFP, a stable cell line was created. The ODD-EGFP HeLa were produced via lenti-viral transduction of a construct containing a fragment that spans 529aa to 652aa of the HIF-1α protein, i.e. part of the oxygen-dependent degradation (ODD) domain containing the second hydroxylation site (p564), fused with EGFP. Live cell imaging of this cell line revealed a similar transient accumulation of the fusion protein in hypoxia, suggesting that the transient accumulation of HIF-1α is not an artefact of ectopic expression.

The oscillatory behaviour observed is indicative of a negative feedback loop and led to the hypothesis that there is an auto-regulatory mechanism controlling the levels of HIF-1α in hypoxia. Mathematical modelling (performed by Joseph Leedale; Mathematics Department, University of Liverpool) based on gene expression data from qPCR and live cell imaging of PHD2 and PHD3 led to the proposal that PHD2 was the principal component of the negative feedback loop. Simulations based on these mathematical models suggested that the transient increase of HIF-1α protein levels would be lost if PHD2 was knocked out and knocking out PHD3 would have little effect. Live cell imaging of shPHD2 and shPHD3 HeLa cell lines ectopically expressing HIF-1α-EGFP confirmed these predictions.

Moreover, in the shPHD2 cells, we observed that levels of HIF-1α continued to accumulate and eventually lead to the cells undergoing apoptosis, suggesting that prolonged HIF activity in hypoxia triggers cell death. To complement this, it was demonstrated that gene
expression relating to apoptosis (PUMA and NOXA) was induced in these cells in hypoxia. This led to the proposal that the PHD2-negative feedback mechanism is in place to protect cells by tightly controlling levels of HIF in hypoxia.

My specific contributions to this paper:

- Single cell imaging and analysis of ODD-EGFP HeLa cells (Figure 1I)
- Analysis of PHD2 and PHD3 gene expression in the ODD-EGFP via qPCR (Figure 1J)
- Creating the shPHD3 HeLa cell line via lenti-transduction of shRNA (Figure 6H)
- Single cell imaging and analysis of shPHD2 and shPHD3 HeLa cell lines transiently expressing HIF-1α-EGFP (Figure 6E and 6I, respectively)
- qPCR experiments looking at the upregulation of VEGF mRNA and genes relating to apoptosis (PUMA and NOXA) (Figure 6E, F and G, respectively)
Author contributions:

James Bagnall

- Conceived and designed the experiments
- Performed the experiments
- Analysed the data
- Wrote the paper
- Prepared figures / tables
- Reviewed drafts of the paper

Joseph Leedale

- Performed mathematical modelling
- Analysed data
- Wrote the paper
- Prepared figures / tables
- Reviewed drafts of the paper

Sarah E. Taylor

- Performed the experiments (Details above)
- Analysed the data
- Reviewed drafts of the paper

David G. Spiller

- Reviewed drafts of the paper

Michael R. H. White

- Reviewed drafts of the paper

Kieran J. Sharkey

- Reviewed drafts of the paper

Rachel N. Bearon

- Wrote the paper
- Prepared figures / tables
- Reviewed drafts of the paper

Violaine Sée

- Conceived and designed the experiments
- Wrote the paper
- Prepared figures / tables
- Reviewed drafts of the paper
Tight Control of Hypoxia-inducible Factor-α Transient Dynamics Is Essential for Cell Survival in Hypoxia

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Background: Hypoxia-inducible factor-α (HIF-α) is the main transcription factor activated in low oxygen conditions.

Results: Single cell imaging reveals pulses in nuclear levels of HIF-α.

Conclusion: The transient nature of the HIF-α nuclear accumulation is required to avoid cell death.

Significance: The duration of HIF-α response depends on cellular oxygenation, and can encode information and dictate cell fate.

Intracellular signaling involving hypoxia-inducible factor (HIF) controls the adaptive responses to hypoxia. There is a growing body of evidence demonstrating that intracellular signals encode temporal information. Thus, the dynamics of protein levels, as well as protein quantity and/or localization, impacts on cell fate. We hypothesized that such transient encoding has a role in HIF signaling and cell fate decisions triggered by hypoxic conditions. Using live cell imaging in a controlled oxygen environment, we observed transient 3-h pulses of HIF-1α and HIF-2α expression under continuous hypoxia. We postulated that the well-described proline hydroxylase (PHD) oxygen sensors and HIF negative feedback regulators could be the origin of the pulsatile HIF dynamics. We used iterative mathematical modeling and experimental analysis to scrutinize which parameter of the PHD feedback could control HIF timing and we probed for the functional redundancy between the three main PHD proteins. We identified PHD2 as the main PHD responsible for HIF peak duration. We then demonstrated that this has important consequences, because the transient nature of the HIF pulse prevents cell death by avoiding transcription of p53-dependent pro-apoptotic genes. We have further shown the importance of considering HIF dynamics for coupling mathematical models by using a described HIF-p53 mathematical model. Our results indicate that the tight control of HIF transient dynamics has important functional consequences on the cross-talk with key signaling pathways controlling cell survival, which is likely to impact on HIF targeting strategies for hypoxia-associated diseases such as cancer progression and ischemia.

In physiological and/or pathological situations where oxygen homeostasis is lost, the oxygen concentration drops and cells experience hypoxia. The cellular adaptation to hypoxia is mediated at the molecular level by the evolutionary conserved transcription factor hypoxia-inducible factor (HIF).2 HIF is a heterodimer composed of α and β subunits. The β subunit is constitutively expressed, whereas the main α subunits of HIF, HIF1α and HIF2α, are regulated in an oxygen-dependent manner. Under normoxic conditions, HIFs are hydroxylated, which promotes its binding to the ubiquitin ligase von Hippel-Lindau protein, thereby targeting it for proteasomal destruction (1). However, under hypoxic conditions, HIF-1 and -2 proteins dehydroxylation decreases, leading to their rapid accumulation. They then activate the transcription of hundreds of genes encoding proteins involved in cell survival and energy metabolism, but paradoxically also ones involved in apoptosis and autophagy (2). This double-edged sword function of HIF in promoting different cell fates has previously been described and depends on the physiopathological context and differential binding to key partners such as p53 (3). However, the switch from a pro-survival to a pro-apoptotic signal is not well understood. This is an important problem with implications beyond basic biology, because it has direct impact on the management of treatments for solid hypoxic tumors.

We postulated that the temporal regulation of HIF might explain its ability to determine two opposite cell fates. The HIF-VEH and p53-mdm2 signaling systems have previously been shown to share similar network structures in terms of degradation/transactivation loops (4). Furthermore, it has previously been demonstrated, using single cell imaging, that the dynamic behavior of p53 varies, depending on the stimulus, which can

The abbreviations used are: HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase; PI, propidium iodide; DOP, enhanced green fluorescent protein; MRE, hypoxia response element; ODO, oxygen-dependent domain; qPCR, quantitative PCR.
HIF-α Dynamics and Mathematical Modeling

influence cell fate decision (5–6). One important component that can lead to pulsatile or oscillatory behavior is the presence of a negative feedback motif. In the HIF system, prolyl hydroxylase (PHD) -2 and -3 have been proposed as potential delayed negative feedback proteins (7–9). Indeed, PHD2 and -3 are responsible for HIF hydroxylation, leading to its subsequent degradation (10). Their activity decreases in hypoxia, resulting in HIF accumulation that can in turn activate the transcription of PHD2 and -3. The subsequent PHD increase can compensate for the decrease of activity in prolonged hypoxia and could potentially bring HIF back to low and undetectable levels (7).

We therefore hypothesized that the potential HIF pulse-oscillatory dynamics due to PHD negative feedback could underlie the different cell fate outcomes that have been observed to result from hypoxia. To test this, we have applied a combination of single-cell imaging and mathematical modeling. We measured HIF levels in single cells with a high temporal resolution over 20 h. We observed discrete single and repetitive transient pulses of HIF-1α and -2α accumulation when cells were exposed to a hypoxic environment (1% O2). We developed a new mathematical model of the HIF-PHD negative feedback loop, which was able to accurately reproduce the single-cell dynamic data, both during a switch from normoxia to hypoxia and during re-oxygenation. We then used the model and experimentation to address the role of the individual PHDs in the generation of HIF dynamics. This demonstrated an essential role for PHD2 in the control of the transient dynamics of HIF and in the prevention of cell death triggered by long lasting HIF-1α levels. Changes in HIF dynamics and levels will likely affect directly HIF transcriptional activity, as well as the activity of its binding partners, for example, mdm2 (11). Indeed, the effects of PHD2 silencing on cell death were correlated with a strong transcription of p53-dependent pro-apoptotic genes in hypoxia. Moreover, coupling of HIF and p53 mathematical models (12) predicted significant effects of the variations of HIF dynamics on the oscillations and levels of the p53 protein, indicating that HIF dynamics not only affects HIF signaling but also its cross-talk with other essential signaling systems involved in the control of cell fate.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

Tissue culture medium was from Invitrogen; fetal calf serum (FCS) from Harlan SerumLab (UK); and pharmacological inhibitor dimethylxaloyglycine from Alexius Biochemicals (Enzolide Sciences, New York). Cycloheximide was from Calbiochem (Merck, Darmstadt, Germany). The antibody against HIF-1α was from BD Biosciences (catalog number 61-0991) and the antibody against HIF-2α was from Santa Cruz Biotechnology (catalog number Sc 13 596). EGFP antibody was from Abcam (catalog number ab296).

Cell Culture and Hypoxia

HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS (v/v) and 1% nonessential amino acids (v/v), at 37°C, 5% CO2. Cells (between passages 8 and 20) were plated at 1 × 105 cells/ml shP HD2 HeLa cells (generous gift from D. Hoogewijk, D. Stiehl, and R. Wengen, University of Zurich, Switzerland) were grown in the same medium as WT HeLa supplemented with 10 μg/ml of penicillin for maintaining the hairpin expression. The C51 colon adenocarcinoma cells pIRESVL (generous gift from S. Lehmann and R. Wengen, University of Zurich, Switzerland) have a stably integrated plasmid containing a minimal SV40 promoter regulated by 3 HRFXs from the human transferrin promoter. There were grown in DMEM high glucose, 10% FCS (v/v), and 400 μg/ml of G418. The pIRES-EGFP Hela cell line was generated by transduction of a HIV-ODD-EGFP.ires d’Tomato lentivirus. For imaging experiments, cells were plated in 35-mm glass bottom dishes (iEssential, Bibby Sterilin, UK). Hypoxic incubation was performed either directly onto the microscope stage equipped with a PeCon incubator with an O2 controller unit or in a hypoxic work station (Don Whitley Scientific, England) for bulk cell experiments (18% O2, 5% CO2, 86% N2).

Immunoblotting

Total protein was extracted with a lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 50 mM NaF 30, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 0.1 mM PMSF, 1/100 protease inhibitor mixture, and 1/100 phosphatase inhibitor mixture). After 1 h at 4°C on a rotating wheel shaker, the lysates were centrifuged at 10,000 × g for 15 min at 4°C and total protein concentration was measured with BCA assay in the supernatant. 40 μg of proteins were resolved by SDS-PAGE (10% gels) and transferred onto nitrocellulose membrane. The membranes were blocked with 5% nwater dry milk in TBST (10 mM Tris-HCL pH 8, 100 mM NaCl, 1% (v/v) Tween 20) and incubated with appropriate primary antibody (overnight, 4°C), followed by incubation with horseradish peroxidase-conjugated secondary antibody (1 h at RT). SuperSignal West Dura Extended Duration Chemiluminescent Substrate was used for the ECL reaction and the signal was detected and quantified using the Gbox gel doc system (Syngene, UK).

Quantitative RT-PCR (qPCR) and Primers

Cellular RNA was purified using Qiagen RNeasy mini kit according to the manufacturer’s instructions. cDNA was synthesized with a Quantitect Reverse Transcription Kit and qPCR was performed using ABI Power SYBR Green PCR master mix according to the manufacturer’s instructions. We used an ABI 7500 Fast Real-time PCR System. Cyclophilin A was used as a calibrator for the relative amplification of genes of interest calculations. Primer sequences used were: cyclophilin A forward, GCTTGTTGTCAGAGAACTTG, reverse, GTGTTGCCAAGATCAGAAG; PDP2 forward, GGAAGATGGAGAAACCTGCTGT, reverse, GCTGTGCTCTCTCCAGTCC; PDP3 forward, AGATGTGAAGAGCCACAGCG, reverse, TCTGCTTTTCTTCACTGTG; PDH forward, ACTGGGAATTTAGGATCGT, reverse, AAATGGACACGGTCATAAAAG; TGF forward, TCTCTAGCGCTTCGTGTG, reverse, ATCTCGCTTGTGATGATTTGA; Pana forward, CTGGAGGCTTCTGACTAAT, reverse, CAACCTAAAGGGCCTACTC; and Nora forward, CGGAGATCAAGCCTGTGCGAC, reverse, ATGAGCTGATGGTTCGTG.
Gene Transfer

Plasmids—Flurorescent HIF-1a and 2a fusion constructs were cloned in the Gateway system (Invitrogen). HIF sequences were amplified by PCR using a plasmid template and cloned into a Gateway Entry vector by recombination. The final EGFP fusion was obtained by recombination of the HIF-ENTRY vector with a EGFP destination vector. PHD1-EGFP and PHD3-EGFP were obtained from the Addgene non-profit making plasmid repository (catalog number plasmids 21400 and 21402), both plasmids were purchased in 2006.

Transfection—Cells were transfected 24 to 48 h before imaging using FuGENE 6 (Roche Applied Sciences, UK) according to the manufacturer’s instructions with a FuGENE/DNA ratio of 2/1.

Viral Transduction—Lentiviral transfer vectors were produced by infection of the fusion of human HIF-1α ODD (amino acids 529–652)-EGFP, amplified from previously made gateway plasmid pG-ODD-EGFP into the lentivector pHIV-infectious Tomato (Addgene plasmid 21374). The pH2D2 lentivirus was obtained from D. Hoogewijs and R. Wengler (University of Zurich). pMD2.G (Addgene plasmids 12259 and Addgene plasmid 12260) was used for packaging.

Time Lapse Confocal Microscopy

Cells were incubated on the microscope stage at 37 °C, 5% CO2, or 20% O2 and observed by confocal microscopy using a Zeiss LSM510 with a Plan-apochromat 63× 1.3 NA oil immersion objective. Excitation of EGFP was performed using an argon ion laser at 488 nm. Excitation of the empty dtds used as a control was performed using a green helium-neon laser (543 nm) and detected both a 545-nm dichroic mirror and a 560-nm long pass filter. Data capture was carried out with LSM510 version 3.0 software (Zeiss, Germany) using the Auto-time series macro (14). For time lapse experiments mean fluorescence intensity was extracted and the fluorescence intensity was determined for each cell using CellTracker version 0.6 software (Zeiss) (15). These experiments were performed three times and ~100 cells were analyzed for each HIF-1α and -2a construct. For promPHD2-PHD3-EGFP, the experiment was performed three times and ~50 cells were analyzed.
HIF-α Dynamics and Mathematical Modeling

A

Hyoxia (h)

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<th>0</th>
<th>4</th>
<th>8</th>
<th>24</th>
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<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>48</td>
<td>3</td>
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<tr>
<td></td>
<td>1</td>
<td>08</td>
<td>37</td>
<td>2</td>
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B

GFP HIF-2α

<table>
<thead>
<tr>
<th>time in hypoxia (h)</th>
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<tr>
<td>NT 0 4 8 24</td>
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<tr>
<td>0 5 10 15 20</td>
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</table>

C

HIF-1α EGFP

D

HIF-1α-EGFP

E

EGFP-HIF-2α

F

Kinetic pattern

G

Peak duration

H

Responding cells

I

J

eIF4E expression (fold induction)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ODD</th>
<th>HIF</th>
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<tr>
<td>21%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
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Flow Cytometry

Cells were seeded in 6-cm dishes at a total density of 100,000 cells and co-transfected with HIF-1α-EGFP and dEdK-ΔP, 1 day before hypoxic incubation as indicated. Upon hypoxic incubation, cells were trypsinized and pelleted and then resuspended in 100 μl of PBS. 100 μl of 4% paraformaldehyde was added (final concentration of 2%) and incubated for 15 min at room temperature. Hypoxic samples were fixed in the hypoxic chamber. Analysis was carried out using a Guava EasyCyte Plus Flow Cytometer (Millipore). The percentage of EGFP and dEdK positive cells in each sample was established using GuavaSoft software (Millipore).

Mathematical Modeling

Fig. 3A describes the minimal model consisting of two coupled ordinary differential equations. HIF-1α (x) is produced through basal synthesis at rate μ, induces the transcription of PHD2 (y) at rate λ, and is degraded via PHD-dependent hydroxylation at an oxygen-dependent maximal rate h with saturation threshold, σ. All models were solved in Matlab R2009a using standard ordinary differential equation solvers. Parameters in the two-component model were optimized for each cell line by minimizing the sum of squared residuals of the ordinary differential equation solution and the experimental data using the built-in Matlab function fminsearch. The ratio of hydroxylation rate in hypoxia to normoxia was taken to be 0.14 based on measured values from the literature for the PHD2 isofrom (16). Initially the free parameter optimization was performed on bell-shaped single-cell data, and median values were obtained. Parameter optimization was then constrained so that h and σ could only vary from the median values by 50%. Parameters λ and σ were unconstrained as experimental protocols such as transfection efficiency or laser intensity may result in variability between cell lines. Cells were classified as good or bad using an error envelope generated from Monte Carlo simulations of the model with PHD2, PHD3, and HIF-1α expression as input (Fig. 3C). The data are consistent with the model predictions for both cell lines showing that PHD2 is induced in both hypoxia and normoxia while PHD3 is only induced in hypoxia. The model predictions are compared to the experimental data in Figs. 3A and B. The model predictions are consistent with the experimental data for both cell lines showing that PHD2 is induced in both hypoxia and normoxia while PHD3 is only induced in hypoxia. The model predictions are compared to the experimental data in Figs. 3A and B.

HIF-α Dynamics and Mathematical Modeling

\[
\begin{align*}
\frac{dx}{dt} &= \mu - \frac{x}{x + y} \sum_{i=1}^{n} k_i x_i \\
\frac{dy}{dt} &= k_y - d_y y \\
\frac{dz}{dt} &= k_z - d_z z \\
\frac{dz}{dt} &= k_z + k_x - d_z z \\
\end{align*}
\]

PHD2 and PHD3 are HIF inducible with induction rates k_y and k_z. The PHD basal degradation parameters, d_i (i = 1,2,3) were taken to be the mean values of measured half-lives (Fig. 5B). The hydroxylation rate parameters, h_i (i = 1,2,3), were based on measured values from the literature (16). In the extended model, it was necessary to introduce basal synthesis of the PHD proteins, as PHD1 is not produced via HIF induction. To estimate the PHD basal synthesis rates, σ_i the steady state ratio of proteins were taken to be 0.25 in Ref. 17. The parameters were optimized by fitting the model to the median cell generated from the 2-component model optimization of de-oxxygenation data.

