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IDENTIFICATION OF NOVEL PATHWAYS OF REGULATION OF AHR AND HIF-1 FUNCTION

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Identification of novel pathways of regulation of
AhR and HIF-1 function

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By

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“It always seems impossible until it’s done”

-Nelson Mandela-

ABSTRACT

Mammalian bHLH-Pas (basic HLH (helix-loop-helix)-PER-ARNT-SIM) proteins belong to the bHLH superfamily of transcription factors. Members of the family have a broad spectrum of functions that among others sense and regulate the cellular response to physiological signals such as low oxygen levels (hypoxia), or environmental signals such as toxins. The bHLH-Pas proteins consist of a signal-induced subunit, for example, aryl hydrocarbon receptor (AhR) or hypoxia inducible factor (HIF). To respond to environmental (AhR) or physiological (HIF) stimuli, the signal-induced subunits dimerize with their ubiquitously expressed, unregulated dimerization partner aryl hydrocarbon receptor nuclear translocator (ARNT), bind to DNA, and induce the activation of a cascade of target genes. At normal oxygen levels (normoxia), proline hydroxylases hydroxylate HIF, and the subsequent interaction of HIF with the von Hippel-Lindau tumor suppressor gene (pVHL) leads to ubiquitination and degradation of HIF. Asparagine hydroxylation by the factor inhibiting HIF (FIH) leads under normoxia to suppressed HIF transactivation. According to the model, proline and asparagine hydroxylation reactions are inhibited under hypoxia.

In **paper I**, we established a mechanism that allows us to explain the molecular switch of the aryl hydrocarbon receptor (AhR) from its transcription factor to its E3 ubiquitin ligase function. We used the breast cancer cell line MCF7 to demonstrate that the availability of ARNT modulates the dual functions of AhR. Upon ARNT availability, the AhR functions as a ligand-induced transcription factor. If, however, other binding partners, such as the repressor of AhR (AhRR), occupy ARNT, the AhR functions as an E3 ubiquitin ligase. In **paper II**, we revealed significantly elevated FIH expression in skeletal muscle compared to other tissues. We also demonstrated that FIH loss leads to an induced oxidative metabolism, and an increased glycolytic capacity, resulting in elevated oxygen consumption. Loss of FIH further correlates with a decreased metabolic efficiency, an increased oxidative rate and an accelerated HIF-mediated response to hypoxia. In **paper III** (manuscript), we investigated the role FIH plays in epigenetic regulation, among others to explain the underlying mechanism behind the results presented in paper II. Muscle cells show the highest FIH levels on tissue level and we observed elevated histone 3 lysine 9 dimethyl (H3K9me2) levels in FIH null mice. Further work showed significantly increased methylation levels at the H3K9 dimethyl repression mark at promoter regions of various metabolic HIF target genes in FIH null mouse embryonic fibroblasts, and subsequently correlations of loss of FIH and Jmjd1a dysregulated protein levels. In the following, despite promising preliminary results to date no FIH-Jmjd1a protein-protein interaction could be demonstrated.

LIST OF SCIENTIFIC PAPERS

I. Luecke-Johansson S,* **Gralla M**,* Rundqvist H, Ho JC, Johnson RS, Gradin K, Poellinger L (2017) A molecular mechanism to switch the aryl hydrocarbon receptor from a transcription factor to an E3 ubiquitin ligase. Mol Cell Biol 37 (13): 1-14.

II. Sim J, Cowburn AS, Palazon A, Madhu B, Tyrakis PA, Macías D, Bargiela DM, Pietsch S, **Gralla M**, Evans CE, Kittipassorn T, Chey YCJ, Branco CM, Rundqvist H, Peet DJ, Johnson RS (2018) The factor inhibiting HIF asparaginyl hydroxylase regulates oxidative metabolism and accelerates metabolic adaptation to hypoxia. Cell Metab 27 (4): 898-913.

III. **Gralla M**, Linke S, Braune EB, Rundqvist H, Gradin K, Lendahl U, Johnson RS, Poellinger L (2018) Factor inhibiting HIF mediates the epigenetic response by regulation of the histone demethylase Jmjd1a. Manuscript.

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CONTENTS

LIST OF ABBREVIATIONS

AhR	Aryl Hydrocarbon Receptor
AhRR	Repressor of AhR
ARD	Ankyrin Repeat Domain
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
ASPP	Apoptosis-Stimulating p53-Binding Protein
B(a)P	Polycyclic Aromatic Hydrocarbon Benzo(a)pyrene
bHLH	Basic Helix-Loop-Helix
bHLH-Pas	Basic Helix-Loop-Helix-PER-ARNT-SIM
BRCA	Breast Cancer Gene
CA9	Carbonic Anhydrase 9
Co-IP	Co-Immunoprecipitation
COMPASS	Complex Of Proteins Associated With Set-1
COX	Cytochrome C Oxidase
CYP1A1	Cytochrome P4501A1
DUB	Deubiquitinating Enzymes
ERα	Estrogen Receptor Alpha
FIH	Factor Inhibiting HIF
GLP	G9a-Like Protein
GSK-3beta	Glycogen Synthase Kinase
HIF	Hypoxia-Inducible Factor
H3K9	Histone 3 Lysine 9
H3K9me2	Histone 3 Lysine 9 Dimethyl
H3K27me3	Histone 3 Lysine 27 Trimethyl
H3K36	Histone 3 Lysine 36
HER2	Human Epidermal Growth Factor Receptor 2
HRE	Hypoxia Response Element
JmjC	Jumonji C

Jmjd1a	Jumonji Domain Containing 1A
L1CAM	L1 Cell Adhesion Molecule
MEF	Mouse Embryonic Fibroblasts
NFR	Nucleosome-Free DNA Region
NLS	Nuclear Localization Sequence
O/O_2	Oxygen
ODD	Oxygen-Dependent Degradation Domain
2OG	2-Oxoglutarate
PCB	Polychlorinated Biphenyl
PER	Periodic Circadian Protein Homologue
PHD	Proline Hydroxylase
Po_2	Partial Oxygen Pressure
PPARα	Peroxisome Proliferator-Activated Receptor Alpha
PPRE	PPAR Responsive Element
PR	Progesterone Receptor
ROS	Reactive Oxygen Species
SIM	Single-Minded Homologue
STF	Stem Cell Factor
TCDD	2,3,7,8-Tetrachlorodibenzodioxin
TAD	Transactivation Domain
UCP1	Uncoupling Protein 1
VEGF	Vascular Endothelial Growth Factor
pVHL	Von Hippel-Lindau Tumor Suppressor Gene
VO_2 max	Maximal Oxygen Consumption
XIST	X-inactive specific transcript
XRE	Xenobiotic Response Element

1 INTRODUCTION

Regulation of gene expression is a complex process that serves to initiate a response to changes in the cellular environment. Critical for the response in this regulatory network are DNA-binding transcription factors. Babu and colleagues use Pfam and SUPERFAMILY to predict the number of DNA-binding transcription factors to be around 2.600 (Babu MM, 2004). E.coli research provides the most detailed information about an organism's transcriptional network. In E.coli, around 75% of transcription factors are two-domain proteins, consisting of a regulatory and a DNA-binding domain, and around one third of the total transcription factors are expressed to regulate other transcription factors (Babu MM and Teichmann SA, 2003). Mammalian basic HLH (helix-loop-helix)-PER-ARNT-SIM (bHLH-PAS) proteins shape a subclass of the bHLH superfamily (Fig. 1). The signal-regulated subunit dimerizes with the unregulated subunit and binds to specific DNA binding sites (motif of 4-6 DNA-binding basic amino acids) to trigger a specific response (Bersten DC, 2013).

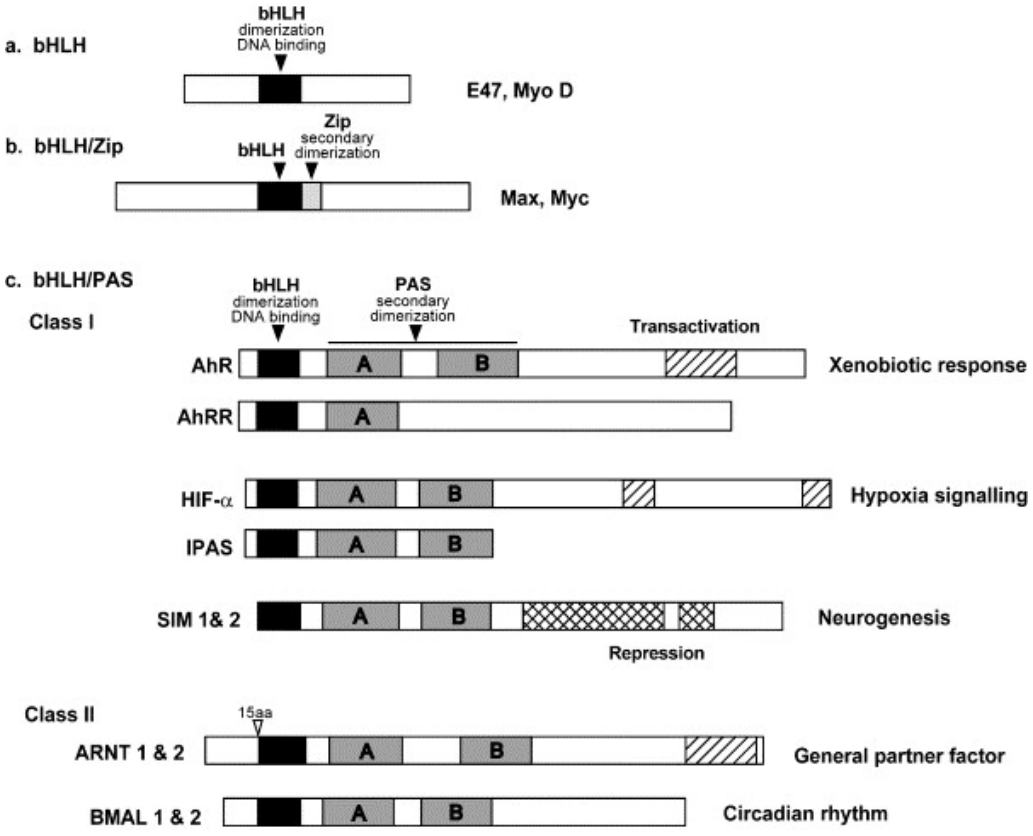

Figure 1: Schematic representation of bHLH-PAS proteins with functional domains (Kewley RJ, 2004).

