Bidirectional crosstalk between Hypoxia-Inducible Factor and glucocorticoid signalling in zebrafish larvae

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ABSTRACT

Hypoxia inducible factor (HIF) and Glucocorticoids (GCs) transcriptional responses play a pivotal role in tissue homeostasis, glucose metabolism and in the regulation of cellular responses to various forms of stress and inflammation. In the last decades few in vitro studies highlighted the potential for crosstalk between these two major signalling pathways. However, how this interplay precisely takes place in vivo is still unclear.

To this end, the aim of this project is to elucidate how and to what degree hypoxic signalling affects the endogenous glucocorticoid pathway and vice versa, using zebrafish larvae (Danio rerio) as an in vivo model organism. Indeed, the use of whole animals allows us to show how these pathways interact at a more systemic and complex level than in cell culture, where interactions between different tissues and cell types cannot be easily modelled.

Previous work from my lab identified glucocorticoids as activators of the HIF pathway (Vettori et al., 2017). Counterintuitively, despite their anti-inflammatory action, it was shown that GR loss of function prevents the transcriptional activity linked to immune response (Facchinello et al., 2017). Finally, the glucocorticoid receptor has been also observed to synergistically activate proinflammatory genes by interacting with other signalling pathways (Langlais et al., 2008, 2012; Dittrich et al., 2012; Xie et al., 2019). Nevertheless, how the crosstalk between hypoxic and glucocorticoid signalling occurs in vivo is still debated.

By taking advantage of both a genetic and a pharmacological approach I altered these two pathways during the first 120 hours post fertilisation of zebrafish embryos. In particular, I used two different mutant lines I have generated (hif1βh544 (arnt1) and
grsh543 (nr3c1), coupled to an already existing vhlhu217/+;phd3:eGFP144/144 hypoxia reporter line (Santhakumar et al., 2012), to study the effect of HIF signalling on glucocorticoid response and vice-versa, through a “gain-of-function/loss-of-function” approach. Phenotypic and molecular analyses of these mutant lines were accompanied by optical and fluorescence microscope imaging.

Firstly, I have demonstrated that in the presence of upregulated HIF signalling, both glucocorticoid response and endogenous cortisol levels are repressed in 5 days post fertilisation larvae. In addition, despite HIF activity being low at normoxia, my data show that it already impedes glucocorticoid activity and levels.

Secondly, I further analysed the in vivo contribution of glucocorticoids to HIF signalling. Interestingly, my results show that both glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) play a key role in enhancing the HIF response.

Finally, I found indications that glucocorticoids promote HIF signalling via multiple routes. Cumulatively, this study suggests a model for how this crosstalk occurs in vivo and, more broadly, paves the way for further in vivo analysis on the extensive interaction behind these two major signalling pathways.
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LIST OF ABBREVIATIONS

3β-HSD – 3β-Hydroxysteroid dehydrogenase/Δ5-4 isomerase
ACTH – adrenocorticotropic hormone
ANOVA – analysis of variance
AP – Alkaline Phosphatase
Asn – Asparagine
arnt – Aryl Hydrocarbon Receptor Nuclear Translocator 1
AVP = Arginine Vasopressin
BAC – Bacterial Artificial Chromosome
BCIP – 5-bromo-4-chloro-3-indolyl-phosphate
bHLH – basis helix-loop-helix
BME – Betamethasone 17,21-dipropionate
BTM – basal transcription machinery
cAMP – cyclic adenosine monophosphate
CBG – corticosteroid-binding globulins
ccRCC – clear cell renal cell carcinoma
CNS – central nervous system
CRF – corticotropin-releasing factor
CRH – corticotropin-releasing hormone
CRISPR – clustered regularly interspaced short palindromic repeats
CRISPant – mutants derived from CRISPR/Cas9-based mutagenesis method
Ct – cycle threshold
cyp11a1 – cytochrome P450 Family 11 Subfamily A Member 1
cyp17a1/2 – cytochrome P450 Family 17 Subfamily A Member 1/2
DBD – DNA binding domain