The HIF-PHD model was compared to a previous model describing the p53-Mdm2 feedback loop (12). In the original p53-Mdm2 model, HIF binds to p53 reducing the rate at which p53 is degraded when in a complex with Mdm2. In the original model this is represented by a switch in the degradation rate following hypoxic stress. Here the model is extended to allow the degradation rate to be explicitly a function of HIF, δ(t) = Ae^{-δn}, with constants A and B chosen so that the degradation rate matches the original model when the HIF levels have attained equilibrium steady state values in normoxia (20% oxygen, low HIF) or hypoxia (1% oxygen, high HIF). Model simulations were initially run with HIF switching between equilibrium levels (steady HIF dependence) to recapitulate the results of Hanzik et al. (12). The model was then coupled to the four-component HIF-PHD model (dynamic HIF dependence).

FIGURE 1. Single cell dynamics of HIF-α nuclear levels and HIF-dependent transcription. A, HeLa cells were transfected with HIF-1α-EGFP or EGFP-HIF-2α (1-132) for the indicated time points. HIF-EGFP fusion protein levels were measured by Western blot using an anti-EGFP antibody and the bands were quantified by densitometry analysis. B, HeLa cells were transfected with HIF-1α-EGFP together with dEdK-ΔP expression plasmid for normalization purposes. C, isolated green fluorescent cells were measured by flow cytometry and plotted as a function of time in hypoxia (transfected control (D) were used for gating). D, representative image of HIF-1α-EGFP and an empty plasmid control. (A) shows the picture to monitor the localization and number of transfected cells. Transfection efficiency was quantified at 10 min. 60% of cells were transfected with HIF-1α-EGFP encoding a monomeric red fluorescent protein. (D) Images were recorded using a fixed time-lapse sequence of the same cell. (E) Nuclear fluorescence levels for HIF-1α (D) and HIF-2α (E) were plotted as a function of time for 4 representative cells. The straight line represents the threshold used for automatic peak detection (see “Experimental Procedures”). All traces for HIF-1α are shown in Supplemental Fig. S1A. Some traces are shorter than the entire time course due to cell death or migration out of the imaging field. F, Classification of the observed HIF-α response kinetics. TEM transients are shown in red, blue, green, and yellow. Each peak was scored with a threshold (see “Experimental Procedures”). The number of cells scored in each category is indicated on the plot. G, duration of the HIF accommodation to transient response. Duration was determined as the time between the point at which HIF fluorescence returned to baseline and the point at which HIF fluorescence levels were 50% of the maximum level. Only cells showing a consistent increase of green fluorescence levels in hypoxia and normoxia were included in the analysis. H, representative plots are shown. (C) HeLa cells were transfected with HIF-1α-EGFP, either stably expressing PDO-EGFP or left untransfected. They were cultured in normoxia or hypoxia (1% O2) for 8 h prior to cell lysis and RNA extraction. mRNA levels for PHD2 and -3 were measured by qPCR and normalized to cyclophilin and mRNAs.
RESULTS

Single Cell Dynamics of HIF-1α and -2α in Normoxia and Hypoxia—To capture HIF-1α and HIF-2α dynamics, we used time lapse confocal imaging of HIF-EGFP (enhanced green fluorescent protein) fusion proteins in an O₂ controlled environment. HIF-1α-EGFP and EGFP-HIF-2α induction in hypoxia was validated by EGFP detection (Fig. 1A). In cells switched from a normoxic to a hypoxic environment, we observed by Western blot (Fig. 1A) and flow cytometry (Fig. 1B), a HIF-1α-EGFP accumulation at 4–8 h that had decayed by 24 h, which was in agreement with previously published results on endogenous HIF (7, 18, 19). HIF-2α was less inducible by hypoxia, and was already detectable in normoxia. For live-cell imaging, observations were initially taken at 20.8% O₂.
Dynamics of HIF-α

HIF-α Dynamics and Mathematical Modeling

A

\[ \frac{dx}{dt} = S - hy \left( \frac{x}{x + \gamma} \right) \]
\[ \frac{dy}{dt} = kx - dy \]

- \( x \): Concentration of HIF-α
- \( y \): Concentration of PHD
- \( S \): Time
- \( h \): HIF-α basal synthesis rate
- \( y \): HIF-α Michaelis constant
- \( k \): PHD induction rate
- \( d \): PHD basal degradation rate
- \( h \): Hydroxylation rate

B

6h 7h 10h 13h

C

D

E

1% O2

0h 11h 20h

G

responding cells

<table>
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<tr>
<th></th>
<th>1% O2</th>
<th>21% O2</th>
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<tr>
<td>responding cells</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>20</td>
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</tbody>
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F

Fluorescence intensity

Time (hours)
Dynamics of HIF-α Dynamics and Mathematical Modeling

tension as a control for 2 h, then, after a switch from 20.8 to 1% 
O₂, cells were further imaged for 20 h. Cells subjected to the 
hypoxic switch showed transient HIF-1α and -2α nuclear accu-
nulation with varied kinetics (Fig. 1, C–E, supplemental Fig. 
S1A and Movie S1 and S2). A single transient bell-shaped pro-
fle was observed in 30% (21 cells) of the HIF-1α and 32% (18 
cells) of HIF-2α responding cells (Fig. 1D), which was of similar 
duration. –2–4 h (Fig. 1E). We observed some spontaneous 
pulses of HIF-1α nuclear accumulation in normoxic cells (19% 
of the transfected cells, Fig. 1H). These levels were higher for 
HIF-2α (30% of the transfected cells), consistent with the stabi-
lization observed by Western blots in normoxic conditions. We 
observed that 22% of HIF-1α (11 cells) and 36% of HIF-2α cells 
(16 cells) had more than 1 peak of HIF nuclear accumulation. In 
most cases the subsequent peaks had increased amplitude (Fig. 
1, D–F). The transient pattern of HIF dynamics was also 
observed in other cell lines (e.g. HEK293T cells, not shown) and 
also observed in a HeLa cell line stably expressing the oxygen 
dergation domain (ODD) of HIF-1α fused to EGFP (Fig. 1F).

Cells visibly expressing ODD-EGFP displayed similar transient 
dynamics, yet contrary to HIF-1α-EGFP the degradation was 
slower. This is likely due to the ODD not possessing transcription-
al activity and so being unable to further increase the PHD 
feedback above endogenous regulation, in contrast to full-
length HIF exogenous expression (Fig. 1G). We also character-
ized the transient dynamics of HIF activity. The HIF-dependent 
transcriptional activity in live cells was assessed by imaging the 
light produced by a hypoxia response element-luciferase 
reporter gene (HRE-luc). We examined C51 cells stably 
expressing a HRE-luciferase (PH3-5XV, C51) (Fig. 2, A and B) 
and HeLa cells transiently transfected with HRE-luc (Fig. 2, C 
and D). In both conditions, we found a transient luciferase sig-
nal, indicating transient transcriptional activity. Some cells dis-
played 2 or more peaks of luciferase expression, which is con-
sistent with the nuclear accumulation of HIF fluorescent fusion 
proteins. Interestingly, the stable C51 cells had some basal 
luminescence signal in normoxia, indicating some degree of 
spontaneous low amplitude luciferase peaks of transcriptional 
activity (not shown) in agreement with the observation of spon-
taneous accumulation of HIF-1α and -2α.

Mathematical Modeling of HIF Nuclear Dynamics during 
Hypoxia and Re-oxygenation—The known negative feedback 
from PHD proteins on HIF is a likely candidate for generating 
the pulses of HIF-1α and -2α (9, 10, 20). To analyze the dynamic 
behavior of HIF, we developed a simple mathematical model 
based on the global HIF-PHD negative feedback loop (Fig. 3A).

We observed heterogeneity in the response time and that HIFα 
responses occurred after cell division in 50% of dividing cells 
(see Fig. 3B for a typical example). This might have been caused 
by transient transfection at the time of nuclear breakdown (21).

Cells were, therefore, artificially synchronized in the cell cycle 
(Fig. 3C), using the mitosis time as t = 0 for mathematical 
modeling.

We initially fitted the bell-shaped single-cell data from HIF-
1α-EGFP, which encapsulate more complex dynamics and 
better mirrors the transience of accumulation observed by 
Western blot, by using models previously described for the p53 
system (22). A model, which included a saturation coefficient 
for hydrogenation provided the best fit (Fig. 3D, and “Experi-
mental Procedures”), 79% (31/39) of the de-oxygenation single 
cell data were then successfully fit to the model, subject to the 
constraint that the parameters k (induced PHD production 
rate) and d (PHD degradation rate) were similar across all cells 
(see supplemental Fig. S1A and “Experimental Procedures”).

The model predicted a slow gradual increase of PHD. This was 
in qualitative agreement with the PHD2 dynamics measured in 
single cells using PHD2-EGFP controlled by the PHD2 proximal 
promoter (Fig. 3, E and F). PHD2 up-regulation was observed in 
40% (20 cells) of the transfected cells switched to 1% 
O₂ (Fig. 3G).

We further assessed the functionality of the experimental 
system and the model by fitting re-oxygenation experiments. 
Therefore, HIF-1α levels were imaged in single cells during 
re-oxygenation after exposing cells to hypoxia for 6 h. Upon 
re-oxygenation, HIF-1α-EGFP-transfected cells displayed a 
rapid loss of fluorescence, presumably due to its degradation 
(Fig. 4A). The kinetics were slower, but consistent with the 
endogenous HIF-1α degradation observed by Western blot 
(Fig. 4B), supporting the validity of the experimental imaging 
results. Interestingly, in some cells, the loss of fluorescence was 
not definitive and these cells had a clear slow return of fluores-
cence 200 min after re-oxygenation. We fitted all cells obtained 
from the re-oxygenation experiment with the parameters k and 
d constrained and good fits were obtained for 78% (31/42) of 
cells (example of 3 cells shown in Fig. 4C; all cells in supplement-
al Fig. S1B).

Role of PHD in HIF Timing and Translucency—A generic “median cell” based on median parameter values was con-
structed and used to test the effects of parameter variation (Fig. 
4D). Varying k (basal synthesis rate of HIF-1α) had a clear effect 
on the amplitude, but not on the kinetics, of the response. This 
could explain the difference in amplitude observed in the single 
cells (visible in Fig. 3C), which are likely to have different copy 
numbers of HIF plasmid due to transfection variability. In con-

FIGURE 3. Mathematical modeling of the generic HIF-PHD feedback loop. A, a description of the model (see “Experimental Procedures”). HIF-α (red) is produced 
at rate k and removed due to PHD (blue) hydroxylation. The maximal hydroxylation rate, d, is oxygen dependent and saturation of hydroxylation is determined by 
the parameter γ, PHD (γ) is produced through induction by HIF-α at rate k and undergoes basal degradation at rate δ. HeLa cells were transiently co-
transfected with HIF-1α-EGFP and an empty plasmid (control plasmid). Cells were imaged every 3 min after a switch from 20.8% (O2) to 1% 
O₂ (c), using time lapse confocal microscopy. The pictures show a typical example of HIF-1α nuclear accumulation occurring after cell division. This was observed in 50% of the cells 
showing a HIF-1α increase. C, HIF-1α levels in hypoxia plotted as a function of time, synchronized based on cell cycle. d, single cell data of HIF dynamics (blue 
line) were fitted computationally using the model (red line). See also supplemental Fig. S1 for more cell fitting. The model PHD profile is in green. The model 
cells initially at an approximate 1% of hypoxia (HIF-1α = 0.048 at t = 0). E, selected de-oxygenation images of HeLa cells transiently 
transfected with the PHD2-EGFP (PHD2-EGFP expression plasmid). Cells were imaged using time lapse confocal microscopy every 3 min in 20.8% O₂, for 1 h 
and then switched to 1% O₂ for 28 h. F, the plots represent the whole-cell fluorescence intensity produced from PHD2-EGFP (PHD2-EGFP expression plasmid) as a function of time. G, the 
percentage of responsive cells is calculated from the number of transfected cells showing an increase of the green fluorescence level over time from 
PHD2-EGFP (PHD2-EGFP expression plasmid) in hypoxia and normoxia. 

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Dynamics of HIF-α
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1. Introduction

2. Methods

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5. Conclusion

6. References

**Figure 1. Single cell dynamics during re-oxygenation and validation of the mathematical model.** A, cells were transfected as described in the legend to Fig. 1A. 24 h after transfection, cells were exposed to 1% O2 for 6 h in the microscope stage and then re-oxygenated to 20% O2. Fluorescence levels were measured in triplicate and plotted as a function of time. B, A cell was cultured in hypoxia for 6 h and then subjected to re-oxygenation. HIF-1α levels were measured by Western blot at the indicated time points. C, densitometry analysis of the bands was plotted as a function of time. **Figure 2.** A, the model described in the legend to Fig. 2. We fitted the single cell kinetics obtained experimentally to A. The model was initially at equilibrium in hypoxia (h = 0.14) and re-oxygenated back into normoxia (h = 1) at k = 0.1. D, parameter sensitivity analysis was conducted by varying one parameter at a time, at a perturbation from the initial condition obtained from the mean parameters (1 = 3.5; 10 ng/ml, y = 2.0610^-10 M, d = 4.71 x 10^-10 min^-1).
Dynamics of HIF-α

HIF-α Dynamics and Mathematical Modeling

A. Cycloheximide

- PHD1
- PHD2
- PHD3

B. Graph showing half-lives of PHD1, PHD2, and PHD3.

C. Graph showing mRNA levels over time.

D. Graph showing HIF-α protein levels over time.

E. Table of parameters and their values:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
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<td>S</td>
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<td>S1</td>
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<td>γ</td>
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</tr>
<tr>
<td>h2</td>
<td>1<em>Normoxia 0.14</em>Hypoxia</td>
</tr>
<tr>
<td>h3</td>
<td>1.25<em>Normoxia 0.13</em>Hypoxia</td>
</tr>
</tbody>
</table>

*Normoxia and Hypoxia indicate conditions.

FIGURE S.A: Measurement of the half-life of PHD1, -2, and -3. HeLa cells were transfected with PHD1, -2, or -3 EGFP. 24 h after transfection, cells were treated with cycloheximide (10 μg/mL), and the PHD levels were measured for up to 24 h by measuring fluorescence intensity. Error bars show the coefficient of variation. The plot is representative of one experiment. The experiment was repeated 4 times. The four component model was used. The model cell is initially at equilibrium in normoxia and is then de-oxygenated into hypoxia at t = 0. E. The parameter sets for the 4 component model are detailed in the table (time units in mins).
**Dynamics of HIF-α**

**HIF-α Dynamics and Mathematical Modeling**

**A** In salico PHD1 knock-out

**B** In salico PHD2 knock-out

**C** In salico PHD3 knock-out

**D** shPHD2

**E** WT

**F** 1% 1%

**G** Fluorescence intensity (a.u.)

**H** WT, shPHD3

**I** HIF-1α levels (a.u.)

**FIGURE 6.** PHD2 knock-down affects HIF temporal profile. A–C, predictions are based on a 4-component model (Fig. S4A) with PHD1, 2, or 3 removed representing the knock-out of PHD1, 2, and 3. The models are initially at equilibrium in normoxia and then de-oxygenated into hypoxia at t = 0. D, Western blot analysis of PHD2 and PHD3 levels in WT and sh-PHD2 Hela cells. E, Western blot analysis of HIF-1α levels in WT and sh-PHD2 Hela cells cultured in normoxia submitted to 3% hypoxia (1% O₂). F, Western blot analysis of HIF-1α levels in sh-PHD2 Hela cells cultured in 1% O₂ for the indicated time points. G, single-cell analysis of HIF-1α-EGFP levels in sh-PHD2 cells exposed to 1% O₂. H, Western blot analysis of PHD3 levels in WT and sh-PHD3 Hela cells. I, single-cell analysis of HIF-1α-EGFP levels in sh-PHD3 cells exposed to 1% O₂.

Subsequent differential binding to other partners and it is, therefore, important to understand the consequences of the timing of HIF1α timing to inform future therapeutic strategies aimed at controlling HIF activity. We have shown that in conditions where transient HIF accumulation was lost, there was a high level of cell death, pointing to the importance of elucidating which component of the HIF-signaling is the guardian of its single-cell imaging data, we predicted that HIF-1α dynamics will display a range of kinetics depending on the hypoxic situation and that it is PHD2 that is specifically involved in the negative feedback responsible for pulsatile HIF levels.

**Single Cell Imaging of HIF: Translucency and Heterogeneity**

Using live cell imaging, we observed a range of dynamics of HIF...
Dynamics of HIF-α

**HIF-α Dynamics and Mathematical Modeling**

**Figure 7.** A. Pictures of a representative field of sh-PHD2 cells expressing HIF-1α-EGFP at the indicated times in hypoxia show cell death associated with high and low exiting levels of HIF-1α. B, the percentage of transfected sh-PHD2 or WT HeLa cells dying within the 20 h of an imaging experiment was calculated for control cells transfected with either an empty EGFP plasmid or with HIF-1α-EGFP in normoxia and hypoxia (n = 40 cells/condition). C and D. HeLa cells (WT, shPHD2 or shPHD3 lines) were imaged simultaneously using a 4-compartment glass-bottom dish (Greiner). They were labeled with Annexin V-FITC (green) and PI (red) 10 min before imaging. Cells were imaged for 2 h in normoxia prior to the switch to 1% O2. Images were recorded every 15 min for 24 h. The number of apoptotic cells (Annexin V labeling) and necrotic cells (PI labeling) was counted out of the total number of cells and plotted C. A typical field of cells at several time points is shown (D). E-G. WT, HeLa cells, or HeLa cells expressing shPHD2 or shPHD3, were cultured in 1% O2 for the indicated time points. VEGF mRNA levels were assessed by qPCR. E. Primers for VEGF mRNA were assessed by qPCR. F. Assay mRNA were assessed by qPCR. G. No mRNA was assessed using qPCR. E-G, the plots represent the average ± S.E. of triplicate samples from a representative experiment. The experiments were performed 4 times. Results are the mean of three independent experiments ± S.E.

***, **, and * indicate statistical difference with p < 0.001, p < 0.01, and p < 0.05, respectively.
nuclear accumulation, including fast and slow kinetics, single and multiple peaks. Although heterogeneity is a common feature of cellular processes (22, 28), this could not be detected using bulk cell analysis approaches and has never been described previously in this system. In the case of the oxygen dependent signaling, inter-cellular heterogeneity may be generated by extrinsic noise such as NO or iron levels, as well as intrinsic noise from transcription (29). We also observed transient HIF accumulation in normoxia (Fig. 1D) and spontaneous peaks of transcriptional activity (not shown), which agrees with the previous suggestion of oxygen-independent mechanisms of HIF stabilization (18, 30).

**HIF Dynamics: Role of Negative Feedback Loops and Their Mathematical Modeling**—Existing HIF models (31–33) have focused on how equilibrium levels of HIF are a function of oxygen levels, in particular probing the possibility of a switch-like behavior in HIF levels in response to hypoxia. Despite being formulated as dynamic models, these previous models have typically focused on the static states of the system. However, the single-cell dynamic data presented here clearly demonstrate that in response to hypoxia, many cells undergo rapid and large amplitude transient dynamics in nuclear HIF, before returning to equilibrium levels, which are comparable with the levels found in normoxic conditions. The model proposed here is highly idealized in that they only consider the dynamics of HIF and the PHD proteins. This simple model might be integrated, in the future, in a recently published model based on HIF transcriptional activity (34). However, even our 2-component model, with the generic PHD, was able to fit a range of the single cell dynamic data and provided a tool to assess the sensitivity of the different model parameters via consideration of a median solution of the system. Furthermore, by expanding the
model to consider separately PHD1, -2 and -3, we were able to test computationally potential specific roles of the PHDs in the control of HIF timing, and demonstrate non-redundancy between PHD1, -2 and -3. We were also able to examine downstream effects by coupling the simple HIF-PHD model to existing biochemical models. Specifically here we coupled HIF to a model of the p38-Mdmb2 feedback loop. We were able to demonstrate the importance of capturing not only equilibrium levels of HIF in normoxia and hypoxia, but also the transient dynamics. Specifically we demonstrated that the overshoot HIF levels observed in transient dynamics lead to higher transitory levels of p33, and a delay in the onset of p33 oscillatory behavior. Furthermore, we determined in silico the HIF-mediated role that PHD2 has in regulating p33 dynamics, explicitly demonstrating that in shPHD2 cells, p35 displays sustained high levels suggesting apoptotic activity.