1.1 Aryl hydrocarbon receptor

1.1.1 AhR biology

Aryl hydrocarbon receptor (AhR) is a member of the bHLH-PAS proteins. AhR gets induced by endogenous factors and a wide range of xenobiotic substances (Bersten DC, 2013; Denison MS, 2011). These can be divided into plant material or pollutants, including natural pollutants such as combustion-derived polycyclic aromatic hydrocarbon benzo(a)pyrene B(a)P, or synthetic pollutants such as hologenated aromatic hydrocarbons, for example, polychlorinated biphenyls (PCBs) or 2,3,7,8-Tetrachlorodibenzodioxin (TCDD).

Upon ligand binding (Fig. 2), the chaperone-bound AhR translocates to the nucleus to dimerize with its dimerization partner aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR-ARNT heterodimer binds to a xenobiotic response element (XRE) to induce transcription of classic detoxification pathway genes for phase I or phase II of xenobiotic metabolism, for example, AhR target gene cytochrome P4501A1 (CYP1A1) (Bersten DC, 2013). AhR null mice develop colonic and intestinal tumors, suggesting a tumor suppressor function of the receptor (Kawajiri K, 2009).

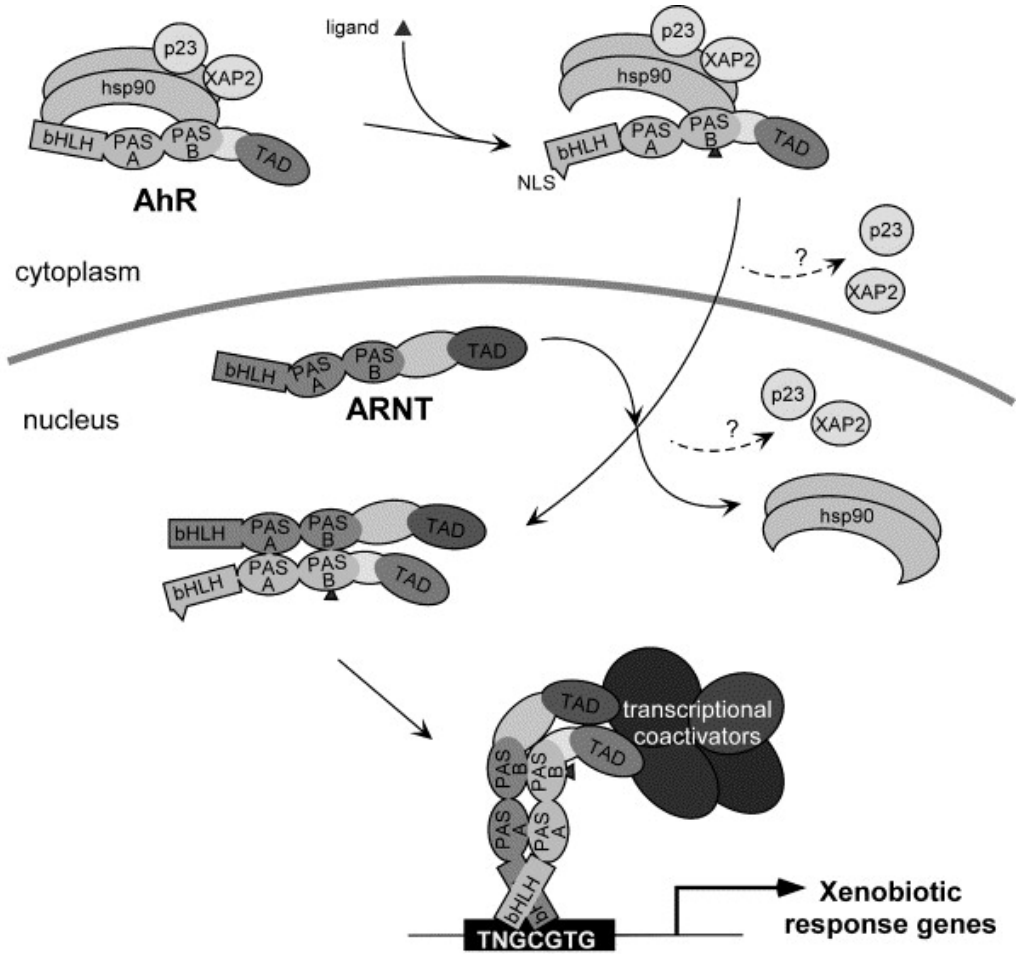

Figure 2: Schematic representation of the AhR signaling pathway. Upon ligand-binding, AhR translocates into the nucleus, binds its dimerization partner ARNT, binds to XREs and triggers the expression of AhR target genes (Kewley RJ, 2004).

Another bHLH-PAS protein is the repressor of AhR (AhRR). The protein lacks the AhR-specific transactivation and ligand-binding domain. For this reason, the AhRR cannot be induced by ligands (Mimura J, 1999). AhR and AhRR competitively utilize ARNT as dimerization partner protein. Oshima and coworkers demonstrated that mutual sumoylation increases AhRR-ARNT interaction and by XRE binding suppresses AhR signaling (Oshima M, 2009). In contrast, hypoxia inducible factor (HIF) – another dimerization partner of ARNT – is not affected by AhRR, as our studies show no effect on HIF target gene expression upon AhRR overexpression, indicating that AhRR does not suppress the HIF signaling pathway (Paper I).

1.1.2 ARNT

ARNT is a nuclear localized protein that serves as dimerization partner for bHLH-PAS family proteins (Eguchi H, 1997). It contains an N-terminal nuclear localization sequence (NLS), a heterodimerization domain and a C-terminal transactivation domain (Whitelaw ML, 1994). Through its NLS, it binds to components of the nuclear pore (Kewley RJ, 2004). In contrast to its signal-regulated dimerization partner, ARNT protein is free from any repressive activity (Whitelaw ML, 1994). ARNT expression was found to be ubiquitous (Abbott BD and Probst MR, 1995).

ARNT is an essential physiological factor. $ARNT^{-/-}$ studies demonstrated its importance in hypoxic regulation by dimerizing with HIF and help initiate transcriptional activation. $ARNT^{-/-}$ mice show embryonic lethality due to abnormal vascular development (Maltepe E, 1997). Furthermore, knock-out studies showed the importance of ARNT during development and in physiology (Kozak KR, 1997; Wang XL, 2009). Ablation of ARNT caused an inaccurate hypoxic response and mouse embryos to die beyond embryonic day 10.5, as a consequence to defects in vascularization (Kozak KR, 1997). A liver-specific ARNT knock-out showed a metabolic phenotype similar to type 2 diabetes, including increased hepatic gluconeogenesis, increased lipogenic gene expression and low serum b-hydroxybutyrate (Wang XL, 2009).

In addition to its well established role as bHLH-PAS dimerization partner, ARNT might have additional roles. ARNT's preferred binding sequence CACGTG as well as its C-terminal transactivation domain might lead to activation of transcription by the CACGTG E-box (Antonsson C, 1995; Huffman JL, 2001).

1.1.3 Breast cancer

Cancer is a complex disease. Abnormal cells divide in an uncontrolled manner, invade nearby tissues and reach through the blood/lymph system other parts of the organism. Tumors show heterogeneous gene expression, histology and clinical outcome. Hanahan and Weinberg described several factors with impact on tumor development (Hanahan D and Weinberg RA, 2000; Hanahan D and Weinberg RA, 2011):

- Self-sufficiency in growth signals
- Insensitivity to anti-growth signals

- Evasion of apoptosis
- Unlimited replicative potential
- Constant angiogenesis
- Tissue invasion
- Metastasis
- Cancer-specific metabolism
- Immune evasion
- Genomic instability
- Tumor-promoting inflammation

In the case of breast cancer, new and malignant growth of tissue (neoplasm) arises in the ducts and lobules of the breast, consequently leading to metastasis in lung, bone and liver (Weigelt B, 2005).

1.1.4 Breast cancer mortality

Cancer is among the leading causes of death worldwide, despite a declining trend. According to the 2018 report of the American Cancer Society, breast cancer is the most frequently diagnosed cancer in women in the United States with over 266.000 estimated new cases. After lung cancer, breast cancer is the second major cause of death (14%). Before the age of 20, brain tumors represent the leading cause of death, breast cancer between 20-59 years of age, and lung cancer thereafter. The 5-year relative survival rate for female breast cancer lies at 90%. The overall breast cancer death rate among women dropped 39% from 1989 to 2015 (Siegel RL, 2018). In line with the US prognosis, the predicted number of deaths for women with breast cancer in 2018 in the European Union are the second highest after lung cancer (Malvezzi M, 2018).