dCAPS – derived Cleaved Amplified Polymorphic Sequence

DEX – Dexamethasone

DMOG – dimethyloxalylglycine

DMSO – dimethyl sulfoxide

dpf – days post-fertilisation

EDTA – ethylenediaminetetraacetic Acid

*eef1a1 – Eukaryotic Translation Elongation Factor 1 Alpha 1*

eGFP – enhanced Green Fluorescent Protein

*egln3 – Egl-9 Family Hypoxia Inducible Factor 3*

epo – erythropoietin

EtOH – ethanol

*fbp1 – fructose-1,6-bisphosphatase 1*

FC – fold change

*FIH – asparagine hydroxylase factor inhibiting HIF*

*fkbp5 – FK506-binding protein 5*

FRET – Fluorescent Resonance Energy Transfer

*g6pc – Glucose-6-Phosphatase Catalytic Subunit*

GC – glucocorticoid

*glut1 – glucose transporter 1*

GR – glucocorticoid receptor

GRE – glucocorticoid-response elements

H – hinge region

HAF – Hypoxia-associated factor

HIF – Hypoxia-inducible factor
hmgcr – 3-Hydroxy-3-Methylglutaryl-CoA Reductase

HPA – hypothalamic-pituitary-adrenal axis

hpf – hours post-fertilisation

HPI – hypothalamic-pituitary-interrenal axis

HR – homologous recombination

HRE – hypoxia-response elements

HSD17B2 – Hydroxysteroid 17-Beta Dehydrogenase 2

HSP90 – Heat Shock Protein-90 kD

IKKβ – IκB kinase beta

IL – interleukin

IL-1RA – interleukin-1 receptor antagonist

il6st – interleukin 6 Signal Transducer

lamb1b – laminin subunit beta-1

LBD – Ligand binding domain

lipca - lipase, hepatic a

MC2R – melanocortin 2 receptor

MeOH – methanol

MGB - minor groove binder

MR – mineralocorticoid receptor

NBT – nitro blue tetrazolium

NF-κβ – nuclear factor-κB

NFQ – nonfluorescent quencher

NHEJ – non-homologous end joining

NH4Ac – ammonium acetate

NLS – N-terminal nuclear localization signal
nr3c1 – nuclear Receptor Subfamily 3 Group C Member 1

NTD – N-terminal domain

ODDD – oxygen-dependent degradation domain

p300/CBP – p300/CREB-binding protein

P450scc – cytochrome P450 cholesterol side-chain cleavage

PAM – protospacer adjacent motif

PAS – Per-Arnt-Sim

PBS – Phosphate Buffer Saline

PBST – Phosphate Buffer Saline + Tween20

pc – pyruvate carboxylase

pck1 – phosphoenolpyruvate carboxykinase 1

PFA – paraformaldehyde

pfkb1 – phosphofructokinase B-type carbohydrate kinase family protein

pfkfb3 – 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3

PHD – prolyl hydroxylases

pi3k – phosphoinositide 3-kinase

PKA – protein kinase A

pomca – proopiomelanocortin-a

Pro – proline

ProtK – proteinase K

PTU – 1-Phenyl-2-thiourea

pVHL – Von Hippel Lindau protein

PVN – paraventricular nucleus

rbx1 – RING box protein-1

ROS – Reactive Oxygen Species
rpm – revolutions per minute

\textit{rps29} – Ribosomal Protein S29

RT – Room Temperature

\textit{RTqPCR} – Quantitative Reverse Transcription Polymerase Chain Reaction

\textit{s.e.m} – standard error of the mean

\textit{siah1a/2} – siah E3 ubiquitin protein ligase 1A

SL – standard length

\textit{slc37a4} – Solute Carrier Family 37 Member 4

\textit{slc2a1a} – solute carrier family 2-member 1a

SSC – Saline-Sodium Citrate

\textit{StAR} – steroidogenic acute regulatory protein

\textit{stat} – signal transducer and activator of transcription

TAD-N/C – N- and C-terminal transactivation domains

TAE – Tris-Acetate-EDTA

TBE – Tris-Boric Acid-EDTA

\textit{Tg} – transgene

\textit{tracrRNA} – trans-activating CRISPR RNA

UPLC – Ultra-Performance Liquid Chromatography

VBA – Visual Background Adaptation

\textit{vegf} – vascular endothelial growth factor

\textit{vhl} – von Hippel Lindau

\textit{vll} – von Hippel Lindau-like

WISH – whole mount in situ hybridisation

\textit{wt} – wild type

\textit{xg} – times gravity
1. INTRODUCTION

1.1. Oxygen homeostasis and hypoxia

Cellular oxygen (O$_2$) is an essential nutrient that is required to maintain metabolism, bioenergetics and to assure the correct functioning and survival of aerobic animals. It is important to note that while ambient air is 21% O$_2$ (150 mm Hg), most mammalian tissues are at 2% - 9% O$_2$ (~40 mm Hg). In vertebrates, oxygen concentration gradients act as signals that guide towards proper development. Indeed, the balance between cell growth and vascularization is able to elicit differential gene expression acting as driving force that directs cell behavior. Moreover, in tissue morphogenesis, the timing of hypoxic exposure can modulate both development and differentiation (Simon and Keith, 2008)