Role of the PHD2 Negative Feedback Loop on HIF Timing and Cell Fate—Berens et al. (23) addressed the question of the multiplicity of the PHDs relative to HIF-1α and showed, by silencing each PHD isoform individually, that only PHD2 controlled the steady state levels of HIF-1α in HeLa cells and other human cells. By extending a simple mathematical model of the HIF-PHD negative feedback loop it was possible to separate the 3 different PHDs on the basis of their levels, stability, and induction. This pointed to a strong role of PHD2 in the control of HIF nuclear accumulation in hypoxia. It is, however, possible that at other O₂ levels, e.g. mild hypoxia or anoxia, the role of the other PHDs will also play an important role and hence contribute to HIF dynamics. The consequences of long lasting HIF-1α levels were observed on VEGF transcription dynamics (7F) and they are likely to also directly affect HIF-binding proteins such as mdm2 (11), hence altering cell survival in hypoxia. The cell death induced by hypoxia in cells in which PHD2 was silenced (Fig. 7, A–D) was in line with the protective role of PHD2 in gliomas against hypoxia-induced tumor cell death (35) and could well represent a new molecular target for cancer drugs. Taken together, our data point to a major role for PHD2 compared with PHD3 in the negative feedback regulation of HIF-1α dynamics, although we cannot rule out the presence of other, non PHD-dependent mechanisms, as previously suggested (36–39). The potential feedbacks, both oxygen-dependent and -independent, and how they affect HIF-1α levels and activity will have to be further investigated.

Cells can experience hypoxia in a wide range of physiological and pathological contexts, where HIF activity is up-regulated and associated with different cell fates. The decision between survival death, proliferation/cell cycle arrest in hypoxia is likely due to differential gene expression, as well as HIF binding to key proteins involved in these mechanisms (e.g. mdm2/p53). In conclusion, we have shown here that one way to trigger hypoxic death is to have uncontrolled high and long lasting HIF levels, and that an important role of PHD2 is to keep HIF on time. Our results show that HIF dynamics have an impact on cell fate through p33 transcriptional activity regulation, and mathematical modeling predictions points to differential p33 dynamics and levels depending on the HIF temporal profile, which will need to be fully investigated in the future.

HIF-α Dynamics and Mathematical Modeling

Acknowledgments—We thank Prof. Melillo for the HBE-huc plasmid, Dr. Stief and Dr. Hengstler for the shPHD2 cells, shPHD4 lentiviruses, and for helpful discussions, and Dr. R. Depping for the PHD2-EGFP plasmid. We thank Bryan Weln for the pHIV-Tra-Ematrix vector and Didier Trzes for the pH2D-G and podA2 plasmids. We thank Hideh Shishido for technical help with qPCR. We also thank Dr. Lehmann and Prof. Wenger for the CS1 stable reporter and helpful discussions and the COST action TD0901 for facilitating these interactions. We thank Dr. Pastek for critical reading and Dr. Levy for fruitful discussions.

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3.3 Additional work

3.3.1 Improving molecular tools for imaging: HIF-1α-GFP stable cell line

Most of the studies into the dynamics of the HIF-α subunits performed so far have required transient transfection of plasmids encoding HIF-1α / HIF-2α fused with fluorescent proteins such as EGFP or dsRed. Transient transfection has many disadvantages such as heterogeneity in expression levels, the protocol can be unreliable and the reagents can be toxic to the cells. In addition to this, the gene is under the control of a promoter that permits constitutive expression, the CMV promoter, which can lead to high levels of expression that are not necessarily representative of those observed for the endogenous promoter, and expression can vary between cell types (Smith *et al.*, 2000). Therefore, to improve the quality of our HIF dynamic studies, we aimed to create stably transfected cell lines for both HIF-1α and HIF-2α.

The initial plan was to create three stable cell lines via lenti-viral transduction, however creating cell lines stably expressing HIF-1α-EGFP and EGFP-HIF-2α proved difficult and we only had success with the ODD-EGFP construct (mentioned earlier). With this cell line, although similar dynamics to HIF-1α-EGFP (ectopically expressed in WT HeLa) in hypoxia were observed, the degradation appeared to be slower. This is could be explained by the fact that the ODD-EGFP is lacking the DNA binding domain and so is not transcriptionally active, resulting in an increase of substrate (ODD domain) in the system with no additional activation of PHD2 transcription to compensate. In addition to this, the ODD-EGFP only has one prolyl residue for hydroxylation which may result in reduced affinity of pVHL binding, thus less efficient targeting of the ODD-EGFP for proteasomal degradation. Although the ODD-EGFP cell line provides some valuable information, there was a need for a cell line stably expressing fluorescently-labelled full-length HIF-1α and HIF-2α. Given the lack of success with the lenti-virus approach to achieve this, I directed my efforts towards alternative strategies. I tried to generate a cell line stably expressing fluorescently labelled HIF-2α using several methods including zinc finger nucleases (ZFNs), but all were unsuccessful (work not shown) and so all work with HIF-2α-EGFP presented in this thesis was carried out using transient transfection. However, I had the opportunity to use a Bacterial Artificial Chromosome (BAC) for HIF-1α-GFP, thanks to the kind gift from Prof B
van de Water’s laboratory (University of Leiden), and was able to create a stable cell line expressing HIF-1α-GFP.

### 3.3.1.1 HIF-1α-GFP BAC stable cell line

A HIF-1α-EGFP cell line was established via stable transfection of HeLa with a BAC encoding the fusion protein. In comparison to the ODD-EGFP cell line, this gene is under the control of its endogenous promoter and BACs can hold large pieces of mammalian DNA (up to 350 Kb, compared to 15 Kb for plasmids and 7 Kb for lenti-viral vectors) resulting in the inclusion of lengthy flanking regions. Therefore a large proportion of the regulatory elements are incorporated and so utilising a BAC provides a more true reflection of endogenous expression (Adamson et al., 2011; Casali, 2003). Another advantage of the BAC cell line is that it was established from a single cell and is therefore a clonal population and so could provide the opportunity to determine whether the heterogeneity observed in Bagnall et al. (2014) is a feature of the HIF signalling system or an artefact of transient transfection.

The HIF-1α-GFP BAC cell line was validated via western blot and imaging. Figure 3.1A shows a strong induction of wild type HIF-1α following treatment with 0.5 mM DMOG (6 h) and a transient induction of endogenous HIF-1α in hypoxia. A second band, approximately 40 kDa higher, follows the same pattern of induction and is presumed to be HIF-1α-GFP. The cells were imaged on a confocal microscope before and after (6 h) treatment with the hypoxia mimic and nuclear accumulation of fluorescent protein was observed (Figure 3.1B). Following these validation experiments, this cell line was utilised to repeat time-lapse experiments that were performed in Bagnall et al. (2014).
Figure 3.1 | Validation of HIF-1α-GFP stable cell line. A) Western blot analysis of HIF-1α-GFP HeLa incubated in different conditions (D = 0.5 mM DMOG, 6 h, N = normoxia, H = hypoxia, number indicates number of hours incubated in hypoxia). Immunoblot was probed with anti-HIF-1α (top) and anti-β-actin (bottom) antibodies. B) HIF-1α-GFP HeLa were imaged on a Zeiss LSM780, before and after treatment with 0.5 mM DMOG (i = brightfield, ii = GFP, iii = merge of i and ii). Scale bar = 10 μ. In pre-treatment cells nuclei are outlined in white.
Figure 3.2 | HIF-1α-GFP BAC time-lapse experiments. A) Confocal images of HIF-1α-GFP BAC cells in 0.5 % O2. Cells were subjected to hypoxic conditions from the second time point onwards. Nuclear boundary highlighted by white dashed line. White arrow indicates perinuclear autofluorescence. B) Quantification of fluorescence signal. Each data series represents the fluorescence measured in the nucleus of a single cell over time.

Upon incubation in hypoxia, the cells showed a transient induction of HIF-1-GFP. After approximately 1 h the levels of fluorescence started to increase, peaked at 3-3.5 h and then started to decrease, reaching levels similar to those at the start of the time-lapse by 6-8 h (Figure 3.2B, M1 on supplemental CD).
Figure 3.3 | Comparison of HIF-1α dynamics in hypoxia Example traces from single HeLa cell ectopically expressing CMV-HIF-1α-EGFP (top) and single HIF-1α-GFP BAC cell line (bottom) in hypoxia. Hypoxic incubation starts at 0 h.

Figure 3.3 compares the typical shape of HIF-1α accumulation in hypoxia in a single cell ectopically expressing CMV-HIF-1α-EGFP (taken from James Bagnall’s data set) and one from the HIF-1α-GFP BAC experiments. In both systems the levels of HIF-1α start to increase at around 1-2 hours and peaks around 3-4 hours. Compared to the stable cell line, the cell transiently transfected with HIF-1α-EGFP shows a sharper increase in fluorescence, reaching much higher levels that then decrease more rapidly. The stable cell line exhibits stabilisation of much lower levels and a softer decline in HIF-1α, similar to what was observed with the ODD-EGFP cell line. The difference in the accumulation pattern might be explained by the difference in HIF-1α levels. The very high HIF levels obtained with transient transfection will induce a stronger negative feedback response compared to the 2 fold increase obtained with the BAC cell line.
Although the BAC cell line should be closer to physiological HIF expression at the mRNA levels as the transgene is under the control of the endogenous promoter and other regulatory elements, the dynamic range of HIF-1α-GFP accumulation in hypoxia is poor i.e. the expression of the transgene are much lower than the endogenous protein in the western-blot (Figure 3.1A). Therefore the accumulation pattern observed with this cell line is unlikely to actually represent the reality any more than transient transfection.

### 3.4 Conclusion

Here we have utilised live single cell imaging to demonstrate that induction of HIF-1α is transient due to an auto-regulatory mechanism. Furthermore we showed that the tight control of HIF-1α dynamics in hypoxia is necessary to prevent cell death. To validate these findings published in Bagnall et al. (2014) I repeated time-lapse experiments with cells stably expressing HIF-1α-GFP.

#### 3.4.1 Imaging tools

The difficulties experienced whilst trying to create a cell line stably expressing fluorescently labelled HIF-1/2α via lenti-viral transduction may be due to the final constructs being quite large (pHIV- HIF-1α-EGFP- IRES- dTomato is 10950 bp and pHIV-EGFP-HIF-2α- IRES- dTomato is 11049 bp). It has been shown that constructs containing larger inserts achieve lower viral titres (units / mL) than smaller constructs (Al Yacoub et al., 2007). Also, the size of the construct can result in reduced recovery of the viral particles following the ultracentrifugation step prior to transduction (Al Yacoub et al., 2007).

These stable cell lines that were successfully made are useful tools for studying the temporal and spatial dynamics of HIF-1α at the single cell level. For example the ODD-EGFP does not have any PAS domains so cannot heterodimerise with HIF-1β and there are no DNA binding domains, therefore there will be no downstream effects of overexpression of the ODD-EGFP as it is not transcriptionally active and therefore any dynamics observed relates purely to the activity of the PHDs. The HIF-1α BAC cell line has other advantages, as the transgene is under the control of the endogenous promoter so any observations take into account regulation at the transcriptional level as well as regulation via protein stability.
Unfortunately, the fluorescent signal in the ODD-EGFP and the HIF-1α BAC cell lines is very dim, which makes quantification of signal difficult and raises issues with acquisition, as higher laser power is required and longer exposure, which can lead to photobleaching and phototoxicity. This poor signal may be due to integration effects. Stable transfection of both lenti-viral vectors and BACs results in the integration of the exogenous reporter gene into the host’s genome (Adamson et al., 2011). However, there is little control over where the transgene is inserted and if it occurs within regions of densely packed, inactive heterochromatin then there will be little or no expression (Dillon & Festenstein, 2002; Adamson et al., 2011). Another possibility is that the transgene integrates downstream of silencing elements. One way around this would be to utilise methods that allow targeting integration such as genome editing nucleases (zinc finger nucleases / ZFNs, transcription activator-like effector nuclease / TALENs, clustered regularly interspaced short palindromic repeats / CRISPR).

Such low fluorescence levels have prevented me from performing longer time-lapse experiments, which would have allowed me to investigate whether there are further oscillations in prolonged hypoxia and if there is a link between the HIF dynamics and the cell cycle (as mentioned in the publication).
Chapter 4: Investigating the spatial localisation of HIF-1α and HIF-2α
When investigating the single cell dynamics of HIF-1α and HIF-2α it became apparent that the two alpha subunits exhibit different sub-nuclear localisation.

**Figure 4.1** | Sub-nuclear localisation of HIF-2α. A) HeLa cells ectopically expressing HIF-1α and HIF-2α EGFP fusions and endogenous HIF-1α and HIF-2α labelled using the immunofluorescent protocol. Images of HIF-1α-EGFP were taken in the presence of DMOG. Scale bar = 5 μm. B) HeLa cells transiently transfected with plasmids encoding clover-HIF-2α (i), dsRED-HIF-2α (ii), HIF-2α-venus (iii) and Halotag-HIF-2α (iv). The cells expressing Halotag-HIF-2α were labelled with the fluorescent Oregon Green Halotag ligand to visualise the fusion protein. C) Confocal images of C2C12 (mouse myoblast; top) and HEK293T (human embryonic kidney cells; bottom) cells ectopically expressing EGFP-HIF-2α. Scale bar = 5 μm.

Figure 4.1 shows the localisation of ectopically expressed EGFP fusions of HIF-1α and HIF-2α. HIF-1α accumulates in the nucleus in a homogenous manner upon exposure to hypoxia or following treatment with a hypoxia mimic (DMOG), whereas HIF-2α localises in punctate foci (Figure 4.1A). Immunofluorescent staining of the respective endogenous proteins in HeLa cells shows a similar sub-nuclear localisation, highlighting that this difference is unlikely to be an artefact of overexpression (Figure 4.1A). Figure 4.1B shows that HIF-2α localises heterogeneously regardless of type or orientation (N- or C-terminal of HIF-2α) of
the tag. This non-homogenous protein expression is also observed in human embryonic kidney, mouse myoblast cells (Figure 4.1C), in neuroblastoma (SK-N-AS), medulloblastoma (D283 & Med8) and mouse primary neuronal cell lines (Bagnall, 2011).

These initial findings suggest that the localisation of HIF-2α is not due to artefacts of ectopic expression, the presence of the tag or the model cell line being used. As localisation is often associated with function, the heterogeneous distribution of HIF-2α was investigated further and is the focus of this study.

**4.1 Is the non-homogenous nuclear localisation of HIF-2α functionally relevant?**

As mentioned previously, spatial organisation within the nucleus is vital for regulating processes and protein function. It has been shown that many nuclear proteins localise in domains or nuclear speckles, which have function as sites of activity, modification or sequestration, for example. Further investigation was carried out to determine the purpose of the localisation of HIF-2α into speckles.

**4.1.1 Characterisation of HIF-2α speckles**

Analysis of images of the HIF-2α speckles revealed that, in normoxia, there is between 10-200 speckles per nucleus (average 64, n = 25) that are 0.24 (± 0.07) μm in size and take up approximately 3% of the nucleus (Figure 4.2A). The size of the speckles did not change in either hypoxia or DMOG compared to normoxia (Figure 4.2B). However, the average number of speckles did increase significantly following treatment with DMOG (Figure 4.2A).

These results could be explained by the exaggerated effects usually observed following treatment with DMOG i.e. a dramatic increase in HIF protein levels and HIF-dependent transcription, far greater than those observed in hypoxia (unpublished observations from V.Sée group). This drug inhibits all three PHD isoforms (and other hydroxylases) and so the HIF-alpha subunits evade oxygen-dependent degradation. So if there is an increase in stabilised HIF-2α molecules, these must go somewhere and these data suggest that the extra HIF-2α localise in additional speckles, either newly formed or speckles that were already present but were undetectable due to low levels of protein.
Spatial localisation of HIF-2α

Figure 4.2 | Measurement of HIF-2α speckles. HeLa cells transiently transfected with EGFP-HIF-2α were imaged on a Zeiss epifluorescent microscope fitted with a very fast and highly sensitive CCD camera (Andor, UK). One thousand frames were acquired per cell in normoxia, hypoxia (1% O2, 16 h) or following treatment with DMOG (0.5 mM, 6 h). The images were analysed using ImageJ. A) The average (±SD) number of speckles per nucleus in each condition was: 64 ± 49 (n=25), 44 ± 24 (n=24) and 96 ± 33 (n=22), respectively. Mean of the sample data represented by the red line. B) The average size of speckles per nucleus over the 1000 frames. The mean values for each condition were: 0.24 ± 0.09 μm (n=25), 0.21 ± 0.07 μm (n=24) and 0.27 ± 0.09 μm (n=22), respectively. The mean values for hypoxia and DMOG were compare to the normoxic values using IBM SPSS statistics software (independent t-test, significance value set at 5%). Mean of the sample data represented by the red line.

4.1.2 Speckle mobility

On closer inspection of EGFP-HIF-2α in time-lapse movies, the HIF-2α speckles could be seen to move (M2 on attached CD). We therefore decided to characterise and quantify this motion further by tracking the speckles. Briefly, the speckles were identified as features in each frame and then these features were linked frame to frame creating trajectories using ImageJ (Sbalzarini & Koumoutsakos, 2005)(Figure 4.3, supplemental movies M3 and M4). The coordinates of these trajectories were exported to Excel for further analysis. Using a macro (see Appendix 1.3) created by Dr David Mason (Image Analyst, CCI, University of Liverpool), the speed, diffusion coefficient and diffusion mode (denoted by the slope of the moment scaling factor aka SMSS) for each trajectory was calculated.
Figure 4.3 | Analysis of the HIF-2α trajectories. A) Schematic of how speckles were tracked using ImageJ i.e. features were identified in each frame and then linked frame to frame to create a trajectory map. B) EGFP-HIF-2α speckle (i) tracked in ImageJ (ii) showing course of speckles over 1000 frames (yellow). Coordinates of these trajectories exported to excel and plotted (iii).