1.2 Hypoxia inducible factor

1.2.1 Oxygen

Energy for animal life is supplied by oxidation/reduction reactions that are coupled to ATP synthesis. Oxygen (O_2) is the only molecule to serve as oxidant in these energy-producing reactions and its deprivation leads to rapid cell death. Due to varied energy requirements in cells, the demand for O_2 varies remarkably among tissues. Oxygen can be incorporated into amino acids, fat, carbohydrates and other molecules, suggesting an important role of oxygen in the organism's structure and regulation (Vanderkooi JM, 1991). In physiology, the amount of oxygen in a tissue is expressed in terms of its partial pressure (Po_2), for example as millimeters of mercury (mmHg). There is no simple way to transform units of pressure to units of concentration as oxygen concentration depends on its solubility in a given fluid at a particular temperature and an ambient pressure of gas.

1.2.2 Hypoxia inducible factors

Hypoxia inducible factor proteins are the master regulators of the hypoxic response (low oxygen). HIFs respond to oxygen changes, as they are regulated by two different oxygen-dependent post transcriptional hydroxylations – hydroxylations of a proline and an asparagine. The three proline hydroxylases (PHD1, PHD2, PHD3) act at the oxygen-dependent-degradation domain (ODD) and lead to binding of HIF to the von Hippel Lindau protein (pVHL). HIF binding to VHL results in polyubiquitination and degradation of HIF at the 26S proteasome. Asparagine hydroxylation controls HIF activity. HIF contains two transactivation domains (TADs) – the N-terminal (NTAD) and the C-terminal TAD (CTAD) (Ema M, 1999). Factor inhibiting HIF (FIH) causes an additional oxygen-dependent hydroxylation reaction, at the CTAD. FIH hydroxylates an asparagine within the HIF CTAD. This reaction prevents binding of the essential p300/CBP coactivators, resulting in a repressed CTAD. Proline and asparagine hydroxylation reactions require oxygen, hence, cellular hypoxia inhibits the reactions and results in stabilization of the HIF proteins, which results in recruitment of coactivators and activation of HIF target gene expression (Hewitson KS, 2002). As PHDs show *in vitro* lower affinity for O_2 than FIH, it is suggested that the proline and asparagine hydroxylations generate a graded, coordinated response to hypoxia (Koivunen P, 2004). This has led to the view that during (Schofield CJ and Ratcliffe PJ, 2005):

- Moderate hypoxia: PHDs are inhibited, thus the NTAD is active and can activate HIF target gene expression
- Severe hypoxia: FIH is inhibited as well, thus the CTAD is active and HIF target gene expression is fully activated.

For HIF however, hydroxylation effects are well-characterized, causing repression of HIF-α activity in normoxia, whereas in hypoxia, when FIH is inhibited, HIF-α is potently activated and can drive expression of hundreds of target genes.

1.2.3 HIF response

HIF-α proteins are members of the bHLH-PAS superfamily of transcription factors and regulate the cellular response to hypoxia. HIFs are degraded and repressed in normoxia, and stabilized and activated in hypoxia (Fig. 3). Upon stabilization, HIFs form DNA-binding heterodimers with their dimerization partner protein ARNT. HIFs bind to the consensus DNA sequence 5'-RCGTG-3' within hypoxia responsive DNA elements (HREs) in numerous target gene promoters, and activate their transcription. The gene products help cells and animals adapt to hypoxia and include vascular endothelial growth factor (vascularization), red blood cell production (erythropoiesis) and numerous glycolytic enzymes (anaerobic ATP generation) (Fedele AO, 2002).

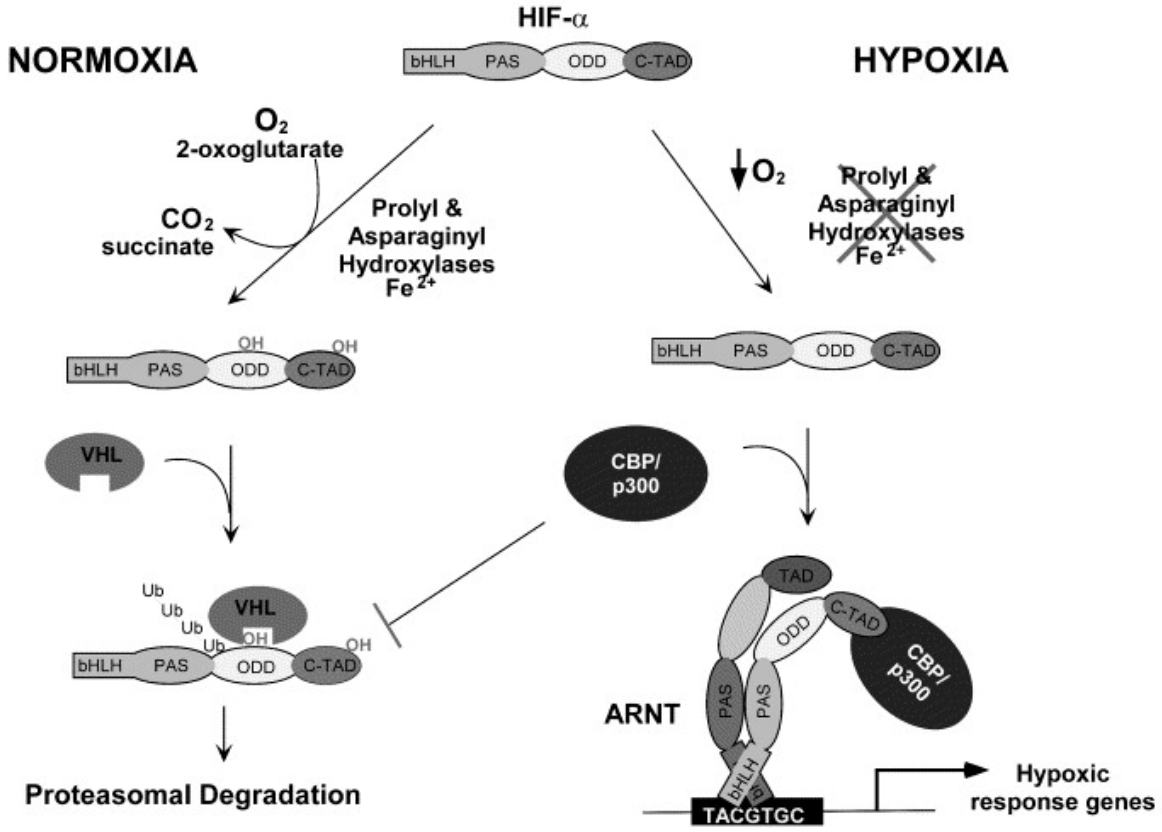

Figure 3: Schematic representation of the HIF signaling pathway. Upon lack of oxygen, HIFs translocate into the nucleus, bind their dimerization partner ARNT, bind to HREs and trigger the expression of HIF target genes (Kewley RJ, 2004).

1.2.4 HIF-α isoforms

HIF-α has several isoforms – HIF-1α, HIF-2α and HIF-3α. HIF-1α and HIF-2α have tissue-specific roles. Both isoforms represent the signal-regulated and tissue-restricted subunit and dimerize with the more ubiquitous and unregulated subunit, known as HIF1ß or ARNT. HIF-1α is ubiquitously expressed, but post-translationally degraded/inactive and upregulated in response to acute hypoxia to drive expression of mainly metabolic target genes. HIF-2α, on the other hand, shows a more restricted tissue distribution and drives target gene expression in response to chronic hypoxia. Besides its metabolic effects and the control of erythropoietin, HIF-2α has been shown to target antioxidant enzymes such as superoxide dismutase 1 and 2, glutathione peroxidase 1 and catalase (Scortegagna M, 2003). The expression of an alternative HIF-3α splice variant (iPAS) functions as negative regulator of HIF-1α and adds another level of complexity to the modes of HIF suppression (Makino Y, 2001).

1.2.5 HIF's role in cancer

HIFs are generally upregulated in a broad range of human cancers. HIF has effects on tumor growth and vascularization, and thus patient survival (Keith B, 2012). However, despite HIF's well established tumor promoting effects, in certain contexts HIFs counterintuitively suppress tumor progression. Multiple HIF target genes play key roles in cancer biology, including metabolic reprogramming by targeting among others Aldolase 1, carbonic anhydrase 9 (CA9), phosphoglycerate kinase 1 (PGK1), or Enolase 1 (Semenza GL, 2012).

In breast cancer cells, HIF-1α and HIF-2α levels are elevated and correlated to mortality (Bos R, 2003; Helczynska K, 2008). Liao and colleagues demonstrated that HIF-1α through its transcriptional activity positively promotes tumor progression and lung metastasis (Liao D, 2007). Metastasis accounts for nearly all breast cancer deaths (Talmadge JE, 2010). Intravasation and extravasation are central processes for breast cancer cells to metastasize, meaning breast cancer cells need to migrate from the primary tumor into a blood vessel and afterwards migrate into a distant metastatic site, for example, the lungs. The HIF target vascular endothelial growth factor (VEGF) has been shown to promote the effects of intravasation (Sullivan R and Graham CH, 2007).