Figure 4.4 | Speed of individual HIF-2α speckles. Speed at which HIF-2α speckles move. Average (±SD) for normoxia = 0.47 ± 0.17 μm s⁻¹ (N=25, n=522), hypoxia = 0.38 ± 0.13 μm s⁻¹ (N=24, n=760) and DMOG = 0.36 ± 0.10 μm s⁻¹ (N=25, n=1402). Independent t-test, significance level 1%; normoxia compared to hypoxia: t₁280=11.238, p<0.001; normoxia compared to DMOG: t₁922=17.954, p<0.001.
The average speed of the speckles was faster in normoxia compared to hypoxia and DMOG, dropping from 0.47 μm s⁻¹ to around 0.37 μm s⁻¹. To provide further insight into the motion of the HIF-2α speckles, the SMSS value was determined for each trajectory (see methods). This value describes the type of movement exhibited by an object (Figure 4.5). For instance, a value of 0 indicates that the object is static, 0.5 indicates Brownian diffusion (the movement of molecules in a suspension caused by random collisions with other molecules) and 1 is indicative of ballistic motion (the object moves in a perfectly straight line). The intermediate types of diffusion are “restricted Brownian” (same as Brownian motion, but the objects movement is restricted within an area) and “directed Brownian” (diffusion is random but has overall directionality e.g. molecular motors transporting vesicles or organelles along the cytoskeleton) (Figure 4.5).

**Figure 4.5. Schematic representation of the different types of diffusion.** Brownian diffusion = random movement of an object in solution in any direction (x, y and z). Restricted Brownian = random movement confined to a specific region. Directed Brownian = random diffusion with gradual movement in a certain direction.
Figure 4.6A shows the SMSS values of individual trajectories of speckles across the three conditions tested. The arbitrary SMSS value ranges attributed to each type of diffusion are depicted on the graph. There was no significant difference between the three conditions, with the average SMSS values being 0.12-0.13. Although the average indicates restricted Brownian motion there are also speckles that do not move. Figure 4.6B highlights that 63% of the speckles fall into the restricted Brownian category, with the remainder being static. The proportion of speckles within each type of diffusion category does not change with oxygen levels or following the inhibition of prolyl hydroxylase enzymes.

Figure 4.6 | Diffusion mode of the HIF-2α speckles. A) Schematic representations of the types of diffusion. SMSS values for individual speckles in normoxia, hypoxia and DMOG. Average SMSS (indicated by red line) was $0.12 \pm 0.07$ (N=25, n=522), $0.13 \pm 0.07$ (N=24, n=760) and $0.12 \pm 0.08$ (N=22, n=1402), respectively. B) Percentage of speckles that fall into each category of diffusion. Average: static = 38%, restricted Brownian = 62%.
These results suggest that these speckles are physically hindered from diffusing out of a localised region. It is likely that they are trapped or, like other nuclear bodies (e.g. Hendzel et al. 2001), tethered to a physical structure (such as the nuclear scaffold).
4.1.3 Protein co-localisation

Immunofluorescence imaging and co-localisation analysis were performed to investigate the spatial relationship between HIF-2α and other proteins (Figure 4.7). To provide a benchmark, we measured HIF-2α co-localisation with its known binding partner HIF-1β, in normoxia and upon DMOG treatment. Quantification using a Manders’ analysis (Manders et al., 1993) revealed that 33% (standard deviation reported in Figure 4.7B) of HIF-2α co-localises with HIF-1β in normoxia which increases to 47% following treatment with DMOG (Figure 4.7; statistically significant; independent t-test; \(t_{95}=13.362, p<0.001\)). As HIF-2α is a transcription factor it was logical to then investigate whether HIF-2α is localising at sites of active transcription. Immunofluorescence was performed using an antibody against phospho(ser5)-RNAPII as a marker for active transcription sites and we found that around 48% of HIF-2α co-localises with the initiating form of RNAPII in normoxia and 54% in hypoxia mimic condition, a marginal yet statistically significant (independent t-test; \(t_{60}=4.242, p<0.001\)) increase.

Both of these results make sense given that treatment with DMOG should increase activity of HIF-2α by inhibiting all PHD enzymes and FIH. They indicate that half of the signal from HIF-2α co-localises with its dimerisation partner HIF-1β in hypoxia mimic conditions (DMOG) and that the same level also co-localises with active transcription sites. However, this type of co-localisation analysis is based on whether both red and green signal is present in the same pixel. It could be worth performing object-based co-localisation analysis to determine if the number of speckles that are co-localising changes. It may also be of interest to look at HIF-1β, HIF-2α and RNAPII simultaneously.
Figure 4.7 | Co-localisation of HIF-2α with HIF-1β and RNAPII A) First image in each panel (far left) is endogenous HIF-2α labelled with Alexa Fluor® 555 (red, pseudocolour). Second image (green, pseudocolour) is a nuclear protein labelled with Alexa Fluor® 488®. Merge = HIF-2α (red) image superimposed onto the image (green) of the adjacent nuclear protein. Coloc = co-localisation channel calculated using ImageJ plugin Co-localisation Threshold. White indicates pixels where both red and green signal is found i.e. co-localisation. Overlay = Merge image with coloc image superimposed. Inlay = magnified region (white square). White arrows highlight regions of co-localisation. Scale bar = 5 μm. Abbreviations: RNAPII = RNA Polymerase II phosphor serine 5. B) Immunofluorescent images were analysed using ImageJ plugin Co-localisation Threshold with uses the Coste et al method to automatically create a threshold prior to calculating the Manders coefficient for both proteins i.e. the percentage of protein A (HIF-2α) that co-localised with protein B (HIF-1β or RNAPII) and vice versa.

4.1.3.1 Other nuclear bodies

We then looked at whether HIF-2α co-localised with other nuclear proteins that are known to localise into nuclear bodies e.g. PML bodies. Previous studies have shown that transcription factors, such as p53 and NRF2, localise into PML bodies leading to the suggestion that one function of this nuclear body is the storage of inactive TFs (Fogal et al.,
However, immunofluorescent labelling of HIF-2α in HeLa cells stably transfected with PML-YFP show very little co-localisation (Figure 4.8A, top row). In the first instance this is not too surprising as PML bodies have been described as typically 0.5 µm in size and usually 10-30 per cell (Spector, 2001) which does not match the characteristics of the HIF-2α speckles. However, as HIF-2α is mainly inactive in normoxia one would think that more than 18% (Figure 4.8B) would be localised at PML bodies in normoxia, if they are in fact the storage site of inactive transcription factors.

Other well-studied nuclear bodies are SC35 domains also known as splicing factor compartments (SFCs) or interchromatin granule clusters (IGC), which house inactive splicing factors, such as SC35. (Wansink et al., 1993; Phair & Misteli, 2000). It was found that only 13% of HIF-2α co-localised with SC35, but 48% of SC35 co-localised with HIF-2α. The biological relevance of this is unclear. If SFCs store inactive splicing factors that move to the site of transcription when required, it would be logical to assume that, in the interest of efficiency, these sites would be within close proximity to these sites. Therefore if the HIF-2α speckles are also near to transcription sites then there is an increase chance of them occurring in similar locations. Then again, it could be down to the simple fact that there is a vast number of HIF-2α and the SFCs take up a large volume, therefore there is an increased chance of the two entities occurring next to each other.

HDAC5 is a histone deacetylase and localises in to domains that have been termed matrix associated deacetylase bodies (Downes et al., 2000). Images of this protein published in Downes et al. (2000) show a sub-nuclear localisation pattern very similar to that of HIF-2α (Figure 4.8A, third row down). We found that 27% of HDAC5-YFP co-localised with HIF-2α and 19% of HIF-2α co-localises with HDAC5-YFP (Figure 4.8B).

Finally, we investigated the localisation of hypoxia associated factor (HAF aka SART1_800). This protein has been demonstrated to interact with HIF-2α (Koh et al., 2011). However immunofluorescent imaging found that only 16% of endogenous HAF co-localises with HIF-2α and 12.5% of HIF-2α co-localises with HAF (Figure 4.8B).
Figure 4.8 | Co-localisation HIF-2α and other nuclear proteins. A) Immunofluorescent images of HIF-2α and other nuclear proteins that are known to localise into nuclear bodies. First image in each panel (far left) is endogenous HIF-2α labelled with Alexa Fluor® 555 (red, pseudocolour). Second image (green, pseudocolour) is a nuclear protein labelled with Alexa Fluor-488® (SC35, HAF) or YFP (PML and HDAC5). Merge = HIF-2α (red) image superimposed onto the image (green) of the adjacent nuclear protein. Coloc = co-localisation channel calculated using ImageJ plugin Co-localisation Threshold. White indicates pixels where both red and green signal is found i.e. co-localisation. Overlay = Merge image with coloc image superimposed. Inlay = magnified region (white square). White arrows highlight regions of co-localisation. Scale bar = 5 μm. Abbreviations: PML = Promyelocytic leukemia protein, HDAC5 = histone deacetylase 5, HAF = hypoxia associated factor, YFP = yellow fluorescent protein.B) Immunofluorescent images (Figure 4.7) were analysed using ImageJ plugin Coloc Threshold with uses the Coste et al method to automatically create a threshold prior to calculating the Manders coefficient for both proteins i.e. the percentage of protein A (HIF-2α) that co-localised with protein B (other nuclear protein of interest) and vice versa.
Overall, the results here indicate that a small proportion of HIF-2α co-localises with each of the nuclear proteins investigated. It is difficult to determine how significant these results are and whether they have any biological relevance. In some instances it is possible that the nuclear bodies are actually separate entities, but diffraction limited microscopy cannot resolve the two so it appears as if they cohabit the same location (such as PML and HIF-2α). Furthermore, we didn’t determine what the majority of HIF-2α is co-localising with, if anything. It is possible that the HIF-2α speckles are a novel nuclear domain and / or that they are specific for HIF-2α (although most, if not all, nuclear speckles that have been described in the literature are cohabited by more than one protein).

Admittedly, only a small range of proteins were investigated here. However, randomly picking proteins that happen to localise into nuclear speckles and performing immunofluorescent staining is a costly and somewhat “needle in a haystack” approach, so we moved on to other methods that could provide information on the function of these speckles. For further co-localisation studies, a more targeted approach should be taken such as performing mass spectrometry on a pull down of HIF-2α or EGFP-HIF-2α. From the resulting list of proteins, suitable candidates (e.g. nuclear proteins reported to localise into speckles etc.) could be identified and then investigated using immunofluorescence.
4.1.4 Molecular Mobility

Next, we investigated the mobility of HIF-2α at the single molecule level. Molecular mobility could provide insight into the function of these speckles, for example no movement of molecules between speckles could indicate a storage or sequestration whereas molecule mobility between the speckles and the rest of the nucleus could indicate a release or a change in protein availability / activity such as being released from storage sites.

4.1.4.1 Nuclear proteins and FRAP

FRAP has been key in determining kinetic information (such as chromatin binding time) of over 50 transcription factors (TFs) (Mazza et al., 2012). Interestingly, it has been found that TFs are highly dynamic and bind transiently to target binding sites within genes leading to an overhaul of the longstanding model that TFs form stable complexes (Hemmerich et al., 2011; Mazza et al., 2012).

FRAP has also been used to study the dynamics of RNAPII in the nucleus. Kimura et al. (2002) found that 75% is highly mobile and 25% is immobile, additionally they found that these two fraction correlate with inactive and actively transcribing RNAPII, respectively. These data back up the theory that active RNAPII is present at transcription factories (see chapter 1.5.1) whilst the inactive form is freely diffusing through the nucleoplasm.

Photobleaching has also been used to study PML bodies. PML and SP100, which co-localised at PML bodies, show no recovery following bleaching within the nucleoplasm or PML bodies themselves whereas CBP, that also localises to PML bodies under certain conditions, is highly mobile and was shown to move in and out of PML bodies rapidly (Boisvert et al., 2001). Thus supporting the conclusion that PML and SP100 play a structural role in PML bodies but also highlighting that components of the same nuclear body can have different mobility, which can correlate with function.

4.1.4.2 Molecular mobility of HIF-2α

Firstly, FRAP experiments were performed on HeLa cells ectopically expressing EGFP-HIF-2α in normoxia, hypoxia and 6 h after treatment with 0.5 mM DMOG. This was achieved by bleaching half the nucleus and measuring the fluorescence levels in this region over time.
(Figure 4.9). The fractional recovery curve of HIF-2α was determined and fitted using a one component exponential equation (see Methods). The mobile fraction (the amount of molecules freely diffusing) and half time (the time taken for the fluorescence in the bleached region to reach half the eventual recovery) of HIF-2α was extrapolated from the fitted curves (see Methods).

Figure 4.9 | EGFP-HIF-2α FRAP. A series of confocal images of HeLa cell ectopically expressing EGFP-HIF-2α in the nucleus that has been photobleached. Images at different time points showing gradual recovery of fluorescence into the bleached region (outlined in white).
Figure 4.10 | Comparing EGFP-HIF-2α mobility in different conditions. A) The percentage of mobile EGFP-HIF-2α molecules (mobile fraction) per nucleus. The average (± SD) mobile fraction of EGFP-HIF-2α was 0.95 ± 0.15 (n=89) in normoxia, 0.99 ± 0.16 (n=115) hypoxia and 0.94 ± 0.13 (n=43) following treatment with DMOG. For HIF-2α-DM-EGFP mobile fraction was 0.9 ± 0.15 (n=23). B) The time taken for recovery to reach half the final recovery (half time / t_HALF) per nucleus in normoxia, hypoxia and DMOG. The average (± SD) half time was 0.56 ± 0.46 (n=89) minutes, 0.68 ± 0.36 (n=115) minutes and 0.40 ± 0.18 (n=43) minutes, respectively. The half time for HIF-2α-DM-EGFP was 0.44 ± 0.31 (n=23) minutes. Independent t-test (α=0.05) was used to compare the mean normoxic half time to those from the hypoxic (t_{200}=2.122, p=0.35) and hypoxia mimic (t_{130}=2.156, p=0.033) conditions. Average represented by black line on graph.
It was found that the average mobile fraction across the three conditions was 96% (Figure 4.10A) suggesting that a significant amount of HIF-2α molecules are freely diffusing through the nucleus. The average half time was calculated to be 34, 41 and 24 seconds in normoxia, hypoxia and hypoxia mimic, respectively (Figure 4.10B). Although the results from the statistical test suggest that the average half times in hypoxia and DMOG for wild type HIF-2α are significantly higher than that for the normoxic sample, the actual values are only marginally different therefore are unlikely to have a great biological impact.

In line with the observation that hypoxia or DMOG has little effect on HIF-2α mobility, FRAP was performed on a constitutively stable HIF-2α (the two prolyl residues within the ODD domain have been substituted for alanines and so cannot undergo PHD-dependent degradation) and the mobile fraction and half time was found to be very similar to that of wild type HIF-2α treated with DMOG.

To confirm the observations made for wild type HIF-2α, a complimentary photoperturbation technique, Fluorescence loss in photobleaching (FLIP), was used. In FLIP experiments the GFP positive cells are continually bleached in the same region and the fluorescent signal in the non-bleached region is measured (Figure 4.11A), therefore measuring the signal decay in the non-bleached region. This technique can be used to determine molecular mobility (like FRAP) but also provides information on the movement of molecules around and between cellular compartments (Mueller et al., 2013). In the case of the HIF-2α speckles, continual bleaching in a specific region of the nucleus resulted in loss of fluorescent signal in the non-bleached region in all three conditions within 15 minutes (Figure 4.11B). The fluorescence loss data were plotted in Matlab and fitted using a one component exponential equation. The resulting curve was used to determine the half times of EGFP-HIF-2α (Figure 4.11C). Overall these results show no difference in the molecular mobility of EGFP-HIF-2α between the three conditions. However, it does highlight that HIF-2α must be continually moving in and out of the speckles (and throughout the nucleus) to have complete loss of fluorescent signal.

It is worth noting that, although not significantly different based on statistical testing, the half time values from the FRAP (Figure 4.10C) and FLIP (Figure 4.11B) experiments follow the same pattern across the three conditions. This could be of biological interest however further experiments would be required to confirm whether this is a coincidence.
Spatial localisation of HIF-2α

Figure 4.1 | Molecular mobility of HIF-2α measured using FLIP. A) Confocal images of a HeLa cell ectopically expressing EGFP-HIF-2α that has been continually bleached in one region (red box). Nucleus outlined in yellow. White arrow highlights the loss of fluorescence in the non-bleached region of the nucleus. B) Overview of the average trend of fluorescence loss in normoxia, hypoxia and DMOG. Y-error bars represent standard deviation. The data was grouped (“binned”) based on time and the X-error bars represent the standard deviation of these bins. C) Half time values were extrapolated from the curves fitted (as in B) for each cell in the three conditions. Average half time (represented by black line on graph) ± SD: Normoxia = 3.47 ± 1.98 (n=53), Hypoxia = 3.96 ± 1.61 (n = 36), DMOG = 2.88 ± 0.88 (n = 23).
4.1.4.3 Molecular mobility of HIF-2α compared to HIF-1α

The molecular mobility of HIF-2α was compared to that of HIF-1α. FRAP experiments were only performed on HIF-1α-EGFP in hypoxia or following DMOG treatment, as there is no detectable fluorescent protein in normoxia (Bagnall, 2011).

It was found that the mobile fraction of HIF-1α-EGFP was 92% in both conditions (Figure 4.12A). This mobile fraction is significantly lower than that of EGFP-HIF-2α in hypoxia (99%, Figure 4.10A). This could be due to HIF-1α activating genes as part of the hypoxic response. It has been proposed that HIF-1α is the dominant transcription factor in the acute response to hypoxia whereas HIF-2α is responsible for regulation of genes in chronic hypoxia (>48 h). As FRAP looks at the whole population of molecules, if sufficient HIF-1α is bound at promoters then a global effect on the mobility could be observed. The difference is significant but small (7%).

Conversely, the difference in half time is much greater (Figure 4.12B). These measurements suggest that HIF-1α-EGFP is more than 10-fold and 5-fold faster than EGFP-HIF-2α in hypoxia and hypoxia mimic conditions, respectively. The rapid recovery of HIF-1α is typical of that seen with other transcription factors (Hager et al., 2009). The slower recovery of mobility of HIF-2α is unusual but could simply be explained by the fact that it is in nuclear speckles which physically impedes its mobility whereas HIF-1α-EGFP is dispersed homogenously throughout the nucleus with only obstructions (such as chromatin) and molecular crowding to hinder its movement.

It should also be noted that although tests were performed in our lab to establish that the orientation of the GFP fusion protein (whether at the N- or C-terminus of the protein of interest) had little influence on the transcriptional activity of HIF-1α or HIF-2α (Bagnall, 2011) the mobility results could be affected by the fluorophore orientation; here the fluorophore is attached at different ends of the two proteins of interest. Wotzlaw et al. (2011) demonstrated using FRET that the orientation of the fluorescent tag can influence hetero-dimerisation of HIF-1α and HIF-1β, so there is a chance that the orientation of the fusion protein could also impact physical interactions of HIF-2α, which would in turn affect molecular mobility.
**Figure 4.12 | Comparing EGFP-HIF-2α and HIF-1α-EGFP mobility.** The average (± SD) mobile fraction and half time for EGFP-HIF-2α are the same as in figure 4.9. Only hypoxia and DMOG conditions are compared as HIF-1α-EGFP can’t be observed in normoxia and so can’t be photobleached. A) Mobile fraction of EGFP-HIF-2α compared to HIF-1α-EGFP. The average (±SD) mobile fraction of HIF-1α-EGFP per nucleus was 0.92 ± 0.11 (n=31) hypoxia and 0.92 ± 0.12 (n=29) following treatment with DMOG (0.5 mM, 6 h). Independent t-test (α=0.05) revealed that the mobile fraction of HIF-1α-EGFP in hypoxia is significantly less than EGFP-HIF-2α (t\(_{144}=2.412, p=0.017\)). B) The half time of EGFP-HIF-2α compared to that of HIF-1α-EGFP in hypoxia and DMOG. The average (±SD) half time was 0.06 ± 0.04 (n=31) minutes and 0.06 ± 0.03 (n=29) minutes, respectively. In both hypoxic (independent t-test: t\(_{34}=9.532, p<0.001\)) and DMOG (independent t-test: t\(_{70}=10.278, p<0.001\)) conditions the half time of HIF-1α-EGFP was significantly less than EGFP-HIF-2α. Average values represented by black line on graph.
For all FRAP experiments, a large variation in the shapes of the recovery curves was observed. This could be due to the inherent heterogeneity of protein expression often seen within a population of transfected cells. A clonal cell line stably expressing a fluorescently tagged HIF-2α or a tuneable expression system where the expression can be increased to desired level using a drug would remove this issue. In addition to this, a major drawback of photobleaching experiments is that only the photo-physical properties are altered via the photobleaching, meaning that the protein of interest is still present and presumed to be functional but can no longer be seen. Therefore, it would be beneficial to utilise photo-convertible proteins such as Dendra2. Here, instead of turning the fluorescent signal off by photobleaching, the signal is changed from green to red using a specific wavelength of light. Using a system such as this would result in the speckles and molecules remaining visible yet distinguishable from the non-converted molecules, therefore providing bi-directional data i.e. one can monitor molecules moving out of the photo bleached region as well as those moving in.