Furthermore, it has been shown that HIFs regulate two critical steps required for extravasation – margination and disruption of the tight interactions between endothelial cells (Padua D, 2008; Zhang H, 2012). Margination describes the process of cancer cells adhering to endothelial cells in the lung vasculature. Zhang and colleagues showed HIF-1α (not HIF-2α)-induced L1 cell adhesion molecule (L1CAM) expression in hypoxic breast cancer cells leads to increased lung metastasis via elevated interaction of breast cancer cells with endothelial cells, which results in margination and consequently extravasation (Zhang H, 2012). To conclude HIFs function as facilitator of breast cancer cell migration from its primary to its metastatic site, hypoxia also regulates cell motility and permeability. Padua and colleagues identified angiopoietin-like 4, a hypoxia-dependent (HIF-1α and HIF-2α) protein that inhibits interactions between endothelial cells and has a demonstrated history in modulating vascular permeability, cancer cell motility and invasiveness. Breast cancer patients with lung metastases show elevated expression levels of angiopoietin-like 4 (Kim SH, 2011; Padua D, 2008).

1.2.6 Interaction between signaling pathways

Signaling pathways are connected on different levels. Recent work discovered interactions of hypoxia, HIFs and oxygen-sensing enzymes with numerous pathways of central function in physiology and disease. The following examples aim to showcase this widespread interaction (Gustafsson MV, 2005; Rodriguez J, 2018; Shareef MM, 2013; Yin G, 2017):

1. Tumor suppressor p53 (Rodriguez J, 2018)

Regulation of p53 tumor suppressor through hypoxia and hydroxylase inhibition has been subject to speculation for a long while. Recent work from Rodriguez and colleagues

demonstrated that PHD3 hydroxylates p53 at proline 359, which is located in the p53-DUB binding domain. The removal of ubiquitin chains is mediated by USP7 and USP10, two deubiquitinating enzymes (DUBs). P359 hydroxylation decreases p53-USP7/USP10 association, and consequently elevates p53 ubiquitination, which leads to reduced p53 levels. The authors conclude to have identified p53 as novel PHD3 substrate and uncovered a direct mechanism of post-translational modification through the oxygen-sensing enzyme to regulate p53 protein stability (Rodriguez J, 2018).

2. Notch (Gustafsson MV, 2005; Shareef MM, 2013)

Gustafsson and colleagues were the first to report interactions between the cellular hypoxic response and Notch signaling (Gustafsson MV, 2005). Notch signaling is conserved across metazoan species. In Notch signaling, the pathway is initiated upon cell-cell-contact. A Notch (Notch1-4) transmembrane receptor-presenting cell is activated by a Notch ligand-presenting cell (DSL including Delta-like and Jagged). Interaction with ligand leads to successive cleavage events, eventually liberating the Notch intracellular domain (NICD) from the plasma membrane. The NICD translocates into the nucleus, recruits a transcriptional activation complex with the DNA-binding protein CSL and co-activators and finally activates transcription of downstream Notch target genes (Bray SJ, 2006; Poellinger L and Lendahl U, 2008). Shareef and colleagues further demonstrated that HIF-1α and Notch 3 (and VHL) interaction is required at the carbonic anhydrase 9 (CA9) promoter for the expression of CA9 in breast cancer carcinoma cells (Shareef MM, 2013).

3. Lipid metabolism (Yin G, 2017)

Metabolic switches allow cells to adapt to different conditions. Hypoxia is an example for such an alteration in environmental condition. While oxidative phosphorylation represents the major source of ATP production in most mammalian cells in normal conditions, during hypoxia, cells decrease their mitochondrial oxygen consumption and among others induce lipid metabolism (Strehl C, 2014). In mammalian cells, the serine/threonine kinase rapamycin (mTOR) is central in sensing of extracellular stimulations and mediating intracellular adaptations (Ricoult SJ, 2013). Yin and colleagues identified mTOR as key player that allows regulation of lipid homeostasis in lymphocytes by mediating the switch from lipid synthesis to lipid oxidation during hypoxia (Yin G, 2017).

1.3 Factor inhibiting HIF

1.3.1 Factor inhibiting HIF hydroxylates ARD-containing proteins

Factor inhibiting HIF (FIH) was originally discovered as an inhibitor of the HIF-α proteins (Mahon PC, 2001). The transcriptional activity of HIFs is partly determined by asparaginyl

hydroxylation (Asn803) by FIH of a C-terminal residue that regulates co-activator recruitment (Lando D, 2002). Hydroxylation of HIFs by FIH inhibits transcriptional activation by blocking association of HIFs with the transcriptional co-activators CBP/p300 (Kallio PJ, 1998). FIH utilizes O_2 and 2-oxoglutarate and contains Fe (II) in its active site (Lando D, 2002). FIH hydroxylates ARD-containing proteins, such as Notch (Coleman ML, 2007; Cockman ME, 2009). Since ARDs are ubiquitous (>5% of eukaryotic proteins), post-translational modification by hydroxylation via FIH seems to be very common in human cells (Kelly L, 2009).

FIH has ubiquitous function in physiology and disease. Three examples are given below (Janke K, 2013; Liu Y, 2013; Peng H, 2013):

1. FIH hydroxylation causes changes in protein-protein interaction (Janke K, 2013)

Protein interactions of ARD-containing substrates can be modified by FIH-dependent hydroxylation. Janke and colleagues identified apoptosis-stimulating p53-binding protein (ASPP) as FIH target. ASPP2 has a single hydroxylation site (N986) located within the ARD. While ASPP2 protein levels and stability were not affected by depletion or inhibition of FIH, ASPP2 binding to Par-3 (cell polarity) was impaired. Furthermore, the study shows relocation of ASPP2 from cell-cell contacts to the cytosol in FIH depleted colon carcinoma cells. Despite the finding that FIH had no effect on ASPP-p53-interaction, apoptosis, or proliferation of cancer cells, it suggests an important role of FIH hydroxylation on protein-protein interaction.

2. FIH-c-kit interaction regulate glycogen metabolism (Peng H, 2013)

Stem cell factor (STF) serves as ligand for tyrosine kinase receptor c-kit. STF-mediated c-kit activation regulates cancer-associated protein kinase B (Akt). Peng and colleagues identified FIH-c-kit interaction as mediator of glycogen metabolism in corneal epithelial keratinocytes and showed diminished glycogen stores through the Akt/Glycogen Synthase Kinase (GSK-3beta) pathway. c-kit targets glucose metabolism via the Akt/GSK-3beta, which is further increased through FIH-c-kit interaction by activating GSK-3beta, resulting in inhibition of glycogen synthase/synthesis.

3. Increased vascularization as result of sequestered FIH (Liu Y, 2013)

Gankyrin is an oncoprotein overexpressed in hepatocellular carcinomas that contains seven ankyrin repeats, which are interacting with multiple proteins such as Cdk4, Mdm2, NF-kB, and Rb. Liu and colleagues demonstrated that transgenic mice overexpressing gankyrin developed hepatic vascular neoplasms. Liu et al. further showed that gankyrin binds to and sequesters FIH, which lead to decreased FIH-HIF-interaction and increased activity of HIF on the VEGF promoter. These effects of gankyrin are more prominent under 3% than 1% O_2.

1.3.2 FIH – a JmjC domain-containing hydroxylase

JmjC domain-containing proteins play a crucial role in the control of gene expression by acting as protein hydroxylases or demethylases. Trewick and colleagues claimed that the fission yeast protein Epe1 is a putative histone demethylase. Similar to FIH, Epe1 contains a JmjC domain (Trewick SC, 2005). Recent work has demonstrated that other 2OG oxygenases (similar to FIH) catalyze histone demethylation (Loenarz C and Schofield CJ, 2011). Another JmjC domain-containing enzyme, Jmjd6, has been shown to act as a histone arginine demethylase as well as a lysine hydroxylase (Chang B, 2007; Webby CJ, 2009). It appears plausible that, in addition to the established oxygen-dependent regulation of HIF function via HIF hydroxylases, there are other targets and steps in the hypoxia signaling pathways that are controlled by other 2-oxoglutarate oxygenases, such as FIH.

1.3.3 FIH knock-out

Mice lacking FIH show no alteration of classical HIF function, such as angiogenesis, or erythropoiesis. Rather, it causes a hypermetabolic phenotype, including reduced body weight, hyperventilation, elevated metabolic rate, and improved glucose and lipid homeostasis. Furthermore, these mice are more resistant to weight gain and hepatic steatosis induced by a high fat diet. A conditional neuron-specific knock-out shows lack of FIH results in reduced body weight, an increased metabolic rate, increased insulin sensitivity and resistance to high-fat-diet induced weight gain (Zhang N, 2010).

1.3.4 Role in disease

FIH is ubiquitously expressed, predominantly cytoplasmic but, in most normal tissues, with significant amounts detectable in the nucleus (Metzen E, 2003; Soilleux EJ, 2005). In tumor cells, however, it more often adopts an exclusively nuclear localization and, furthermore, its subcellular localization has been correlated with patient survival (Kroeze SG, 2010; Shaida N, 2011; Soilleux EJ, 2005; Tan EY, 2007). It has been reported for breast and renal tumors that high levels of nuclear FIH predict longer patient survival, whereas the lack of nuclear localized FIH predicts a more adverse outcome (Kroeze SG, 2010; Shaida N, 2011; Tan EY, 2007). On the other hand**,** in prostate tumors, the trend is the opposite (Kroeze SG, 2010; Shaida N, 2011; Tan EY, 2007). The mechanism for these correlates is unknown and whether FIH acts causally or purely as a silent marker is also not known.

1.4 Epigenetics

Regulation of gene expression has evolved from characterizing transcription factors and their binding sites into a complex collection of post-transcriptional events, including microRNA, DNA methylation, or histone modifications that package DNA.

1.4.1 Chromatin

Histones are nucleoproteins in eukaryotic cells. Their net positive charge allows them to associate with negatively charged core DNA to help condense it into chromatin. They package and order DNA into structural subunits called nucleosomes (Bernstein BE, 2007). Nucleosomes form a pack called chromatin (two chromatins form a chromosome) and consist of the following histone types: H2A, H2B, H3, and H4. H1 serves as nucleosome connector and represents the least tightly bound histone to chromatin. Nucleosomes are the first level of DNA organization and consist of two copies of four core histones (H2A, H2B, H3, H4), called octamer. 146 base pairs (bp) DNA are wrapped around one histone octamer. Linker or spacer DNA (8-114bp) connect histone octamers (Kornberg RD and Lorch Y, 1999; Richmond TJ and Davey CA, 2003).

1.4.2 Chromatin modification

In addition to sequence-specific activators and repressors, chromatin modification and remodeling play crucial roles in regulation of gene expression. Residues within the histone tails and the histone globular domains can be modified in various ways, including phosphorylation, acetylation, ubiquitination, and methylation (Lee JS, 2010). Histone modifications have context-dependent effects and can act in multiple ways (Fig. 4).

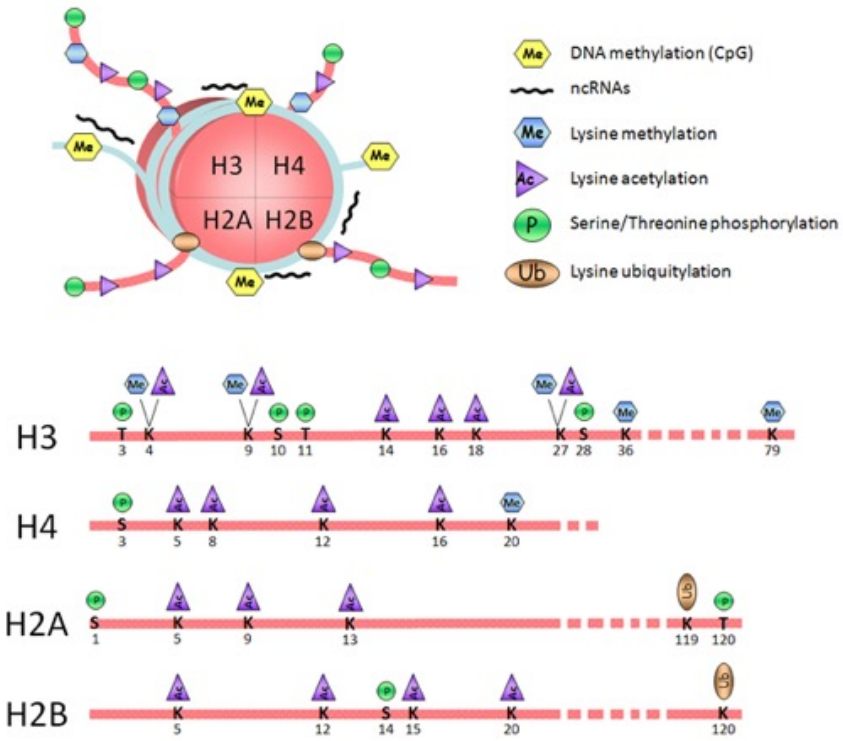

Figure 4: Schematic representation of histone modifications (biochem.slu.edu/).

According to Suganuma and colleagues three main cross-regulatory mechanisms appear to exist (Suganuma T, 2008):

(1) A histone modification initiates an increased activity in a histone-modifying enzyme,
(2) Coordination of different histone-modifying enzymes present in the same protein complex, and
(3) A histone modification mediates the cleavage of the N-terminal tail of histone H3, leading to an irreversible modification.

For example, it has been shown that H2B ubiquitination is required for histone 3 lysine 4 methylation by H3K4 methylase complex COMPASS (complex of proteins associated with Set-1) (Shilatifard A, 2006). To add another level of complexity, deubiquitination also affects histone 3 methylation, hence, H2B ubiquitination and deubiquitination act as master switches, mediating numerous histone 3 methylation events (Wyce A, 2007).

These depending scenarios can be facilitated through complexes histone-modifying enzymes bind to, therefore modification of specific binding sites may lead to access of another enzyme to a nearby residue. This interplay can lead to facilitation or prevention of recognition of another substrate (Guccione E, 2007; Martin DG, 2006).

1.4.3 Chromatin modifying enzymes

Histone modifications influence central cellular events by regulating chromatin structure and gene transcriptional activity. The different modifications are correlated to either transcriptional activation or repression. Numerous enzymes control these modifications in a dynamic manner to add and remove these modifications.

Jumonji Domain Containing 1A (Jmjd1a; also known as JMJD2A/KDM4A) is a histone lysine demethylase that targets H3K9 and H3K36 (Berry WL and Janknecht R, 2013). Jmjd1a specifically targets H3K9me2 reactions, which is associated with transcriptional repression. Jmjd1a regulates metabolic genes related to energy homeostasis, including anti-adipogenesis, fat storage, glucose transport, and type 2 diabetes. The histone demethylase plays further an important role in DNA damage response, cellular differentiation, and male germ cell development and nuclear hormone receptor-mediated gene activation (Berry WL and Janknecht R, 2013). Furthermore, studies have shown that Jmjd1a is overexpressed in tumors, among others breast, colorectal, lung, and prostate cancer, and that it is required for efficient cancer cell growth (Guerra-Calderas L, 2015).

Jmjd1a mouse knockout models identify the histone demethylase as an important regulator of the expression of metabolic genes. Tateishi and colleagues demonstrated ablation of Jmjd1a results in obesity and hyperlipidemia in adult mice by disrupting beta-adrenergic-stimulated glycerol release and oxygen consumption in brown fat, decreasing fat oxidation, and diminishing glycerol release in skeletal muscles. The authors demonstrated peroxisome proliferator-activated receptor alpha (PPARα) and uncoupling protein 1 (UCP1) – genes involved in controlling energy balance – are regulated and therefore direct targets of Jmjd1a. This was achieved by decreased levels of H3K9me2 at the PPAR responsive element (PPRE)

in response to beta-adrenergic activation-induced binding of Jmjd1a to PPRE of the ucp1 gene (Tateishi K, 2009).

Tateishi and colleagues' findings are in line with a study conducted by Inagaki et al. They identified Jmjd1a as overall crucial for regulating the expression of metabolic genes related to energy homeostasis including anti-adipogenesis, regulation of fat storage, glucose transport, and type 2 diabetes. Loss of Jmjd1a function phenotypically results in adult onset obesity, hypertriglyceridemia, hypercholesterolemia, hyperinsulinemia, and hyperleptinemia – hallmarks of the metabolic syndrome – and fasted-induced hypothermia indicating reduced energy expenditure, as well as a higher respiratory quotient indicating less fat utilization for energy production. Inagaki and colleagues also observed suppressed global H3K9 demethylation and germ cell developmental defect in males due to ablation of Jmjd1a (Inagaki T, 2009).

Furthermore, Okada and colleagues identified Jmjd1a as essential factor in spermatogenesis, in the regulation of fat metabolic gene expression in muscle and brown fat tissue, and in obesity and hyperlipidemia. Infertility is achieved by impaired postmeiotic chromatin condensation through direct binding of Jmjd1a to core promoter regions of genes involved in sperm nuclear condensation (Okada Y, 2010).

H3K9 methylation is a pivotal epigenetic mark known to be transcriptional repressing. Hypoxia increases global H3K9me2 levels, which results in elevated histone methyltransferase G9a protein expression and enzyme activity (Chen H, 2006). G9a is a H3K9 specific methyl transferase and essential for embryonic development (Tachibana M, 2002). Tachibana and colleagues further identified a related methyltransferase, the G9a-like protein (GLP), to be important for H3K9 methylation of mouse chromatin. GLP-deficiency led to severe decrease in H3K9me1/me2 levels and lethality in embryos. The authors conclude that G9a and GLP work cooperatively in complexes to function as a methyltransferase in vivo (Tachibana M, 2005).

Inactive X-chromosomes are rich in histone 3 methylation at lysine 9 and lysine 27. Ohhata and colleagues therefore suggested a role of G9a in X-inactivation by studying the X-inactive specific transcript (Xist). Despite the absence of G9a, Xist is properly regulated and the X chromosome inactivated. The authors consequently conclude, ablation of G9a is not essential for X-inactivation (Ohhata T, 2004). Tachibana and colleagues, on the other hand, demonstrated dramatic effects of G9a ablation. Not only observed the authors drastically reduced H3K9 methylation levels mostly in euchromatin regions, but more importantly severe growth retardation and early lethality, implicating H3K9 methylation regulated by G9a has important roles in early embryogenesis through transcriptional repression of developmental genes (Tachibana M, 2002).

1.4.4 Chromatin and HIF

Hypoxia increases global H3K9me2 in several mammalian cell lines, which is correlated with an increase in histone methyltransferase G9a protein and enzyme activity (Chen H, 2006). Furthermore, it is proven that the histone demethylase genes Jmjd1a, Jmjd2b, and Jarid1b are

HIF targets, suggesting an indirect influence of HIFs on gene expression at the level of histone methylation under hypoxia (Krieg AJ, 2010). Epigenetic regulatory mechanisms play a crucial role in the cellular response to hypoxia. Modulation of the hypoxic response by epigenetic mechanisms seems to be multilayered (Watson JA, 2010):

(I) Stabilization of HIF proteins is influenced by epigenetically controlled expression of VHL and PHD3.