We have performed some spatial analysis on the FRAP experiments by tracking the speckles during bleaching and have observed some cases of recovery in the same place (Supplemental movies M5, M6 and M7), complementing the SMSS data and the possibility that these speckles might be tethered structures in specific locations. However, photo-convertible proteins would allow the switched speckles to remain visible during recovery, thus allow continued monitoring of speckle location and provide more conclusive evidence.

Finally, it would be worth comparing both N- and C-terminally tagged HIF-2α and HIF-1α to make sure that this does not have an influence on the molecular mobility.
4.2 Conclusion

Here we have shown that both endogenous and exogenous HIF-2α display heterogeneous distribution in the nucleus. We observed a small change in the number and movement of these speckles when comparing different conditions. Although statistically significant, the differences are marginal and the biological relevance of these results is difficult to determine. Photobleaching experiments revealed that HIF-2α is freely diffusing in and out of the speckles and throughout the nucleoplasm. Finally, there is some indication that these could be domains that are associated with or ‘tethered’ to a structure such as the nuclear matrix.

Hendzel et al. (2001) proposed that there are “transcription factor-enriched foci” within the nucleus that act to: concentrate functionally-related proteins in order to streamline assembly of macro-molecular complexes; and, control the concentration of the proteins / complexes in the nucleoplasm. One thought was that the HIF-2α speckles could have a purpose similar to the latter. Stemming from the fact that both endogenous and ectopically expressed HIF-2α is detectable in normoxia (HIF-1α is not), we hypothesised that perhaps HIF-1α is regulated in an oxygen-dependent manner via protein stability (as widely accepted) but HIF-2α is regulated spatially i.e. the alpha subunit accumulates in nuclear speckles and is physically impeded from activating genes in normoxia. However, this has proved somewhat difficult to verify.

In this study, we have shown that EGFP-HIF-2α is continuously moving in and out of speckles, regardless of oxygen levels or PHD enzyme activities. Therefore, it would seem unlikely that these speckles are storage sites, as one would expect a change in the mobile fraction as HIF-2α is released to find and bind to promoters of hypoxia inducible genes. However, it is possible that HIF-2α is localising at a number of sites that have different functions. CREB binding protein (CBP) has been shown to localise with p300 in small foci in the nucleus (Hendzel et al., 2001) but also to transiently localise at PML bodies (Boisvert et al., 2001). So, if one imagines that there are different sub-populations of HIF-2α e.g. some are activating genes, some are forming transcriptional complexes and some are being sequestered, then the results from the co-localisation study become more meaningful.
To probe the function of the HIF-2α speckles further, more advanced techniques such as single molecule tracking (SMT) should be employed which forms the basis of the following chapter.
Chapter 5: Development of tools for single molecule tracking in living cells
We have utilised advanced confocal microscopy techniques, FRAP and FLIP, to investigate the dynamics of HIF-2α and found no considerable changes in the molecular mobility under different conditions. However, these techniques look at the global behaviour of the protein being studied and the ‘averaging effects’ of these methods may be masking the dynamics of individual molecules. In addition to this we have not established the residence time of HIF-2α molecules in the speckles and whether this changes with oxygen potential. Therefore, the aim was to label HIF-2α with gold nanoparticles and image in the nucleus of living cells, using photothermal heterodyne imaging (PHI). This methodology facilitates the tracking of single gold nanoparticles (and, therefore, single molecules), as demonstrated by Duchesne et al. (2012). The single molecule data generated using this technique would provide information on: the movement of HIF-2α between speckles; movement in and out of the nucleus / speckles; and, provide information on velocity and degradation. All of which, could contribute to the elucidation of the function of the HIF-2α nuclear bodies.

As discussed in the introduction (Section 1.7.2.5), SMT can be performed using fluorescently probes such as dyes, proteins or semi-conductor nanocrystals (aka quantum dots), however, this approach has drawbacks. Firstly, probes such as fluorescent dyes and proteins photobleach and only short trajectories of ~10 s can be acquired, even with very careful illumination. Secondly, quantum dots emit a fluctuating and sporadic fluorescent signal. This temporary loss of signal causes issues when tracking molecules (Kuno et al., 2001). Moreover, for optimum results, as much out of focus light as possible must be removed during acquisition, which has led to research groups using more specialist custom-built microscopes, such as those reviewed in Mueller et al. (2013).

Gold nanoparticles are a useful and complementary alternative to fluorescent labels. They have several features that makes them ideal for SMT. Firstly, they are optically stable and do not suffer from the equivalent of photobleaching. Single nanoparticles can be imaged for as long as required (or as long as the stability of the microscope permits) without loss of any signal (Lasne et al., 2006). Secondly, the photothermal signal from gold nanoparticles is continuous. Thirdly, the photothermal signal from gold nanoparticles has little or no background, except for a weak and distinguishable signal from mitochondria (Lasne et al., 2007). Taken together, all these features make gold nanoparticles an ideal probe for single molecule tracking.
Gold nanoparticles also offer imaging versatility as they can also be visualised with an electron microscope, thus providing more information on sub-cellular localisation at molecular scale resolution. More importantly, however they can be specifically and stoichiometrically functionalised, as described in Duchesne et al. (2008), Duchesne et al. (2012) and Nieves et al. (2014). Briefly, gold nanoparticles require passivation i.e. the synthesis of a monolayer composed of small ligands (peptides, alkane thiol ethylene glycols with or without peptidols) on their surface that prevents non-specific binding (Lévy et al., 2004; Duchesne et al., 2008). The self-assembled ligand shell can also be functionalised by inserting other biological molecules, such as a nuclear localisation sequence (NLS) that will target the gold nanoparticles to the nucleus. Here the aim was to insert a ligand (Halotag ligand) that will allow the attachment of a protein of interest (POI) via the Halotag protein, facilitating the single molecule dynamics to be monitored by tracking the gold nanoparticles.

Single molecule imaging using gold nanoparticles has previously been successfully utilised in Liverpool to measure how fibroblast growth factor-2 (FGF2) moves through the extracellular matrix via its heparan sulphate binding sites (Duchesne et al., 2012; Nieves et al., 2015). Purified, recombinant HisTag-FGF2 was attached to gold nanoparticles via Tris-Ni\(^{2+}\)NTA. Although this method was able to provide useful insight into the single molecule dynamics of FGF2, the Tris-Ni\(^{2+}\)NTA on the surface of the gold nanoparticles can be prone to exchange with histadine patches on the surface of endogenous proteins, thereby disrupting the bond between the POI and the probe and so confounding single molecule tracking results. Therefore, the aim here was to utilise the Halotag\(^{®}\) as a means to develop a novel method for covalent and stoichiometric (1:1) conjugation of proteins to the surface of a gold nanoparticles.

The Halotag\(^{®}\) (Promega) is a modified haloalkane dehalogenase protein that has been designed to bind synthetic ligands that carry a chloroalkane (Los et al., 2008). This reaction is highly specific, as there is no corresponding activity in mammalian cells, and irreversible, since a mutation (His272Phe) in the catalytic centre means that the reaction proceeds no further than the covalent intermediate of substrate-enzyme (Los et al., 2008). The strategy was to incorporate the Halotag ligand (a chloroalkane ligand, see Appendix 1.5) onto the surface of the gold nanoparticle and react this with a Halotag fusion protein. Figure 5.1 gives an outline of the approach.
Figure 5.1 | Overview of GNP-Halotag plan. Flow chart highlighting the key steps to tracking single molecules in living cells. 1) The aim is to incorporate the Halotag ligand into the ligand shell of gold nanoparticles (10 nm gold core) and 2) express and purify recombinant Halotag fusion protein. 3) The GNP-Halotag and Halotag fusion will react to form GNP’s carrying the protein of interest (GNP-Halotag-POI). 4) The GNP-Halotag-POI will be delivered to the cells. For extracellular proteins such as FGF-2 (used for proof of principle experiments), the GNP-Halotag-FGF2 are added to the medium and bind to the surface of cells. For intracellular proteins such as HIF-2α, this requires delivery into the cells via microinjection. 5) The single GNPs are visualised and imaged over time using photothermal microscopy. Individual GNPs are tracked allowing the molecular dynamics of the proteins to be determined.
The initial challenges were:

1) The synthesis of gold nanoparticles carrying the Halotag ligand

2) The conjugation of the gold nanoparticles to a protein of interest

3) The expression and purification of a full length recombinant HIF-2α

The work described in this chapter was a collaborative effort between me and members of both Prof. D. G. Fernig’s and Dr R. Lévy’s research groups (University of Liverpool, UK). The contributions by each individual were:

- Changye Sun
  - Together we expressed and purified Halotag-FGF2
  - Made the pET14B-Halotag-FGF2 construct
  - Provided advice and guidance on expression of Halotag-HIF-2α and contributed to troubleshooting
  - Contributed reagents

- Dr Umbreen Shaheen
  - Made GNPs carrying Halotag ligand for Halotag-FGF2 work
  - Guided me in making Halotag ligand GNPs for Halotag-HIF-2α work

- Dr Raphaël Lévy
  - Contributed reagents and equipment
  - Provided advice about gold nanoparticle synthesis and conjugation

- Prof Dave Fernig
  - Contributed reagents and equipment
  - Provided advice and guidance regarding protein expression and chromatography

- Dr Daniel Nieves
  - Performed photothermal imaging
  - Provided advice and guidance on protein purification and gold nanoparticle synthesis

- Dr Joanna Wnetrzak (CCI, University of Liverpool)
  - Performed microinjection
5.1 Can the Halotag be used for conjugation of a protein to a gold nanoparticle?

5.1.1 Testing the Halotag approach for labelling a protein of interest with gold nanoparticles

To investigate whether the Halotag can be used to conjugate a protein of interest to a gold nanoparticle, we employed an N-terminal Halotag-fibroblast growth factor 2 (FGF2) fusion protein. We chose to use FGF2 to carry out preliminary experiments, as our collaborator Prof. D. G. Fernig (University of Liverpool) and his research group have substantial experience in expressing and purifying recombinant FGFs (in particular FGF2). Since FGF2 is an extracellular protein that binds to components of the extracellular matrix such as heparan sulfate, obtaining biological validation is easier, as it does not require delivery into the cell. As a proof of principle, we would express and purify Halotag-FFG2, synthesise gold nanoparticles carrying the Halotag ligand and conduct experiments to test whether FGF2 can be labelled with gold-nanoparticles via the Halotag reaction. The work on the production of Halotag-FGF2 has been published (Sun et al., 2015).

5.1.1.1 Expressing recombinant Halotag-FGF2

A bacterial expression vector, pET14B-Halotag-FGF2, encoding the Halotag fused to the N-terminus of full-length FGF2 via a linker region was made by Changye Sun (Prof. D. G. Fernig research group, University of Liverpool). Figure 5.2A illustrates the key characteristics of the Halotag-FGF2 fusion protein.
Although Prof Fernig’s group have worked with many recombinant FGFs, the addition of the Halotag can affect both the bacterial expression and purification of the recombinant protein. Cultures of C41 DE3 *E. coli*, a strain commonly used for expression of recombinant proteins, transformed with the Halotag-FGF2 construct were incubated for 4 h at 37 °C following the induction of protein expression (via the addition of IPTG) (Xu *et al.*, 2012). Analysis by SDS PAGE and coomassie staining showed a large induced band close to 50 kDa in both samples (Figure 5.2B, lanes labelled I) compared to the control culture (no IPTG added, lane C).

In order to determine the solubility of the expressed Halotag-FGF2, the bacteria (induced sample) were lysed via sonication. Following centrifugation, the soluble (supernatant) and insoluble (pellet) proteins were analysed by SDS PAGE. A band at 50 kDa was observed in both the pellet and supernatant suggesting that both soluble and insoluble Halotag-FGF2 is present (Figure 5.2B, lanes P and S, white arrows). Taking together the fact that the purification of soluble protein is a more straightforward process compared to that of insoluble proteins and that there was good expression of Halotag-FGF2 in the soluble fraction, the fusion protein was purified from the supernatant (Figure 5.2, lane S).
5.1.1.2 Optimisation of Halotag-FGF2 purification protocol

Purification of FGF2 utilises its affinity for heparin, so heparin affinity chromatography was employed for the initial purification of the Halotag-FGF2 fusion protein. Figure 5.3 shows preliminary chromatography of the soluble protein fraction. Analysis via SDS PAGE shows that a protein corresponding to the expected size of Halotag-FGF2 is present in each eluted fraction and that the contaminants are gradually removed resulting in a final eluate that was judged to be reasonably pure Halotag-FGF2 (Figure 5.3B, Blue Box).

Figure 5.3 | Trial purification of Halotag-FGF2. A) The steps taken to purify Halotag-FGF2. B) The soluble protein fraction was applied to Heparin Affinity Gel (BioRad, UK). The eluted protein underwent buffer exchange via gel filtration (G25) chromatography and then applied to a CM cation-exchange column (IEX). Finally, the eluate from IEX was applied to a second heparin affinity column and eluted with a gradient of NaCl (using a BioRad Econo pump (EP-1) fitted with UV monitor; set up as follows: 0-70%, 70 minutes. Buffer B = PBS set at 6%, Buffer A = 1.4M NaCl) 29 fractions were collected, fractions 5-29 contained with protein (eluted at approximately 34-68%) and were combined for analysis via SDS PAGE (H(2)). (S = soluble fraction, FT = flow through, W = wash with 0.5 M NaCl, E = fraction eluted with 1.4 M NaCl, GF = Sample following buffer exchange via gel filtration chromatography, IEX = Sample eluted from CM resin column with 0.4 M NaCl, black arrows = contaminants, blue box = reasonably pure Halotag-FGF2)

To investigate further the binding of Halotag-FGF2 and to determine which additional chromatography steps could be used and the sequence to use them in to further purify the fusion protein, small–scale purification was then performed on the Halotag-FGF2 (Figure 5.3B, blue box). Three chromatography methods: heparin affinity, cation-exchange (CM resin, negatively charged) and anion-exchange (DEAE resin, positively charged), were
tested. Each of these purification methods exploits a different feature of the recombinant protein, namely the binding affinity of FGF2 to heparin, the strong positive charge / very basic pI of FGF2 and, the negative charge / acidic pI of the Halotag. The chromatography was performed with Halotag-FGF2 and TEV-digested Halotag-FGF2.

Analysis via SDS PAGE shows that the Halotag-FGF2 bound to heparin, DEAE and CM resins (Figure 5.4) highlighting that all three methods can be used for purification of the fusion protein. The samples digested with TEV show that these results are due to FGF2 binding to the heparin and CM resin (Figure 5.4A and B, red arrows), and the Halotag binding to the DEAE resin (Figure 5.4C, yellow arrow), as predicted.

Based on these findings the Halotag-FGF2 was purified using all three chromatography steps in the following order: heparin affinity, anion-exchange and cation-exchange chromatography. Details of the final protocol can be found in Sun et al. (2015).
**Figure 5.4 | Halotag-FGF2 purification tests.** Halotag-FGF2 was incubated with TEV protease overnight (digested) and applied to a heparin column (A). Digestion results in additional bands of approximately 30 kDa, 27 kDa and 18 kDa, which represent the Halotag, TEV enzyme and FGF2. Samples of undigested and digested Halotag-FGF2 samples were applied to a CM (B) resin column (FT = flow through, W = PBS wash, 0.3 M = 0.3 M NaCl wash, 2 M = 2 M NaCl wash) and DEAE (C) resin column (FT = flow through, W1=PB wash, W2 = PBS wash, 0.3 M = 0.3 M NaCl wash, 2 M = 2 M NaCl wash).
5.1.1.3 Testing the functionality of purified Halotag-FGF2

Following purification, it was important to determine that both parts of the fusion protein were folded correctly and had retained their function. The fact that the protein bound to heparin and eluted similarly to native FGF2 indicated that the FGF2 possessed an intact primary heparin binding site. Since this is formed from amino acids that are physically adjacent in the tertiary structure, but distant in the protein sequence, it was likely that the FGF2 was correctly folded. Other data demonstrating that the HaloTag-FGF2 protein retains the same activity as the native FGF2 such as stimulating the phosphorylation of p42/44MAPK are described in Sun et al. (2015).

Figure 5.5 | Testing the activity of Halotag-FGF2. A) Halotag-FGF2 and Halotag-FGF2 digested with TEV protease were labelled with Oregon Green fluorescent Halotag ligand and analysed via SDS PAGE. The fluorescently labelled protein was visualised with an ImageQuant LAS 4000 imager (Sybr green settings). B) 3 µM Halotag-FGF2 was labelled with the red fluorescent (TMR Halotag) ligand (7.5 µM) for 30 minutes at room temperature. The TMR-Halotag-FGF2 was added to Rama 27 fibroblasts (final concentration 2 nM), incubated at room temperature for 1 hour and imaged using a Zeiss LSM 710 confocal microscope. The images here were taken with C-apolochromat 63×/1.20 Oil immersion objection, 2.1 zoom. Scale bar represents 10 µm.

To determine whether the Halotag is functional, the Halotag-FGF2 and Halotag-FGF2 digested by TEV were labelled with a Halotag ligand conjugated to a fluorescent dye (Oregon Green). Figure 5.5A shows a fluorescent band at approximately 50 kDa in the undigested sample and a one at 30 kDa following digestion with TEV, which represent the Halotag-FGF2 and Halotag, respectively. This highlights that the Halotag is functional (i.e. can bind its ligand) when it is part of the Halotag-FGF2 fusion protein and after cleavage from FGF2.

As it was determined that both parts of the chimeric protein were functional, the Halotag-FGF2 was reacted with a Halotag ligand carrying a fluorescent dye (TMR) before labelling Rama 27 cells. Figure 5.5B shows that the TMR-Halotag-FGF2 associated with the surface of the cells. The lack of signal in the regions in between the cells highlights that the TMR-
Halotag-FGF2 has specifically bound to the cells, likely through the binding of FGF2 to heparan sulfate present in the pericellular matrix.

Following the acquisition of these images, the cells were washed with 2M NaCl to see if the interactions between FGF2 and heparan sulfate could be disrupted. Images were acquired using the same settings and a reduction in TMR-Halotag-FGF2 signal was observed, further contributing to the suggestion that the labeling of the cells observed in Figure 5.5B is due to FGF2 binding heparan sulfate.

5.1.1.4 Labelling Halotag-FGF2 with gold nanoparticles via the Halotag ligand

Since it was shown that TMR-Halotag-FGF2 could bind to the pericellular matrix of cells, the next step was to substitute the fluorescent label with gold nanoparticles. Gold nanoparticles carrying Halotag ligands (chloroalkane, see Appendix) on the surface were prepared by Dr. U. Shaheen (Raphaël Lévy group, University of Liverpool). Following conjugation, approximately 5 nM Halotag-FGF2 labelled with gold nanoparticles (GNP-Halotag-FGF2) was added to the medium of Rama 27 cells (Labelling protocol same as for TMR-Halotag-FGF2). Figure 5.6 (right panel) shows the cells clearly labelled with the gold nanoparticles. As a control, Rama 27 cells were also incubated with nanoparticles lacking Halotag-FGF2 and almost no photothermal signal was observed (Figure 5.6, left panel). These results strongly suggest that the Halotag can be utilised as method of conjugating a recombinant protein of interest to gold nanoparticles, thus providing a platform for single molecule imaging.
In this section, it has been shown that: Halotag-FGF2 can be expressed in C41 DE3 E. coli and purified using a three step chromatography protocol; both the FGF2 and Halotag parts of the purified protein are functional; and, FGF2 can be labelled via the Halotag with fluorescent dyes and most importantly GNPs. The work described here contributed to a paper entitled: “Halotag is an effective expression and solubilisation fusion partner for a range of fibroblast growth factors” (Sun et al., 2015).

5.2 Expression & purification of Halotag-HIF-2α

The next step was to follow the strategy used for FGF2 with HIF-2α. By expressing and purifying Halotag-HIF-2α from E.coli it would then be possible to conjugate it to gold nanoparticles carrying the Halotag ligand.

A DNA vector (pFN21AB4384) containing the Halotag-HIF-2α gene was purchased from Kazusa DNA Research Institute, Japan. In order to check the spatial localisation of the Halotag-HIF-2α in cells, this plasmid was transiently transfected into HeLa cells and labelled with the Oregon-Green Halotag ligand (Promega, WI, USA). Figure 5.7A shows that the Halotag-HIF-2α fusion protein localises into discrete foci in the nucleus, as observed with fluorescent protein fusions and endogenous HIF-2α. Figure 5.7B shows analysis of protein extracts from HeLa cells transiently transfected with CMV-Halotag-HIF-2α via SDS PAGE and

Figure 5.6 | Photothermal images of FGF2-Halotag-nanoparticles. Photo thermal images of Rama 27 cells labelled with unconjugated nanoparticles (Control; left) or Halotag-FGF2 conjugated gold nanoparticles (GNP-Halotag-FGF2; right). The control GNPs or the Halotag-FGF2-GNPs were added to the medium and after 1 hour the cells were washed and imaged on a photothermal microscope (imaging carried out by Dr D. Nieves, Raphaël Lévy group, University of Liverpool, UK). Scale bar represents 10 µm.
western blot. The samples were labelled with the Oregon Green Halotag ligand prior to separation on SDS PAGE and fluorescent bands were observed at 150 kDa (Figure 5.7B, top panel). Following this, the proteins were transferred onto nitrocellulose membrane and probed with a HIF-2α antibody. Western blot revealed one band at around 100 kDa and another at 150 kDa, representing endogenous HIF-2α and the ectopically expressed Halotag-HIF-2α, respectively (Figure 5.7B, bottom panel). These data show that the Halotag-HIF-2α ectopically expressed in mammalian cells is 150 kDa, can react with the Halotag ligand and exhibits HIF-2α immuno-reactivity. Further to this, confocal microscopy confirmed that fluorescent labelled Halotag-HIF-2α and EGFP-HIF-2α co-localise. All of these results suggest that the Halotag is functional and does not interfere with the spatial localisation of HIF-2α.

![Figure 5.7][1] Halotag-HIF-2α recombinant protein in HeLa cells. A) Live HeLa cell transfected with the Halotag-HIF-2α plasmid and labelled with the fluorescent Oregon Green Halotag Ligand. Fluorescence is localised in nucleus in speckles. Scale bar = 5 μm. B) Protein extracted from HeLa cells transfected with pFN21A-Halotag-HIF-2α. Samples were labelled with the fluorescent Oregon Green Ligand and analysed via SDS PAGE. (N = normoxia, H = hypoxia, D = 0.5 mM DMOG). C) Confocal images of HeLa cells co-transfected with EGFP-HIF-2α and Halotag-HIF-2α and labelled with the TMR conjugated Halotag ligand.
5.2.1 Expressing Halotag-HIF-2α in bacteria

pETM-11-Halotag-HIF-2α was transformed into three bacterial strains that are commonly used for protein expression: C41 DE3, BL21 DE3 and BL21.pLysS DE3. Figure 5.8 shows gels stained for total protein (left panel) and HIF-2α (western blot, anti-HIF-2α, right panel) for the three strains tested. Different protein expression conditions (variation in temperature and time post induction with IPTG) were trialled. The coomassie staining (Figure 5.8, left) shows no strong induction of protein expression in the cultures treated with 1 mM IPTG compared to the control cultures in all three bacterial strains. Immuno-reactivity was detected in some, but not all, of the induced samples across the three strains. Two bands very close in size are detected at approximately 150 kDa, the expected size for Halotag-HIF-2α, and some additional bands at lower molecular weights (Figure 5.8, right). The 29.8 °C, 16 h sample in the BL21 and BL21.pLysS E.coli showed very strong immuno-reactivity, yet this was not correlated with any signal obtained by coomassie staining, suggesting that there is little Halotag-HIF-2α protein present.
A faint induced band could be seen at around 120 kDa (Figure 5.8, left, black arrows), which is slightly smaller than the expected size of Halotag-HIF-2α. However, analysis of bacteria transformed with different pETM11 constructs shows an induced band at around 120 kDa in all samples treated with IPTG, which suggests that this band corresponds to an endogenous protein (Figure 5.9).
Figure 5.9 | Induction of protein expression from different pETM-11 constructs. BL21.pLysS DE3 E.coli transformed with an empty pETM-11, pETM-11-Halotag-HIF-2α and pETM-11-FGF1 were incubated for 4 h at 37 °C, post induction of protein expressing via addition of IPTG. An additional condition of 16 h at 29.8 °C was included for Halotag-HIF-2α. A control sample of Hela cells ectopically expressing Halotag-HIF-2α was run alongside the samples. A) Coomassie Yellow arrows = induced protein. B) Fluorescent Oregon Green Halotag ligand. Image inverted to aid visualisation of the bands. Red arrows = proteins labelled with Halotag ligand.
Although a band corresponding to recombinant Halotag-HIF-2α cannot be seen with coomassie staining, analysis via western blot with an anti-HIF-2α antibody highlights that there is potentially some Halotag-HIF-2α present, albeit in small amounts (Figure 5.10A & B). Samples from the soluble (supernatant) and insoluble (pellet) fractions of BL21.pLysS (37 °C, 4 h) were labelled with the fluorescent Oregon Green Halotag ligand and analysed by SDS PAGE (Figure 5.10C). The fluorescent bands on the gel suggest that there is protein present that reacts with the Halotag ligand, but none of these are the expected size of 150 kDa (Figure 5.10C). There is a strong band at around 40 kDa which is likely to be the Halotag (Figure 5.10C, Lane S, black arrow). In the insoluble fraction (Lane P) there are multiple bands (Figure 5.10C, white arrowheads), suggesting either the expression of proteins of different sizes or protein degradation or incomplete transcription/translation. Either way, these results indicate that the Halotag is being expressed, but the HIF-2α part of the fusion protein is not complete.

Figure 5.10 | BL21.pLysS DE3 E.coli expression and solubility test. BL21.pLysS DE3 E.coli incubated at 37 °C for 3 h post IPTG addition (+). Control sample (−) = no IPTG added but incubated in the same conditions. A) Coomassie stained SDS Page. B) Protein was transferred to nitrocellulose membrane and probed with anti-HIF-2α antibody. C) The induced BL21.pLysS culture was sonicated and the soluble (supernatant) and insoluble (pellet) proteins were separated by centrifugation. Samples from the pellet (P) and the supernatant (S) were labelled with Oregon Green Halotag ligand and analysed via SDS PAGE. Black arrow = fluorescent bands likely to correspond to the Halotag. Image inverted to aid visualisation of the bands. White arrowheads = fluorescent bands likely to correspond to several truncated versions of Halotag-HIF-2α.
In the three bacterial expression systems used here it appears that several things could be happening. Firstly, full length recombinant HT-HIF-2α is expressed at such low quantities that it can only be seen via western blot and although small amounts of protein could potentially be purified, for the envisaged application it would not be sufficient. Secondly, it is being degraded due to endogenous proteases. Or, truncated forms are being translated, possibly due to the high number of codons rarely used by *E. coli* in the coding region of HIF-2α.

### 5.2.1.1 Bacterial Expression System: Troubleshooting

To address some of the potential causes of the poor Halotag-HIF-2α protein expression discussed above we tried several other strains of *E. coli*.

*E. coli* have different preferences for codons compared to mammals (Zhang *et al.*, 1991). Many codons found in mammalian genes are rarely found in bacterial genomes hence the term ‘rare codons’. Rosetta™ DE3, a strain of BL21 *E. coli* engineered for the expression of eukaryotic proteins containing a high proportion of rare codons (Merck Millipore, Germany), was tried. Figure 5.9A shows the total protein for induced (I) versus non-induced (C) for each condition, with an induced band at approximately 150 kDa. The sample grown at 37 °C following induction was further analysed. The bacteria were lysed, centrifuged and the proteins present in the pellet and supernatant separated via SDS PAGE. A band at around 150 kDa is present in both fractions, suggesting that there is both insoluble and soluble protein (Figure 5.11A, Lane P & S).
Figure 5.11 | HIS-Halotag-HIF-2α expression in Rosetta DE3 E.coli. 5 mL cultures of Rosetta DE3 transformed with the pETM-11-Halotag-HIF-2α vector were incubated for 18 h at 16°C or 4 h at 37°C. A) Coomassie stained SDS PAGE. (C = control, I = induced protein expression with 1 mM IPTG, P = pellet / insoluble proteins, S = supernatent / soluble proteins). White arrowheads highlight induced protein observed at around 150 kDa. B) Trial purification was performed on a sample of the supernatant (S). A column was prepared with 0.6 mL ProBond™ nickel-chelating resin (Invitrogen, CA, USA), the resin was washed with 300 mM imidazole and sonication buffer prior to adding the sample. 3 mL of filtered supernatant was applied to the column and the flow through (FT) collected, any additional unbound protein was removed from the column with PBS washes and the bound fraction (E) eluted with 300 mM imidazole. The eluted protein (E) was then applied to a column of heparin agarose (as described for Halotag-FGF2). Samples (20 µL) were labelled with 4 µL Oregon Green Halotag ligand (50 µM) and analysed by SDS PAGE. Left = coomassie, right = fluorescence SDS PAGE. Orange box = faint fluorescent band at around 150 kDa. Red box = several strong fluorescent bands around 40 kDa.

Trial purification of the protein from lane S (Figure 5.11A) was performed. The bacterial expression plasmid, into which the Halotag-HIF-2α gene was cloned, resulted in the addition of a poly-histidine tag to the N-terminal end of the recombinant protein, allowing
it to bind to a nickel chelation resin. The supernatant was filtered and applied to a nickel chelation column. The eluted fraction (presumed to contain Halotag-HIF-2α) was then applied to a heparin column, due to the presence of a DNA binding domain HIF-2α should bind to this sulfated polysaccharide. The flow through (FT) and eluted (E) fractions were labelled with the fluorescent halotag ligands and analysis by SDS PAGE from which two observations can be made. Firstly, a 150 kDa protein (presumed to be Halotag-HIF-2α) was present in the supernatant and the flow through of the nickel column (Figure 5.11B, Orange box) indicating that this protein did not bind to the nickel resin, suggesting that either the Halotag-HIF-2α is missing the poly histidine tag or the tag is not exposed. Secondly, several fluorescently labelled proteins of varying sizes between 35 – 40 kDa (Figure 5.11B, red box) were observed in the supernatant. These bands were also present in the fraction eluted from the nickel column, but were in the flow through from the heparin column, indicating that these proteins were able to bind the Halotag ligand and to the nickel resin but not heparin, suggesting that these are truncated or partly degraded forms of the His-Halotag-HIF-2α protein where the His-tag and Halotag portion has remained intact and functional.

Even though there appears to be some protein that is of the predicted size that binds the Halotag-ligand, the amount of protein is very low. This is a problem, as during protein purification the yield of protein tends to diminish with increasing chromatography steps, therefore, it is best to start with a large amount of target protein. In this case, it is likely that the protein yield after protein purification would be far too low for the experiments planned.

Other bacterial strains, such as Lemo21, useful for difficult (e.g. toxic, membrane, insoluble) proteins and SoluBL21(DE3), were used. However, the yield of full-length Halotag-HIF-2α could not be improved.

**5.2.1.2 Expression of Halotag-HIF-2α in E.coli: Conclusion**

To conclude, the expression of recombinant Halotag-HIF-2α in bacteria did not yield sufficient protein for further purification and analysis. Possible reasons for this could be due to degradation by endogenous bacterial proteolytic enzymes. For instance, the full length recombinant protein could be expressed, but the protein is being degraded from the HIF-2α end, leaving the Halotag intact. However, this contradicts the fact that the bacteria used for protein expression tend to be engineered to have fewer endogenous proteases to prevent
this from happening. Another, (possibly more likely) scenario is that the bacteria are expressing truncated forms of the recombinant protein, i.e. the translation starts at the poly-histidine / Halotag end and proceeds unperturbed until the ribosome reaches the HIF-2α region where translation is stalled. Furthermore, it is likely that the induced band observed at around 120 kDa is a bacterial protein involved in transcription or translation, as this band is only present in cultures that have been induced with IPTG and is present regardless of the recombinant protein being expressed.

In the interest of time and due to exhausting a substantial number of routes for bacterial protein expression, it was decided to express Halotag-HIF-2α by other means.

### 5.2.2 Cell-free protein expression

A second approach for the expression of recombinant proteins was tested, the PureExpress in vitro system (NEB), was tested. This kit contains all the components (as purified recombinant his-tagged proteins) from bacteria that are required for protein expression, thus removing any chance of proteolysis. Although this system gives a lower yield than bacteria, it may produce sufficient amounts of full-length Halotag-HIF-2α for the present purpose.

Figure 5.12(1) shows a western blot of the products of the in vitro translation. There was immuno-reactivity to the HIF-2α antibody at around 150 kDa. However, the fluorescent Oregon Green Halotag ligand signal was detected at a range of sizes. To test whether this was non-specific binding due to using a high concentration of Halotag ligand, the reaction was re-run and samples were labelled with various concentrations of ligand ranging from 2 µM to 8 µM. All concentrations showed a similar band pattern (data not shown). These results suggest that the protein is being translated, but some products are truncated, i.e. the Halotag part is being translated and then the ribosome is stopping or falling off before reaching the end of the coding part of the mRNA, so full length HIF-2α is not produced. This could result in many truncated proteins that react with the Halotag ligand, but only a few that will exhibit immuno-reactivity against the HIF-2α antibody. This method of protein production was, therefore, also abandoned.
5.2.3 Mammalian expression system

As previously shown, the Halotag-HIF-2α can be expressed in mammalian cells (Figure 5.7). However, using mammalian cells for recombinant protein expression has drawbacks, such as it is more expensive, the yield of protein is much lower and purification is difficult due to the vast number of endogenous proteins present. Therefore, we chose to take a slightly different approach that does not involve purification of the protein via chromatography, which I have termed in vitro and in vivo conjugation (Figure 5.13). Briefly: the HL-GNPs would be added to the cell lysate of HEK293t ectopically expressing Halotag-HIF-2α (in vitro conjugation), following incubation to allow conjugation to occur the lysate would be centrifuged to “pull down” the GNP-labelled protein, which would then be microinjected into cells for imaging. Alternatively, live HeLa cells expressing Halotag-HIF-2α would be microinjected with HL-GNPs and imaged (in vivo conjugation).
5.2.3.1 in vivo conjugation

The protocol for in vivo conjugation has several drawbacks such as once injected it relies on the nanoparticles finding and reacting with the Halotag-HIF-2α in the cells. Also, there is no way to control non-specific interactions. However, the primary issue was identifying cells for microinjection, as the pFN21A-Halotag-HIF-2α construct does not have a fluorescent marker. To overcome this, a fragment of DNA encoding an internal ribosome entry site preceding the fluorophore dTomato (IRES-dTomato) was inserted at the C-terminus of Halotag-HIF-2α to create a construct that expresses the Halotag-HIF-2α and dTomato separately (Figure 5.14). Thus providing a way to identify cells to be microinjected with HL-GNPs and bypass the need for co-transfection.
5.2.3.2 in vitro conjugation

Figure 5.15 shows a protein sample taken from HEK293T cells transiently transfected with pFN21A-Halotag-HIF-2α that has been analysed by SDS-PAGE, western blot and fluorescence. A strong band of the expected size (150 kDa) that exhibits immuno-reactivity against the anti-HIF-2α and anti-Halotag antibody was observed. The lysate was incubated with the fluorescent (Oregon Green) Halotag ligand prior to denaturation and analysis by SDS PAGE. The fluorescent band in lane 1 (Figure 5.15) indicates that a functional Halotag makes up part of this the 150 kDa.

Gold nanoparticles carrying the Halotag ligand were incubated with the lysate of HEK293T cells that were transfected with Halotag-HIF-2α (transfected lysate). As a control HL-GNPs were also incubated with the lysate of non-transfected cells (control lysate) or with just PBS (- Lysate). Samples from each condition were spotted onto nitrocellulose membrane and
probed for HIF-2α. Alongside these experiments, gold nanoparticles with a ligand shell consisting of only polyethylene glycol (PEG-GNPs) were tested as negative controls. The dot blots in figure 5.16 show that there is HIF-2α non-specifically bound to HL-GNPs and PEG-GNPs in the transfected and control lysate pre-wash. However, following a wash step to remove weakly bound proteins, a faint signal is only seen for the sample from the HL-GNPs incubated with the transfected lysate, suggesting that HIF-2α has specifically bound to the surface of the gold nanoparticles via the Halotag ligand (Figure 5.16C, red circle).