(II) Epigenetic modifications regulate HIF binding by inducing or maintaining an active chromatin confirmation within and around HIF binding site regions.

(III) HIF-1α targets the transcription of histone demethylases, such as Jmjd1a.

(IV) Global changes in histone modifications and DNA methylation occur in response to low oxygen levels.

(V) Nucleosome-free DNA regions (NFRs) are established while hypoxia-induced transcription takes place in hypoxia-inducible promoters (Suzuki N, 2018).

2 AIMS

The main purpose of my PhD study was to elucidate mechanisms of gene regulation in response to external factors - specifically by the transcription factors HIF (oxygen) and AhR (toxins). In order to better understand HIF's role in hypoxia-dependent transcriptional activation, we focused on the regulation of HIF signaling by the enzyme FIH. The specific aims of my thesis were to:

1. Identify the molecular mechanism that modulates the dual functions of AhR in breast cancer cells by analyzing the role of ARNT;
2. Characterize FIH's role in regulation of the oxidative response and its role in accelerating metabolic adaptations to hypoxia in muscle cells; and
3. Unravel the mechanism of FIH action in an oxygen-dependent manner to induce an epigenetic response in muscle cells.

3 RESULTS AND DISCUSSION

3.1 Identification of a molecular switch mechanism shifting the aryl hydrocarbon receptor from an E3 ubiquitin ligase to a transcription factor (Paper I)

The present study was carried out in order to describe a molecular mechanism that allows AhR to switch between its transcription factor and E3 ubiquitin ligase function. To date, AhR is the only bHLH-PAS protein identified to possess these dual functions. Central for the regulation of the switch process proved to be the ubiquitously expressed protein ARNT, which itself is regulated by the bHLH-Pas protein AhRR. Follow-up work with AhRR overexpression resulted in reduced tumor mass, arguing for a tumor-suppressive-like function of AhR.

ERα degradation by the $CUL4B_{AhR}$ complex is not dependent on ARNT.

We chose MCF7 cells to study the transcription factor versus proteasomal degradation function of AhR, as this specific human breast cancer cell line expresses a functional ERα signaling pathway. Our initial experiments showed that the AhR functions as a ligand-activated E3 ubiquitin ligase targeting ERα for proteasomal degradation by using immunoprecipitation and RNA interference assays. As demonstrated before, AhR protein itself was degraded upon ligand treatment (Davarinos NA, 1999). Ablation of ARNT in TCDD treated MCF7 cells resulted in significantly decreased ERα (higher levels of ubiquitinated ERα) and CYP1A1 levels, indicating that the ubiquitously expressed dimerization partner of AhR is critical for AhR's transcription factor function.
In previous work by Ohtake and colleagues, ARNT has been identified as part of the $Cul4b_{Ahr}$ transcription complex along with Cullin-4B, Rbx1, DDB1, TBL3, the 19S regulatory particle, and activated AhR (Ohtake F, 2007). In contrast to Ohtake's findings, we could not identify ARNT as part of this multi-component complex upon TCDD treatment (Co-IP with Cul4B antibody). However, while we used Flag-tagged ARNT in MCF7 cells and neither found ARNT nor Cul4B or ERα as complex components, Ohtake and colleagues used Flag-tagged AhR overexpressed in HeLa cells. We believe their conclusion might be the result of a detected mix of $Cul4B_{AhR}$ E3 ubiquitin ligase and AhR-ARNT transcription factor complex, indicating their results show ARNT within the AhR-ARNT transcription factor complex and not within the E3 ubiquitin ligase complex.

Degradation of ligand-activated AhR is dependent on ARNT.

AhR levels are subject to a tight control mechanism. Previous work by Ma and Baldwin showed that AhR when induced by ligands is regulated in a time- and dose-dependent way. Ma also showed that the ligand-induced ubiquitinylation of AhR is dependent on ARNT (Ma Q, 2000). We confirmed these data, indicating ARNT plays a central role in ligand-activated AhR degradation.

DNA binding disruption of AhR and by that negative regulation of AhR transcriptional function in cells overexpressing AhRR.

AhR and AhRR both operate by dimerizing with ARNT (Oshima M, 2009). To further analyze TCDD-induced AhR signaling, we developed stably overexpressing Myc-tagged AhRR MCF7 cells. We then examined the effect of overexpressed AhRR on CYP1A1 and found decreased expression levels and enzyme activity, and impaired AhR recruitment to the CYP1A1 XRE DNA binding site, indicating overexpression of AhRR show disrupted transactivation potential and DNA binding of ligand-induced AhR.

MCF7 cells stably overexpressing AhRR show decreased estrogen responsiveness and ERα protein levels.

To further investigate the role of AhRR in the process that allows AhR to switch between its dual functions, we next analyzed whether overexpression of AhRR:

a) changes the E3 ubiquitin ligase function of AhR, and
b) shows effects on estrogen treatment (AhR targets ERα for proteasomal degradation)

Our results obtained show that AhRR overexpression leads to reduced ERα target gene levels (cathepsin D and progesterone receptor). Previous work by Brunnberg et al. identified ARNT as cofactor for estrogen-dependent transcription, opening up the possibility that coactivator competition might have an effect on the responsiveness of ERα target genes to estrogen treatment (Brunnberg S, 2003). The observation of decreased estrogen responsiveness was supported by the finding that ERα protein levels were lower and E3 ubiquitinylation was increased in cells overexpressing AhRR. AhRR-ARNT interaction, as previously shown by Oshima and colleagues, apparently (shown in our study) results in switching the AhR receptor towards its E3 ubiquitin ligase function (Oshima M, 2009).
In conclusion, despite the potential effects AhRR overexpression might have on other signaling pathways that regulate ERα function, our data argue in favor of ARNT being the mediator of the molecular switch of AhR as transcription factor and E3 ligase.

MCF7 xenograft tumors stably overexpressing AhRR show reduced tumor volume and ERα expression.

The effect of AhR E3 ubiquitin ligase function on ERα protein levels may have therapeutic implications for the treatment of estrogen-dependent tumors, hence, we conducted an *in vivo* study to investigate tumor growth in MCF7 breast cancer cells with high levels of AhRR. AhRR overexpressing MCF7 cells show significantly lower proliferation rates, as previously described by Zhang and colleagues in estrogen-dependent cell lines (Zhang S, 2009). The estrogen-independent cell line MDA-MB-468 on the other hand showed no effect on cell proliferation. Our xenograft studies show decreased ERα protein levels (similar to our *in vitro* data) and a reduction of tumor growth in SCID mice that received

AhRR overexpressing cells, indicating a tumor-suppressive role of AhR in MCF7 xenograft tumors. Again, our data clearly point towards a tumor-suppressive function of AhR. Recent literature argues in favor and against these findings. While AhR was identified to possess protumorigenic function in liver and stomach tumors, Kawajiri and colleagues suggest an antitumorigenic role of AhR (Andersson P, 2002; Kawajiri K, 2009; Moennikes O, 2004). Standard cancer therapy includes surgery, radiation therapy, hormone therapy, and chemotherapy – often used in combination. Hormone-dependent tumors, for example, breast cancer, are widely subject to hormone therapies, including aromatase inhibitors (inhibiting E2 production) or estrogen receptor antagonists (e.g. tamoxifen). Resistance to these approaches represent a major obstacle in the treatment plan and might result in the development of a more aggressive phenotype.
In summary, our data support a central role of ARNT in the modulation of the molecular switch of AhR towards its transcription factor or E3 ubiquitin ligase function targeting ERα for proteasomal degradation. The availability of ARNT seems to direct AhR towards one of its dual functions. In addition, we also show that AhRR overexpression in MCF7 cells results in reduced levels of ERα protein levels, reduced growth rates, and reduced estrogen-dependent xenograft tumor growth, implying that AhRR protein levels modulate ARNT availability and thereby AhR function. Whether or not AhR E3 ubiquitin ligase function can be utilized for treatment of estrogen-dependent tumors, is unclear. It is certain, however, that our findings point towards a tumor-suppressor role of the E3 ubiquitin ligase function and there is a need for therapeutic treatment that targets the cellular levels of activated ERα. More *in vivo* data will shed light on the mechanisms around AhR and reveal its cancer therapeutic potential.

3.2 Identification of the factor inhibiting HIF as regulator of the oxidative metabolism and driver of the metabolic response to hypoxia (Paper II)

In the current study, we characterize the role of FIH as metabolic mediator in response to changes in energy demand in skeletal muscle. We further compared FIH and VHL function and show synergistic actions of these two key post-translational HIF regulators to modulate metabolism.

Metabolic effects in FIH and VHL Null Cells

We performed microarray analysis and showed that in contrast to loss of VHL loss of FIH can operate both as activator and suppressor of metabolic genes in MEFs under normoxic oxygen levels (deletion of both factors has distinct effects compared to their single deletions). The observed differences might be due to additional functions FIH has when HIF levels are low. The following differences were observed in an qRT-PCR approach:

- Loss of VHL: decreases levels of oxidative genes and increases expression of most glycolytic genes under normoxia and early hypoxia
- Loss of FIH: increases levels of glycolytic gene expression and "locks" oxidative gene expression under normoxia and early hypoxia

Next, we examined intracellular aqueous metabolites (lactate, alanine, glucose, and glutamine) and found clear differences in FIH null and control cells. The above findings are supported by previous studies that demonstrated an upregulation in glycolytic genes as a consequence of loss of FIH (Sakamoto T, 2011; Wang E, 2014).

FIH modulates mitochondrial activity

Several observations in the present study point towards a function of FIH in regulating mitochondrial activity. The following findings support this conclusion:

1. Metabolic differences in FIH and VHL Null Cells
   Due to its high affinity to oxygen, FIH can act as a modulator of the hypoxic response at low oxygen levels. For example, FIH null MEFs showed elevated lactate production and glucose uptake during prolonged hypoxic conditioning but not during normoxia. Interestingly, ATP levels were elevated under 21% O_2, arguing in favor of the presence of alternative oxygen-dependent pathways, possibly as a result of the formation of metabolic intermediates activated by loss of FIH. Data from VHL null MEFs (elevated ATP levels) support a correlative function of these two post-translational HIF regulators.

2. FIH suppresses production of hypoxia-induced mitochondrial ROS
   Furthermore, FIH loss does not alter mitochondrial superoxide production at normal oxygen levels. Previous findings by Rasbach and colleagues show regulation of superoxide dismutase by HIF-2α, which contributes to the control of the antioxidant defence mechanism and limits the production of reactive oxygen species (ROS) (Rankin EB, 2007; Rasbach KA, 2010). In the current study, we demonstrate prolonged hypoxia generated an increase in ROS production in wild-type cells, which is suppressed by loss of FIH – another indication that native FIH activity is not totally suppressed at low oxygen levels.

3. No effect of loss of FIH on the mitochondrial membrane potential
   The data generated until this stage allows the conclusion that the roles of VHL and FIH in mitochondrial regulation are clearly differential. While FIH mediates mitochondrial ROS production and apoptosis in hypoxia, VHL does so in normoxia. We further showed that lack of VHL diminished mitochondrial membrane potential in normoxia. In contrast, loss of FIH shows no effect on mitochondrial potential in normoxia/hypoxia.

4. Mitochondrial levels and activity of FIH null MEFs
   Further studies demonstrated that lack of FIH stimulate the hypoxic effects on mitochondria, indicating diminished mitochondrial functioning and stimulating a shift to a glycolytic metabolism. Loss of FIH increases cytochrome c oxidase (COX) activity in normoxia, while chronic hypoxic O_2 levels reduce COX activity again, suggesting loss of FIH plays a role in oxidative metabolism.

FIH effects on glycolytic metabolism and oxygen consumption

In order to gain further insights into a better understanding of the metabolic response in the knock-out MEFs, we studied oxygen consumption and proton generation in these cells under normoxic oxygen levels.

Loss of FIH showed a significantly elevated glycolytic reserve in normoxia, suggesting a sort of preparedness for hypoxic glycolytic metabolism. Loss of both FIH and VHL drives cells into an ultimate glycolytic state, meaning cells generate energy solely from glycolosis in contrast to oxidation.

As shown in previous work by Hervouet and coworkers, VHL null MEFs show the predicted oxygen consumption response, meaning complete usage of the spare respiratory capacity (Hervouet E, 2005). In contrast to previous findings, FIH null cells, on the other hand, stimulate an increased basal respiration rate in normoxia, which is erased by chronic hypoxia, indicating a suppressive function of the hypoxic response (Fukuda R, 2007). The increased oxygen consumption in FIH null cells is not a result of a switch to fatty acid oxidation, but instead glucose is channeled through pyruvate to the mitochondria.

FIH effects in skeletal muscle

Loss of FIH in skeletal muscle reveals two main functions – acceleration of hypoxic metabolic shifts and stimulation of hypoxic adaptations.

The data provided in this study clearly point towards a role for FIH as mediator of the response to rapid changes in tissue's metabolic activity due to varying oxygenation status. Exclusively skeletal muscle show the by far highest FIH expression levels, both fast and slow twitch muscle. Our *in vivo* data were generated in global FIH deletion animals (global VHL deletion is postnatally mortal), as they show higher basal metabolic rates, as previously demonstrated by Zhang and coworkers, or skeletal muscle-specific deletions of FIH, VHL, or both (Zhang N, 2010). The following findings were observed:

- Loss of FIH: no shifts in basal metabolic rate, higher nocturnal VO_2, increased VO_2 in skeletal muscle during exercise
- Loss of VHL: higher nocturnal VCO_2, decreased VO_2 in skeletal muscle during exercise, increased myoglobin expression in skeletal muscle
- Loss of FIH/VHL: sharp fall-off in VO_2 at higher running speeds, fiber type switch away from fast twitch towards slow twitch, which is a more oxidative fiber type

In sum, lack of FIH increases oxygen uptake, while lack of VHL suppresses it. Lack of FIH/VHL causes an accelerated response that drastically lowers oxidative metabolism.

The maximal aerobic capacity (or maximal oxygen consumption; VO_2 max) serves as key measure of an individual's endurance capacity during sustained, prolonged exercise. Skeletal muscle-specific FIH knock-out cells show an increased oxygen dept after exercise, supporting the finding that FIH null cells have an elevated oxidative metabolism. We further showed that FIH null mutant mice have an increased maximum rate of oxygen consumption during incremental exercise compared to baseline VO_2, and reach their VO_2 max at a lower speed, indicating FIH null mutants maintain glycogen reserves to utilize at higher intensities. The diminished oxygen efficiency at low intensities seems to not have an effect on the overall long-distant running performance, as we observed no reduced endurance compared to the control mice (in contrast to VHL and FIH/VHL mutants). We further observed:

- Increased relative COX activity,
- Elevated overall perfusion of the FIH mutant muscle,
- Significantly increased serum lactate levels,
- Suppressed VO_2 with increased exercise intensity,
- Decreased VO_2 under lower oxygen levels (12%; inhalation),

indicating an accelerated metabolic response that is characterized by elevated VO_2 during early exercise and subsequent suppression of oxygen uptake shifting the aerobic into an anaerobic metabolism at higher exercise intensities. These hypoxic adaptations might explain the examined decrease in oxygen efficiency despite continued exercise performance.

Conclusions drawn from our findings can picture the following model: lack of FIH accelerates oxidative processes > decreases intracellular oxygen levels > inhibits PHDs > accumulates HIF > suppresses oxidative metabolism via HIF. This model might be especially relevant for tissues that are subject to rapid changes in oxygen levels to keep the cells in an oxidative state for as long as possible and subsequently to quickly induce the shift to anaerobic metabolism through the accumulation of HIF. As shown in our study, skeletal muscle shows highest FIH protein levels. This is supported by previous findings that demonstrate elevated FIH expression levels in elite endurance athletes (Lindholm ME, 2014). To maintain an oxidative state, FIH might alternatively use other oxygen storages, for example, oxygen bound to myoglobin in the muscle cell surface (Takahashi E and Asano K, 2002).

This study sheds further light on FIH action, even though the mechanisms of action stay elusive. For example, we have not addressed the hydroxylase function of FIH, potential interactions via its JmjC domain, to which extent FIH function is dependent on oxygen and HIF, the spatial localization of FIH, or hypothetical non-HIF targets of FIH. Further studies will be needed to understand FIH action in more detail to unveil its relevance in physiology and disease.

3.3 The asparagine hydroxylase factor inhibiting HIF correlates with epigenetic changes and targets the histone demethylase Jmjd1a (Manuscript)

FIH is a protein involved in the regulation of HIF-α (Mahon PC, 2001). The by far highest expression of FIH protein is found in skeletal muscle, indicating key regulatory roles the asparagine hydroxylase holds in this tissue (Zhang N, 2010). In the current study, we drew attention to a potential role FIH might play in epigenetic regulatory processes in response to low oxygen levels in FIH null muscle cells.

Role of FIH as histone modifying enzyme?

The canonical role of FIH as an asparagine hydroxylase of the HIF CTAD does not imply an obvious role for FIH in the nucleus. However, due to its small molecular weight its main form of transport is passive diffusion, and it is known that FIH can be actively transported to the nucleus when nuclear ankyrin proteins, such as Notch, are overexpressed (Zheng, 2008). This indicates that FIH hypothetically can access chromatin, and play a role in altering gene expression.

FIH possesses a JmjC domain that is identical to domains histone demethylases own, supporting the hypothesis that FIH might function as a histone demethylase and by that mechanism regulate HIF target genes in a promoter-specific manner (Mahon PC, 2001; Trewick SC, 2005). The analysis included histone methylation marks known to be transcriptional repressors such as H3K9me2/me3, and transcriptional activators such as H3K4me2/me3, and we also monitored the H3K9ac epigenetic activation mark. And indeed, we identified significantly elevated methylation levels at the repression mark histone 3 lysine 9 dimethyl at promoter regions of various metabolic HIF target genes (PHD3, PGK1, Aldolase A, Enolase 1, PFKFB3, Dec1) in FIH null mouse cells at normoxia and acute hypoxia, indicating FIH might specifically target H3K9me2 that is associated with transcriptional repression of potentially active genes. Note, muscle cells show the highest FIH expression levels in tissues (Zhang N, 2010).

In line with these findings, we observed elevated global H3K9me2 levels in quadriceps muscles of female FIH null mice. Subsequent analysis led us to analyze FIH's potential to act as a histone demethylase. With the help of an *in vitro* carbon dioxide capture assay, we analyzed the catalytic activity of FIH towards histone 3 and histone 3 lysine 9 dimethyl peptides. FIH cannot function as histone demethylase on its own. The not observed capacity of FIH to demethylate histones might be explained by incorrectly folded histones, deprivation of iron when mixed with histones, lack of an essential cofactor, or the fact that FIH plainly might not directly demethylate histones.