To confirm the size of the protein attached to the surface of the nanoparticles, the ligand shell was stripped from the gold core with DTT (Chen et al., 2012) and analysed via western blot. Following several washes, to remove aspecifically bound proteins, the nanoparticles were treated with 50 mM DTT for 36 h. Figure 5.16D shows that upon stripping, a strong HIF-2α immunoreactivity at 150 kDa was observed (Lane labelled DTT), suggesting a clear conjugation of HIF-2α to gold nanoparticles (last lane). As a control, samples of from the transfected cells (size control for HT-HIF-2α) and from the wash steps were separated on the same gel. HIF-2α was detected only in the first wash, suggesting any non-specifically bound HIF-2α was removed by subsequent washes. Altogether, these results suggest that Halotag-HIF-2α is covalently bound to the gold nanoparticles via the Halotag ligand that is incorporated into the ligand shell.
Figure 5.16 | Conjugation of HL-GNPs to Halotag-HIF-2α from mammalian cell lysate. A) Lysates from HEK293T cells not expressing (control) and expressing Halotag-HIF-2α were spotted onto a nitrocellulose membrane and probed with a HIF2α antibody (Abcam, UK). B) Fluorescence image of 200 μg of lysates from (A) labelled with 10 μM of HaloTag Oregon Green ligand resolved via SDS PAGE. C) Nanoparticles after overnight incubation with lysates from (A) or without lysate were spotted onto nitrocellulose membrane and probed with anti-HIF2α (Abcam, UK). Dot blot shows before (pre) and after (post) four washes with PBST to remove unbound / weakly bound protein. D) To determine the size of the protein attached to the gold nanoparticles the ligand shell and, therefore, anything bound, was stripped from the gold core and analysed via western blot. HL-GNPs were incubated with HT-HIF-2α lysate. The nanoparticles were washed four times in PBST to removed weakly bound proteins. After washing, the nanoparticles were incubated with 50 mM DTT for 36 h. The ligand shell was separated from the gold via centrifugation and the presence of HT-HIF-2α was determined immunoblotting (anti-HIF-2α).

Although this result was very promising, we observed variability in success rates of conjugation between different batches of HL-GNPs and experienced difficulties in consistently reproducing the results observed in figure 5.16. Taking this into consideration, along with the development of new approaches for nanoparticle conjugation to proteins
(Leduc et al., 2013), we decided to pursue a different strategy for coupling HIF-2α to gold nanoparticles.

5.2.3.3 Anti-GFP Nanobodies: A compromise?

Nanobody is a term that refers to a single variable domain (VH) derived from heavy chain only antibodies (IgG) found in camelpid species such as alpaca (Hamerscasterman et al., 1993; Muyldermans et al., 1994; Muyldermans & Lauwereys, 1999). These nanobodies are a fraction of the size of conventional antibodies (full length 160 kDa), with a molecular weight of around 15 kDa (Doshi et al., 2014). Their small size, 2.5 x 4.2 nm, gives them certain advantages over antibodies (Revets et al., 2005; Muyldermans et al., 2009), such as a much smaller minimal linkage distance between the tag and the target, which is of particular value for super-resolution imaging and single molecule tracking (Ries et al., 2012; Leduc et al., 2013).

Leduc et al. (2013) have already shown that nanobodies to GFP can be used as a means for labelling proteins of interest with gold nanoparticles (Leduc et al., 2013). This method requires an EGFP fusion of the protein of interest, which we already had available for HIF-2α and was, therefore, appealing to enable the study of HIF-2α at the single molecule level.

Preliminary experiments show that highly specific labelling of EGFP-HIF-2α with GFP nanobodies (conjugated with fluorescent dye ATTO594) can be achieved using standard immuno-fluorescent protocols (Figure 5.15). Figure 5.16 shows an example of a HeLa cells ectopically expressing EGFP-HIF-2α that has been microinjected with the anti-GFP-ATTO594 nanobodies. Nuclear colocalisation between the green and red channels can be observed (Figure 5.16iv.). These promising results suggest that ectopically expressed EGFP-HIF-2α...
could potentially be labelled with GNPs via anti-GFP nanobodies in live cells using microinjection as the delivery method.

**Figure 5.18 | Microinjection of anti-GFP nanobodies into cells ectopically expressing EGFP-HIF-2α.**

Live HeLa cells transiently transected with EGFP-HIF-2α were injected with anti-GFP nanobodies. i) Brightfield, ii) EGFP-HIF-2α, iii) anti-GFP ATTO594 nanobodies, iv) merge of ii & iii.

Incorporation of the anti-GFP nanobodies onto the surface of the gold nanoparticles requires a different protocol to that of the Halotag-ligand which would need optimisation. As I was approaching the end of my time in the laboratory, and the size and scope of this work was unknown, the GFP-nanobody project was passed onto another member of the laboratory and is currently under development.

### 5.3 Conclusion

The aim of the work described in this chapter was to develop a method of labelling single molecules of HIF-2α with gold nanoparticles to facilitate their tracking in live cells. The initial plan was to use the Halotag, an enzyme that specifically and irreversibly binds to chloroalkanes. In collaboration with Promega, a Halotag ligand was specifically designed so that it could be incorporated into the ligand shell of a gold nanoparticle but protrude far enough so that it could react with a Halotag fusion protein.

Preliminary ‘proof of principle’ experiments were conducted with fibroblast growth factor-2 (FGF2). Recombinant Halotag-FGF2 was expressed in bacteria and purified using a three-step chromatography protocol. We showed that FGF2 could be coupled to gold nanoparticles via the Halotag technology.

However, when we moved on to the protein of interest, HIF-2α, several issues were encountered. Despite work published in Sun, C. et al (2015) suggesting that having the Halotag at the N-terminal of the fusion protein can aid bacterial expression of recalcitrant
protein and although many different bacterial strains and variations of protocols were trialled, expression of full-length recombinant Halotag-HIF-2α in useful amounts was not achieved. The poor expression observed could be due to the large size of the protein or the large proportion of rare codons in the coding sequence HIF-2α. The latter interpretation is supported by the marginal improvement in expression observed in the Rosetta strain. At present there are no crystal structures of full length HIF-2α available, only structures of the HIF-2α PAS-B domain (Erbel et al., 2003; Card et al., 2005; Scheuermann et al., 2009; Key et al., 2009; Scheuermann et al., 2013; Rogers et al., 2013; Guo et al., 2015), suggesting that others may have also struggled to express full-length recombinant HIF-2α.

Secondly, we encountered problems with reproducibility with regards to nanoparticle production / conjugation. Although it was shown that FGF2 could be conjugated to gold nanoparticles via the Halotag, we were unable to consistently conjugate HIF-2α to HL-GNPs. One possible reason for this is that the Halotag-FGF2 / nanoparticle conjugation reaction occurred in more favourable conditions (in vitro) i.e. as we are able to express & purify recombinant Halotag-FGF2, the reaction occurred in excess of Halotag-FGF2 and with no interference from other proteins. In the case of Halotag-HIF-2α, we seemingly could not express full-length Halotag-HIF-2α in mammalian cell. However, any protein expressed was only visible via western blot (an amplification technique) and not on an SDS PAGE stained with coomassie blue. It is difficult to calculate the concentration of fusion protein that is present in the lysate, but we can be sure that it is far less than that of the purified Halotag-FGF2. In addition to this, there is competition from other endogenous proteins that may non-specifically interact with the surface of the nanoparticle, blocking Halotag-HIF-2α from reacting with the Halotag-ligand. Both of these factors would reduce the chances of successful conjugation.

Another explanation for this could be that the Halotag ligand is inaccessible to the Halotag protein. The Halotag ligand was designed to protrude far enough out of the ligand shell so that it can react with the Halotag protein, however, there are hydrophobic regions along the ligand (for ligand structure see Appendix 1.5) and it is possible that this causes the Halotag ligand to reorient itself so that these regions are immersed in the ligand shell, thus resulting in the Halotag protein being unable to react with the chloroalkane part of the ligand.
Despite lack of reproducibility, the results presented here suggest that it is possible to conjugate HIF-2α to gold nanoparticles via the Halotag protein. With improvements to the design of the Halotag ligand and optimisation of a robust protocol for making Halotag ligand gold nanoparticles, this would be an invaluable technique for labelling proteins of interest for single molecule tracking.

Nanobodies are another promising and versatile method for labelling a protein of interest with gold nanoparticles. The anti-GFP nanobodies can be used with any GFP (and GFP derivative)-fusion proteins. They have been shown to have uses in numerous microscopy techniques, not only for single molecule tracking but also for super-resolution microscopy (Leduc et al., 2013; Ries et al., 2012)
Chapter 6: Discussion
In this thesis, we have demonstrated that the HIF-1α subunits transiently stabilise in hypoxia and that this dynamic accumulation is the result of a negative feedback mechanism that ensures a controlled HIF response and is specifically controlled by PHD2. We have further demonstrated that HIF-1α and HIF-2α exhibit different sub-nuclear distributions, with HIF-2α being localised in nuclear foci. I have shown that HIF-2α freely diffuses in and out of the nuclear speckles and through the inter-speckle space. We initially hypothesised that HIF-2α might be regulated by molecular availability, as opposed to protein stability, yet my results show no clear difference in HIF-2α molecular availability or diffusion rate in normoxia and hypoxia. However, the diffusion of HIF-2α molecules is much slower than HIF-1α irrespective of oxygen tension. All these results were obtained using live cell imaging and for this project, I have generated several useful tools for imaging from the nano-scale to the cellular scale. I have developed a method for conjugating gold nanoparticles to a protein of interest for single molecule tracking and I have also created several lentiviral plasmids and stable cell lines for quantitative imaging of HIF-1α/2α.

6.1 Utilising fluorescent confocal microscopy to investigate protein dynamics in living cells: Reflection and future prospects

6.1.1 Stable cell lines

We have utilised fluorescent fusion proteins to monitor and quantify a protein of interest. This method however has drawback, for example: delivery of the construct encoding the fusion protein which can be variable and lacks control; the fusion protein is under the control of an exogenous promoter such as the CMV promoter which can provide heterogeneous levels of expression between experiments and cell types (Smith et al., 2000); and, it is not the endogenous protein that is being observed.

To overcome some of these problems we have endeavoured to make cell lines stably expressing fluorescent fusions of HIF-1α and HIF-2α. Although some success was had via the use of a BAC for HIF-1α, the resulting cell lines were still less than ideal as the signal was very weak. Many of the drawbacks of BACs were discussed in chapter 3, one of which is the lack of control over where the transgene integrates into the genome. One way around this is to utilise genome editing nucleases such as zinc finger nucleases (ZFNs), Transcription
activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats (CRISPr). These technologies facilitate directed integration of the transgene into the host’s genome. There are two options: (1) insert an exogenous fusion of the protein of interest (e.g. HIF-1α-EGFP) at a predetermined site (e.g. AAVS1 locus) or (2) insert an exogenous tag (e.g. EGFP or Halotag) downstream of the gene of interest. The latter option means that the endogenous protein is transcribed with the tag attached under the control of endogenous promoter; it also means that any other regulatory elements are also still having an effect and therefore any observations made are more physiologically relevant.

6.1.2 Data analysis

One difficulty with microscope experiments, particularly time-lapse experiments, is turning the qualitative information into quantitative data that can be analysed and interpreted. A particular problem with analysing live cells is that they can move and/or change shape over the course of the experiment. So in order to measure the fluorescent signal from the nucleus of one cell, one has to track the nucleus through the time course. If the protein of interest is not visible for the entirety of the experiment (such as HIF-1α), then automated detection and tracking is difficult as the loss of signal means software (such as Imaris, Bitplane) loses the object during these periods. This can be overcome by using a nuclear marker, however with regards to the HIF-1α-GFP BAC cell line, finding a spectrally suitable, non-toxic nuclear marker that will last the duration of the experiment (>24 h) proved difficult. This meant that for these experiments there was no way to automatically track the region of interest, resulting in the tedious task of manually drawing the boundaries of each cell nuclei and moving through each frame of the time-lapse checking for cell movement and adjusting the boundaries accordingly. To make the analysis of future experiments less laborious, I would create a construct encoding either lamin or histone-2b (H2B) fused with a red fluorescent protein such as dTomato or mCherry.

6.1.3 Investigating molecular dynamics

Although work continues with investigating methods (e.g. nanobodies) of labelling HIF-2α in vivo with gold nanoparticles, it would be worth considering other methods of SMT. I believe a useful tool for investigating the molecular dynamics of the HIF-α subunits would be a fusion of HIF-1α or HIF-2α with a photo-switchable/convertible protein. This could provide
more comprehensive information for FRAP such as bi-directional diffusion and can be utilised for super-resolution techniques such as fPALM and single molecule tracking.

Continually, the Halotag can be specifically and irreversibly labelled with a cell-permeable Halotag ligand carrying a synthetic dye (e.g. TMR) and has been utilised by several groups for SMT (Abrahamsson et al., 2013; Mazza et al., 2012). So we could utilise the Halotag-HIF-2α construct and the TMR ligand for SMT in living cells. This work would also benefit from the opportunity to use the equipment at the Advanced Imaging Centre (AIC) at Janelia Farm, USA. This facility has many custom-built state of the art pieces of equipment designed for super-resolution single molecule tracking and throughout the year they run a call for proposals where external researchers can bring their own samples to the facility for imaging.

6.2 HIF-1α versus HIF-2α

6.2.1 Normoxic stabilisation of HIF-2α

The literature suggests that both HIF alpha subunits are regulated in an oxygen-dependent manner whereby they are hydroxylated by the PHD enzymes, which results in association with VHL and subsequently degradation via the proteasome. However, in contradiction with this model, we observe normoxic stabilisation of both endogenous and ectopically expressed HIF-2α in a variety of cells line (HeLa, HEK, SK-N-AS cells) via different methods (live cell imaging, immunofluorescence and western blot) and so are confident that is that this is not an artefact.

One possibility is that the cell lines used have reduced activity of the components involved in the oxygen-dependent hydroxylation and VHL-dependent degradation, such as the cofactors required for hydroxylation of the prolyl residues located within the ODD domain (Figure 6.1). However, as we do not observe normoxic stabilisation of HIF-1α this is unlikely to be the case. This also suggests that it is something specific to HIF-2α.
Discussion

Figure 6.1 | PHD enzymatic reaction. The reaction that takes place when the PHD proteins hydroxylate the prolyl residues located within the ODD domain. Essential co-substrates and co-factors are highlight in the blue box.

There is evidence that there are deubiquitinases (DUBs) that modify HIF-1α (e.g. ubiquitin specific peptidase 29 / USP29) and positively influence its stability (Edurne Berra, CIC bioGUNE, Spain; unpublished; communication at the Hypoxia: From Basic Mechanisms to Therapeutics (E3) Keystone meeting 2015). If there are also DUBs that specifically modify HIF-2α, then this would result in evasion of proteasomal degradation and give rise to normoxic stabilisation (Figure 6.2). It could also be possible that there is an enzyme that reverses the actions of the PHD enzymes, which would prevent association of pVHL with the alpha subunit and the subsequent targeting to the proteasome. Lastly, there could be a protein similar to a chaperone protein that protects HIF-2α from degradation by physically preventing PHDs or pVHL interacting with and modifying the subunit.

Figure 6.2 | Theory of normoxic stabilisation of HIF-2α.

6.2.2 Not just a backup system

For a long time HIF-2α has been considered the "under-study" of HIF-1α. However, interest in HIF-2α is growing as there is more and more evidence that this isoform is not just HIF-1α’s fall back, but that it has specific roles and unique target genes. However, one of the main conundrums is how does gene specificity arise. David Mole and colleagues have performed genome wide analysis of HIF (HIF-1α/HIF-1β or HIF-2α/HIF-1β) and found that
when comparing HIF-1 (HIF-1α + HIF-1β) and HIF-2 (HIF-2α + HIF-1β) specific binding sites there is no obvious difference in the consensus sequence and proximal sequences, therefore the specificity does not lie within the sequence that the two isoforms bind to (Schödel et al., 2011). Peter Ratcliffe and colleagues have found that when HIF-1α and HIF-2α are both present, then certain genes will be preferentially bound by HIF-1α, but when HIF-1α is knocked down then HIF-2α is found to bind these genes. This highlights that both isoforms can bind to the “HIF-1α-specific gene” but there is competition for binding (Peter Ratcliffe, University of Oxford, unpublished, communication at the Hypoxia: From Basic Mechanisms to Therapeutics (E3) Keystone meeting 2015), suggesting that gene specificity arises due to HIF-1α having a stronger affinity for these genes or due to mobility i.e. HIF-1α diffuses more quickly than HIF-2α so arrives at the gene first, which would fit with our FRAP results.

6.3 Final remarks

The hypoxia signalling pathway is a vital oxygen deprivation survival mechanism that plays a role in many normal physiological and developmental processes. It has also been implicated in various diseases and there is a large focus on HIF as a potential therapeutic target in cancer, stroke and transplant rejection (for example). Therefore, it is important to gain a full understanding of how these two transcription factors and their activity is regulated and so further investigation is a needed into what causes the discrepancies, such as gene specificity, protein stabilisation and spatial localisation, between HIF-1α and HIF-2α.
References


colorectal adenocarcinomas is associated with wild-type TP53 but not with BNIP3 promoter hypermethylation or apoptosis. British Journal of Cancer 99, 727-733.


References


Appendix

1.1 Amino acid sequences of each domain for HIF-1α and HIF-2α

>bHLH_HIF1a
RRKEKSRDAARSRSKESEVFYELAHQLPLPHNVSSHLDKASVMRLTISYLRLVR

>bHLH_HIF2a
RRKEKSRDAARCRRSKETEVEFYELAEHELPLPHSVSSHLDKASIMRЛАISFLRTH

>PAS_A_HIF1a
KAQMNCFYLKALDGTVLTDGGDMDIYISDNVNKYMGLTQFELTGHSVDFTHPCDHEEMREMTHRNGLKV

>PAS_A_HIF2a
DQQMDNLKALEGFIAVTVDGDMLSENISKFMGLTQVLTGHSVDFTHPCDHEEIRENLSKLNGSG

>PAS_B_HIF1a
PHPSNIEPLDKTSRHSNLDKFSYCDERITELMGYEPEELLGRSIYYYHALDSHDHTKTHHDFTKG

>PAS_B_HIF2a
QHPHMDLDPDKTSRHSNLDKFSYCDERITELIGYHPEELLGRSAYEYYHALDSENMTKSHPQNLCCTKG

>NTAD_HIF1a
FKLELVEKLFEDTEAKNPSTQDTDLDEMLAPYIPMDDDFQLR

>NTAD_HIF2a
LKIIEVIEKLAMDTEADQCTQTDFNDELLETLAPYIPMDGEDFQL

>CTAD_HIF1a
SMDESGLPQLTYSYDCEVNAPIQGSRNLLQGEELLRALDQVN

>CTAD_HIF2a
SFESYLLPTERYDCEVNPVLGSSTLQQGDDLRALDQAT

>NLS_HIF1a(711-730)
LALQNAQRKRMEHDGSLFQ

>NLS_HIF2a(731-750)
STSHLMWKRMKNLRRGSCPL

>ODD_HIF1a
APAAGTDTSLDGSSNDTTDDQOLEEELVNMLPSPNEKLQININLAMSPLPTAETPKLRSSADPNLQVALK
LEPNPESELSFTRPQDQTPSPDDGSTQSSFPEPNSEYCFYVOSDMVNEFKLELVEKLFAEDTEAKNPSTQDT
DLDLMLAPYIPMDDDFQLRSFDLQSPLESSASASPQSTVTIFQ

>ODD_HIF2a
TQTDFNEDLLELTAPYIMPDGEDFQLSPICPEERLAEQPSTQPQHCFSAMTNQPLAPVAPHPFLLDKFQQLES
KKTEPHERPMSSIFFDAGSKASLPPCCGQASTPLSSMGGRSNTQWPPDPPLHFPTKAWGDQRTLEFLGAAPLGP
PVSPPHVSTFKTR
1.2 Alignment of HIF-1α and HIF-2α ODD domain protein sequence

Sequence alignment of ODD domains using BlastP:

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<td>Range 2: 1 to 100</td>
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<td>--------</td>
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Sequence alignment of ODD domains using MUSCLE:

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</tbody>
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1.3 Explanation of macro used for tracking HIF-2α speckles

Tracking macro (step-by-step)

1) User Input
   - Get user input.
     - calibration in x & y [xyCalib]
     - z [zCalib]
     - temporal interval [tCalib]
     - minimum track length [intMinTrackLen]

2) Pre-processing
   - Find number of trajectories (from last trajectory number in Column B) [intTotalTracks]
   - For each trajectory
     - Check if trajectory is shorter than intMinTrackLen. If so, move onto next.
     - If longer, Copy Trajectory out to a new sheet named [tXX] where XX is trajectory number

3) Analysis of Trajectory for τ=1
   - If absent add a summary sheet
   - Spatially calibrate the data (x,y,z)
   - For each timepoint:
     - Calculate the euclidean distance (displacement) between the coordinates of t (x,y,z) and t+1 (x₁, y₁, z₁).
       \[ d = \sqrt{(x - x₁)^2 + (y - y₁)^2 + (z - z₁)^2} \]
     - Record a running sum of displacements for dx where x=1 to 6
   - Record the trajectory length [trajectoryLength]
   - Calculate the whole trajectory displacement (using equation above with start and finish coordinates)
   - Calculate the instantaneous velocity for a given window [intWindow] as the sum of step distances divided by the calibrated time.