Role of FIH as modifier of histone modifying enzymes?

To further investigate the role FIH might play in epigenetic regulation, we examined whether histone demethylases or methyl transferases might be targets of FIH. Recent work has demonstrated that other 2OG oxygenases catalyze histone demethylation reactions (Loenarz C and Schofield CJ, 2011). Another JmjC domain-containing enzyme, Jmjd6, has been shown to act as a histone arginine demethylase as well as a lysine hydroxylase (Chang B, 2007; Webby CJ, 2009). Therefore, it appeared plausible that, in addition to the established oxygen-dependent regulation of HIF function via HIF hydroxylases, there are other targets and steps in the hypoxia signaling pathways that are controlled by FIH. Another report identified histone 3 lysine 9-specific demethylase Jmjd1a and methyl transferase G9a as HIF target genes (Beyer S, 2008).

Given the histone methylation changes we observed at promoter regions of numerous genes, we hypothesized that FIH might regulate epigenetic changes by recruiting other proteins, such as the demethylase Jmjd1a, to HIF target gene promoters. Our findings demonstrate a correlation of loss of FIH and changes in Jmjd1a expression, indicating the protein hydroxylase and the histone demethylase might interact to regulate the epigenetic response observed at the histone 3 lysine 9 repression mark. Whether or not these changes result in direct FIH-Jmjd1a protein-protein interaction, has been subject to subsequent work. Preliminary studies showed no interaction of endogenous FIH and Jmjd1a. Further investigation is required to better understand the mechanisms that sequester FIH and restrict its cellular hydroxylase activity.

Conclusion

The work presented here shows fundamental restrictions. In this study, we have neither considered a wide range of other histone modifying enzymes nor a crosstalk that involves multiple histone-modifying complexes nor have we tried to identify FIH as part of such a complex. A key concern is FIH action has not been addressed as a question of context and time, hence, at the present, it seems premature to draw any conclusions about FIH's involvement in the epigenetic response to changing oxygen levels. Lee and colleagues drew the conclusion that it is not sufficient to just look at a pattern of chromatin modifications at a locus to determine its gene expression status, indicating a crosstalk of modifications depend on each other. For example, it has been proposed that chromatin modifications establish a complex code recognized by transcription factors to determine the transcriptional state of a gene (Lee JS, 2010). The complexity seems overwhelming, as Zippo and colleagues report that in addition to histone modification crosstalk, the histone tails H3 and H4 seem to also interact. Furthermore, histone modifications appear to recruit opposing enzymes, for example, H3K4 and H3K36 methylation recruit both deacetylases and acetyl transferases (Zippo A, 2009). Through the present study, we have gained a new perspective of FIH metabolism. Unfortunately, more questions remain unanswered than new insights have been provided. Further work is essential to deepen our understanding of FIH as epigenetic modulator, involving possibly wider consequences as, for example, PHD3 has been reported to be specifically methylated in breast cancer cell lines. As abnormal profiles of histone

modifications result in a variety of pathological disease, future work might have implications beyond our understanding of FIH's molecular action.

4 SUMMARY AND FUTURE PERSPECTIVES

The present study was carried out in order to shed light on mechanisms of bHLH-Pas transcription factor regulation, specifically the AhR and the HIF signaling pathways. The main findings can be summarized as follows:

(I) To date, the AhR receptor is the only transcription factor in the bHLH family that possesses a second function. The receptor functions as a ligand-activated transcription factor or E3 ubiquitin ligase. The current study identified ARNT as key player in the modulation of the switch. Previous work by Ohtake and colleagues identified AhR and ARNT as part of the ubiquitin ligase complex. Our findings are contrary, as we fail to identify ARNT as part of the complex. AhRR plays another crucial role as it competitively dimerizes with ARNT and changes its availability, and thereby disrupts the transcriptional function of AhR. AhRR further reduces ERα protein levels and diminishes estrogen-dependent xenograft tumor growth.

(II) 50-fold higher FIH levels in skeletal muscle compared to other tissues make the asparagine hydroxylase a perfect target for studying hypoxic effects on the cell's changing energy demands. Interestingly, we found that FIH can even function as a regulating factor under hypoxia. This is mainly due to its high affinity to oxygen. Furthermore, FIH seems to "prepare" cells for a state of increased glycolytic energy generation caused by hypoxia. And indeed, FIH null cells show elevated glycolytic stores at normoxia and drive cells into a glycolytic metabolism in hypoxia. FIH has further effects during prolonged exercise. In normoxia, FIH shows an elevated oxidative metabolism, while at low oxygen levels we observe a shift from aerobic to an anaerobic metabolic state at increased exercise intensities.

(III) FIH serves as key regulator of the hypoxic response to low oxygen environments. However, FIH has been shown to possess HIF-independent functions, for example, FIH hydroxylates ARD-containing proteins. Furthermore, FIH protein contains a JmjC domain homologous to domains possessed by histone demethylases. FIH indeed seems to have a mediating function as we observed significantly increased methylation status at the transcriptional repression mark histone 3 lysine 9 dimethyl in promoter regions of metabolic HIF target genes in loss of FIH cells at normoxic and acute hypoxic oxygen levels. Finally, we observed correlations of loss of FIH and Jmjd1a expression.

Further investigation will be key to deepen our understanding in the underlying mechanisms, including identification of specific HIF1α and HIF2α regulating pharmaceuticals, further characterization of co-activators, co-repressors, or collaborating transcription factors, and the role of ARNT. More work will be critical to elucidate the mechanism (a) how AhR's ubiquitin ligase works, (b) how FIH can act as metabolic mediator, and (c) whether or not FIH plays a pivotal role in the epigenetic response to environmental changes. Further studies will reveal whether AhR or FIH can serve as therapeutic targets and whether we can develop therapeutic treatment regimen for various diseases, among others estrogen-dependent tumors (in case of AhR).

5 MY ACADEMIC ORGANIZATION

The following is my very own point of view based on the experiences I gained during the last 15 years at three different universities in three different countries – Sweden, Germany, and the US. My thoughts are not a reflection of the work at my previous labs, but rather general observations I made within the mentioned period of time. In all fairness, I have had insights into aspects of leadership training organized by the universities, but not in completion.

If we ignore for a minute the special role a public university has (financed by tax payer's money, creation of knowledge as central goal, aiming for scientific advancement, work for the good of mankind instead of financial interests), a university is nothing else than any other organization, in which a management team develops a strategy and establishes a certain working culture.

As supporter of people first, my university as organization should have four major functions:

(1) Establish a transparent hiring process,

(2) Develop a well-functioning onboarding program,

(3) Support its team leaders, and

(4) Create an organization culture to thrive.

1) Establish a transparent hiring process

To date, I have not experienced any sort of organizational recruitment routine. I envision a university, in which employees are recruited not only dependent on their past merits, but also based on certain personality traits such as kindness, curiosity, innovative mindset and a high level of integrity.

2) Develop a well-functioning onboarding program

It must not only be in the university's interest to recruit the right people, but upon acceptance, to help them become innovative leaders of their projects. I can only see this becoming a practical approach, if the university offers a program on innovation, entrepreneurship and design thinking before the start of the doctoral studies. To give the students the opportunity to master the literature of the given topic, this onboarding time may also include a thorough lecture-series on scientific principles, methods and topic-related studies.

3) Support their team leaders

A scientific career is special and the path unique. Scientists become group leaders based on their individual achievements. Before long researchers run a team of 5 to 10+ people. Team leaders face so many challenges, hence, building a well-functioning team might at times get lower attention. Here I wished the university would support team leaders and help focus on developing their teams. Such support may include (but it is not restricted to) raising awareness about communicating a vision, establishing short-term but also long-term goals, performance appraisals, and team roles. Actual project management tools might be a great help to structure the student's goals and help set key performance indicators (KPIs). The combination of a bottom-up and top-down approach might contribute to a higher level of responsibility, as students design projects and set goals by themselves. One last note on team development, scientific teams are built under the cover of "independent working", which means nothing else than it is the sole responsibility of the student/scientist to address a scientific question in practice. I find it hard to understand why teams do not approach a certain question collaboratively. The base might be set by an individual before the whole team's expertise combines in early stages (not only during a revision process) to then finish the project. In addition, a team approach guarantees a shared knowledge base, supports employee bonding, creates a shared standardized technological infrastructure, and establishes a common purpose. All this results in the formation of an empowered and self-managing team.

4) Create an organization culture to thrive

Culture is the single most important factor to run an organization successfully. Survival of the fittest is one way to run a scientific organization, another way is to put people first, communicate and honor scientific breakthrough, support young group leaders and group leaders in general, be fair, and create an environment of innovation and entrepreneurship. To all academic leaders out there, be passionate, innovative, social, creative, entrepreneurial, inspirational, and visionary! Your employees will pay back with their blood, sweat and tears.

Finally, a complete change of the established academic system is such a radical idea that it won't happen. However, it is fun to speculate how another approach might look like. The next level university might ensure full control over processes and roles by the university itself. The idea is based on the fact that the various roles in the lab could not be more different. Just imagine Master programs would address this and educate for purely lab-related roles (methods, literature, presentation skills), writing roles (grant application, manuscript writing) and scientific team management roles (innovation, design thinking, speaking skills, people management, scientific literature).

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