4) Calculate Displacements for τ>1
   - For each time window (τ) from 2 to trajectoryLength-1, calculate the displacements and (as above) record the running sum of displacements for dx where x=1 to 6
   - Divide the running sums by the number of frames to get the moments of the mean displacements (i.e. mean displacement, mean squared displacements, mean cube displacements ...)
   - Make a note of the MSD with the calibrated time.
5) **The Moment Scaling Spectrum**
- Plot a double log graph of Tau vs Moment for moments 1-6
- Calculate the Scaling Coefficients from the slopes of the double log plots and plot (below) and calculate the slope of the moment scaling spectrum (sMSS) by fitting a trendline.

Note: Variables used in macro given in brackets
Figure 1. Analysis of individual trajectory. Screenshot of the output generated by the macro for a single trajectory.
Figure 2. Depiction of the difference between distance and displacement (left) and what “Tau = x” represents.

Figure 3. Graphs calculated by macro. A) Mean squared displacement. B) Log/log plot. C) Moment scaling spectrum.
1.4 Matlab code for FRAP analysis

%%FRAP ANALYSIS

% For analysis of FRAP data in excel spreadsheet format or CSV format with
% Time, Bleached Region, Non-bleached Region and Background.
% By using the code, the end product
% will be the value of Y at time infinity, the rate of the
% and goodness of fit of the curve.

%% Written By Si Seet Chan, March 2015
%% v5+ Revisions by Dave Mason [dnmason@liv.ac.uk]

%% VERSION HISTORY
%
% v3: Added ability to read in csv, xls, xlsx, txt files
% v4: Final version for SiSeet's project. Can process multiple
% sheets at once.
% v5: Added option for a master log of results
% v6: v5 breaks with multisheet excel files - reads xls and xlsx
% with "xlsread" and others (csv, txt) with
% importdata as v5. Also changed figure background to white on
% initialisation. Altered title to include filename
% (without extension) and cancel Latex Interpretation.
% v7: Reduced bleach threshold to 30%
% v7.1: Error messages changed to start with 'Dave says'
% Clear the workspace and command history
%c
% -- Ask if there's a master file
useMaster=questdlg('Do you have a master file? Select No to make a new one or Cancel not to bother','Master');
if strcmp(useMaster,'Yes')
    [mastFile,mastPath]=uigetfile('**','Select Master File');
    % -- Load in existing data
    [~, ~, mastData]=xlsread([mastPath mastFile]);
elseif strcmp(useMaster,'No')
    mastPath=uigetdir('Select a folder in which to make a new Master File');
    mastFile='masterFRAP.xls';
    %-- No file, so store a new header
    mastData={'Timestamp','Path','Filename','Plateau','Rate','R squared'};
end
% -- catch empty master path here if useMaster is no or cancel
###

% To get data from the workspace
% In the form of Time, Bleach (ROI1), Control (ROI2) and Background (ROI3)
% Find data in different format
Appendix

[inFile, inPath]=uigetfile({'*.*'; '*.xls'; '*.txt'; '*.csv'; '*.xlsx'}, 'select file(s)', 'multiselect', 'on');
if inPath<0;
    disp('DAVE SAYS - Error in finding file')
    return
end
% For multiselection of files to occur, need code to see whether it is multiple files or single files. Multiple files = char, single file = cell
if iscell(inFile)
    for n=1:length(inFile)
%-- Get extension then test. If Excel, only import first sheet
[tempPath tempFile tempExt]=fileparts([inPath inFile{n}]);
if strfind(tempExt, 'xls')>0
    D=xlsread(fullfile([inPath inFile{n}]),1);
    Data=D(:,1:4);
else
    D=importdata(fullfile([inPath inFile{n}]));
    Data=D.data;
end
    Time=Data(:,1);
    ROI1=Data(:,2);
    ROI2=Data(:,3);
    ROI3=Data(:,4);
end

% csv, old xls and txt format is 3, xlsx format is 4 (the code count the number to delete to replace the format
% Set Variables
% Number of PREBLEACH values
for i=1:size(ROI1,1)-1
    NUM_PREBLEACH=i;
    if (ROI1(i)-ROI1(i+1))/ROI1(i)>0.3
        break
    end
    if NUM_PREBLEACH>round((0.5*(size(ROI1))))
        disp('DAVE SAYS - Error in finding prebleach value')
        return
    end
end
% NUM_PREBLEACH=input('How many pre-bleach values?');

%Find the mean of Background
Mean_ROI3=mean(ROI3);

% Background Subtraction for ROI1 and ROI2
BGSUB_ROI1=ROI1-Mean_ROI3;
BGSUB_ROI2=ROI2-Mean_ROI3;

% Pre-bleach values for both ROI1 and ROI2
Pre_ROI1=ROI1(1:NUM_PREBLEACH);
Pre_ROI2=ROI2(1:NUM_PREBLEACH);

% Mean of Pre-bleach value for both ROI1 and ROI2
Mean_PreROI1=mean(Pre_ROI1);
Mean_PreROI2=mean(Pre_ROI2);

% Divide BG substraction for ROI1 and ROI2 with mean of Pre-bleach value
Norm_ROI1=BGSUB_ROI1/Mean_PreROI1;
Norm_ROI2=BGSUB_ROI2/Mean_PreROI2;

% Correct for non-specific bleaching (Rt)
Rt=Norm_ROI1./Norm_ROI2;

% First Post Bleach Value (Rp)
Rp=Rt(NUM_PREBLEACH+1);

% Correct for Bleach Function
Bleach_Fraction=(Rt-Rp)/(1-Rp);

% Correct time for value after bleaching
Norm_Time=Time(NUM_PREBLEACH+1:end)-Time(NUM_PREBLEACH+1);

% Get the Norm recovery data from first post bleach value till end
Norm_Recovery=Bleach_Fraction(NUM_PREBLEACH+1:end);

% Curve Fitting
Equation='(a-b)*exp(-c*x)+b';
% where a= Value of Y where line cross Y axis
% b= Value of Y a infinity
% c= rate constant for graph

% Plotting of graph and set the starting point for the equation to fit the
% graph for normalised recovery
[fit1,fit1_gof,fit1_alg]=fit(Norm_Time,Norm_Recovery,Equation,'Start Point',[1 1 0.01]);

% Flagged low Rsquare value (less than 0.7 percent)
% plot residuals here against NormTime - don't forget to label axes and
% give the graph a title - use Figure to make a new figure
fit1_alg.residuals
if fit1_gof.rsquare<0.7
    figure('Color',[1 1 1]);
    plot(Norm_Time,fit1_alg.residuals);
    xlabel('Time (min)');
    ylabel('Residuals');
    title([{'Residuals - ' inFile{n}(1:end-(h+1))},
    'Interpretation',none]);
    legend('Residuals','location','northeast')
    axis('square')
    disp('DAVE SAYS - Low Rsquare value for graph')
    F=getframe(gcf);
    imwrite(F.cdata,[inPath inFile{n}(1:end-(h+1)) '_residuals.png'],
    'png');
end

% Flagged negative Rsquare value
if fit1_alg.exitflag<0
    disp('DAVE SAYS - Fail to fit equation')
    return
end

% Plotting of graphs (Raw Data and Normalised Recovery)
% Put both graphs into one plot
figure('Color',[1 1 1]);
    % Set the figure position and size of the figure
set(gcf,'position',[100 100 960 430])
movegui(gcf,'center') %-- Do this to make sure that the figure isn't cut off (or including other windows) when exporting

% Plotting Raw Data - plot(Time,ROI1,Time,ROI2,Time,ROI3)
% Labeling of graph and axis, graphs are in square format so that it is easier to look at datapoints
subplot(1,2,1);
plot(Time,ROI1,Time,ROI2,Time,ROI3);
title(['FRAP Data - ' inFile{n}(1:end-(h+1))],
'Interpreter','none');
xlabel('Time (min)');
ylabel('Fluorescence Intensity (a.u.)');
legend('Bleach','Control','Background','location','northeast')
xlim('auto')
ylim('auto')
axis('square')

% Plotting Normalised Recovery graph - plot(fit1,Norm_Time,Norm_Recovery)
% Labeling of graph, axis and setting limits to y axis
% Putting the graphs in square format so that it is easier to look at the datapoint
subplot(1,2,2);
plot(fit1,Norm_Time,Norm_Recovery);
title(['Normalised Recovery Graph - ' inFile{n}(1:end-(h+1))],
'Interpreter','none');
xlabel('Time (min)');
ylabel('Fractional Recovery');
legend('Normalised Recovery','Curve Fitting','location','southeast')
xlim('auto')
ylim([0 1.2])
axis('square')

% Save figure in png format
F=getframe(gcf);
imwrite(F.cdata,[inPath inFile{n}(1:end-h),'.png']);

% Save workspace in mat format
save([inPath inFile{n}(1:end-h),'.mat']);

%-- If selected, write out data to master
if strcmp(useMaster, 'Yes') || strcmp(useMaster, 'No')
c=fix(clock);
c=strcat(int2str(c(1)), '-', sprintf('%02d',c(2)), '-', sprintf('%02d',c(3)), '-', sprintf('%02d',c(4)), sprintf('%02d',c(5)));
%-- Append new data
mastData=vertcat(mastData,{c inPath inFile{n} num2str(fit1.b) num2str(fit1.c) num2str(fit1_gof.rsquare)});
%-- Write out master
xlswrite([mastPath 'mastFile'], mastData);
%-- Running batch to a master file, you probably don't want the images to stay open so...
close all
end

% Report data needed for analysis
disp(['----- ' inFile{n} ' -----'])
disp(['Y value at time infinity = ' num2str(fit1.b)])
disp(['Rate Constant = ' num2str(fit1.c)])
disp(['R Square value for fitness of the equation=' num2str(fit1_gof.rsquare)])
end

%-- Single file selected
else
%-- Get extention then test. If Excel, only import first sheet
[tempPath tempFile tempExt]=fileparts([inPath inFile]);
if strcmp(tempExt, 'xls')>0
    D=xlsread(fullfile([inPath inFile]),1);
    Data=D(:,1:4);
else
    D=importdata(fullfile([inPath inFile]));
    % Data=D.data;
end

Time=Data(:,1);
ROI1=Data(:,2);
ROI2=Data(:,3);
ROI3=Data(:,4);

% csv, old xls and txt format is 3, xlsx format is 4 (make it into a % variable so that the code could find out whether is it csv,xls,txt or % xlsx format
h=length(inFile)-strfind(inFile,'.'));

% Set Variables
% Number of PREBLEACH values
for i=1:size(ROI1,1)-1
    NUM_PREBLEACH=i;
    if (ROI1(i)-ROI1(i+1))/ROI1(i)>0.3
        break
    end
    if NUM_PREBLEACH>(0.5*(size(ROI1)))
        disp('DAVE SAYS - Error in finding good prebleach value')
        return
    end
end
% NUM_PREBLEACH=input('How many pre-bleach values?');

%Find the mean of Background
Mean_ROI3=mean(ROI3);

% Background Subtraction for ROI1 and ROI2
BGSUB_ROI1=ROI1-Mean_ROI3;
BGSUB_ROI2=ROI2-Mean_ROI3;

% Pre-bleach values for both ROI1 and ROI2
Pre_ROI1=ROI1(1:NUM_PREBLEACH);
Pre_ROI2=ROI2(1:NUM_PREBLEACH);

% Mean of Pre-bleach value for both ROI1 and ROI2
Mean_PreROI1=mean(Pre_ROI1);
Mean_PreROI2=mean(Pre_ROI2);

% Divide BG substraction for ROI1 and ROI2 with mean of Pre-bleach value
Norm_ROI1=BGSUB_ROI1/Mean_PreROI1;
Norm_ROI2=BGSUB_ROI2/Mean_PreROI2;

% Correct for non-specific bleaching (Rt)
Rt=Norm_ROI1./Norm_ROI2;

% First Post Bleach Value (Rp)
Rp=Rt(NUM_PREBLEACH+1);

% Correct for Bleach Function
Bleach_Fraction=(Rt-Rp)/(1-Rp);

% Correct time for value after bleaching
Norm_Time=Time(NUM_PREBLEACH+1:end)-Time(NUM_PREBLEACH+1);

% Get the Norm recovery data from first post bleach value till end
Norm_Recovery=Bleach_Fraction(NUM_PREBLEACH+1:end);

% Curve Fitting
Equation='(a-b)*exp(-c*x)+b';
% where a= Value of Y where line cross Y axis
% b= Value of Y a infinity
% c= rate constant for graph

% Plotting of graph and set the starting point for the equation to fit the
% graph for normalised recovery
[fit1,fit1_gof,fit1_alg]=fit(Norm_Time,Norm_Recovery,Equation,'Start Point',[1 1 0.01]);

% Flagged low Rsqaure value (less than 0.7 percent)
plot residuals here against NormTime - don't forget to label axes and
% give the graph a title - use Figure to make a new figure
fit1_alg.residuals
if fit1_gof.rsquare<0.7
    figure('Color',[1 1 1]);
end
Appendix

plot(Norm_Time,fit1_alg.residuals);
xlabel('Time (min)');
ylabel('Residuals');
title(['Residuals - ' inFile{n}(1:end-(h+1))],
'Interpreter','none');
legend('Residuals','location','northeast')
axis('square')
set(gcf,'position',[100 100 450 450])
movegui(gcf,'center') %-- Do this to make sure that the figure isn't
cut off (or including other windows) when exporting
disp('DAVE SAYS - Low Rsquare value for graph')
F=getframe(gcf);
imwrite(F.cdata,[inPath inFile(1:end-(h+1))
'_residuals.png'],'png');
end

% Flagged negative Rsquare value
if fit1_alg.exitflag<0
    disp('DAVE SAYS - Fail to fit equation')
    return
end

% Plotting of graphs (Raw Data and Normalised Recovery)
% Put both graphs into one plot
figure('Color',[1 1 1]);
% Set the figure position and size of the figure
set(gcf,'position',[100 100 960 430])
movegui(gcf,'center') %-- Do this to make sure that the figure isn't
cut off (or including other windows) when exporting

% Plotting Raw Data - plot(Time,ROI1,Time,ROI2,Time,ROI3)
% Labeling of graph and axis, graphs are in square format so that it
% is easier to look at datapoints
subplot(1,2,1);
plot(Time,ROI1,Time,ROI2,Time,ROI3);
%title('FRAP Data');
title(['FRAP Data - ' inFile(1:end-(h+1))],'Interpreter','none');
xlabel('Time (min)');
ylabel('Fluorescence Intensity (a.u.)');
legend('Bleach','Control','Background','location','northeast')
xlim('auto')
ylim('auto')
axis('square')

% Plotting Normalised Recovery graph - plot(fit1,Norm_Time,Norm_Recovery)
% Labeling of graph, axis and setting limits to y axis
% Putting the graphs in square format so that it is easier to look at the
% datapoint
subplot(1,2,2);
plot(fit1,Norm_Time,Norm_Recovery);
%title('Normalised Recovery Graph');
title(['Normalised Recovery Graph - ' inFile(1:end-(h+1))],'Interpreter','none');
xlabel('Time (min)');
ylabel('Fractional Recovery');
legend('Normalised Recovery','Curve Fitting','location','southeast')
xlim('auto')
Appendix

ylim([0 1.2])
axis('square')

% Save figure in png format
F=getframe(gcf);
imwrite(F.cdata,[inPath inFile(1:end-h),'png']);

% Save workspace in mat format
save([inPath inFile(1:end-h),'mat']);

%-- If selected, write out data to master
if strcmp(useMaster,'Yes') || strcmp(useMaster,'No')
    c=fix(clock);
    c=strcat(int2str(c(1)),'-',sprintf('%02d',c(2)),'-',
            sprintf('%02d',c(3)),'-',
            sprintf('%02d',c(4)),'-',
            sprintf('%02d',c(5)));
    %-- Append new data
    mastData=vertcat(mastData,{c inPath inFile num2str(fit1.b)
num2str(fit1.c) num2str(fit1_gof.rsquare)});
    %-- Write out master
    xlswrite([mastPath '\mastFile'],mastData);
end

% Report data needed for analysis
disp(['----- inFile -----'])
disp(['Y value at time infinity = ' num2str(fit1.b)])
disp(['Rate Constant = ' num2str(fit1.c)])
disp(['R Square value for fitness of the equation='
num2str(fit1_gof.rsquare)])
end

%-- Clear the variable used to write out
clear mastData
1.5 Structure of Halotag ligand
1.6 Legends for supplementary movies

**M1: Timelapse of HIF-1α-GFP BAC cells in hypoxia.** HIF-1α-GFP BAC HeLa cells were plated in a 35 mm glass-bottom dish and incubated on the microscope stage in 1 % O₂. Images were taken every 5 min. Total length of movie is 17 h. Frame rate = 8 frames / s.

**M2: Timelapse of EGFP-HIF-2α speckles.** HeLa cells were transiently transfected with EGFP-HIF-2α and plated in a 35 mm glass-bottom dish 24h prior to imaging. Cells were placed on the microscope stage in normoxic conditions. Timelapse shows the movement of the HIF-2α speckles.

**M3: Example of EGFP-HIF-2α speckle trajectories (1).** EGFP-HIF-2α speckle (highlighted by white circle) tracked using ImageJ and the trajectory recorded (white line). Highlights a speckle exhibiting more restricted / confined movement.

**M4: Example of EGFP-HIF-2α speckle trajectories (2).** EGFP-HIF-2α speckle (highlighted by yellow circle) tracked using ImageJ and the trajectory recorded (yellow line). Highlights a speckle exhibiting less restricted movement.

**M5: Example of tracking locations of EGFP-HIF-2α speckles during photobleaching experiment.** Location of EGFP-HIF-2α speckles during FRAP experiment was analysed using ImageJ. Speckles were identified prior to bleaching and tracked during experiment to determine if recovery of fluorescence occurs in the same location.

**M6 and M7: Example of individual speckles tracked during photobleaching experiments.** Two examples that suggest that EGFP-HIF-2α recovers into pre-existing speckles (speckles that were present prior to bleaching).