Hypoxia, or decreased oxygen levels, is also a common pathophysiological condition which has profound effects on the cellular transcriptome. Generally, it is characterized by a reduced oxygen availability (below 2%), whereas severe hypoxia or “anoxia” by O$_2$ levels below 0.02%, to either cells, tissues or organs in the body (Cummins and Taylor, 2005; Bertout, Patel and Simon, 2008; Semenza, 2013). In particular, hypoxia may occur either as a consequence of improper blood supply (i.e. during embryonic development, or in solid tumors) or as a result of the environment (i.e. poorly oxygenated waters or at high altitude) (Webb, Coleman and Pugh, 2009). Cumulatively, hypoxia acts both as a crucial developmental stimulus and physiological stressor to which cells must rapidly respond in order to assure homeostasis.
In all organisms, including vertebrates, homeostasis is the state of optimal internal, physical and chemical conditions, which is maintained by several regulatory apparatus aimed to assure both survival and correct functioning of the body (Biddlestone, Bandarra and Rocha, 2015). One of the main cellular mechanisms that aerobic organisms developed during the course of evolution to cope with decreased oxygen availability is controlled by the hypoxia-inducible factor transcription factors (HIF) family. Being a widely action family of transcription factors, it is made up of primary regulators of the cellular response to low oxygen levels, which mediate several biological functions (i.e. haematopoiesis, angiogenesis, maintenance of vascular tone, etc.) in order to provide tissues with blood and oxygen. In addition to that, HIF can also rapidly coordinate a series of metabolic changes (i.e. from aerobic to anaerobic metabolism) that enable the body to prevent both metabolic shutdown and death (Bertout, Patel and Simon, 2008; Semenza, 2013; Ebersole et al., 2018). If these mechanisms fail or are inadequate, they can lead to the development of a wide range of pathological conditions which includes tissue ischemia, inflammation, stroke and the growth of solid tumors (Elks et al., 2015).

As previously mentioned, since cells require oxygen to survive, hypoxia acts as a potent cellular stressor. Indeed, oxygen deprivation leads to reduced mitochondrial oxidative phosphorylation, augmented lactate production and elicits an excessive accumulation of free radicals which further fuel the hypoxic stress (Chandel et al., 2000; Klimova and Chandel, 2008; Solaini et al., 2010). To avoid that, cells must rapidly adapt to match metabolic, redox and bioenergetic requirements to a reduced O₂ supply. In particular, at a protein level these hypoxic responses can be sorted into two different groups: acute and chronic phase. Acute phase responses are prompt and transient and
are mainly based on post-translationally modifications of existing proteins, whereas chronic phase reactions are more persistent and intense.

These changes consist of altering gene transcription and protein biosynthesis to enable cells to temporarily block the cell cycle at G1/S of the interphase. This is usually accompanied by the secretion of survival and proangiogenic factors (mainly in cancer) and by the reduction of energy consumption (Majmundar, Wong and Simon, 2010). Consequently, understanding the molecular mechanisms by which cells sense and respond to hypoxia may provide the basis for novel therapeutic approaches to many disorders.
1.2. The Hypoxia-Inducible Factor pathway

The progression of the cell cycle is an energy requiring process that demands a refined metabolic regulation to occur. Indeed, it is well known that cells must overtake an energy restriction checkpoint during G1 phase, in order to progress through the cell cycle. In this regard, as aerobic organisms require oxygen for metabolically converting nutrients into energy, \( O_2 \) represents a vital signalling molecule directing the cell fate. Consequently, assuring oxygen homeostasis is a critical task that must be precisely managed by cells in order to perform correctly and survive in a hostile environment (Semenza, 2011b).

In mammalian cells, this is primarily carried out by the Hypoxia-Inducible Factors (HIFs) which are a family of transcription factors that react both to environmental oxygen and cellular energy alterations (i.e. hypoxia) (Semenza, 2001). HIFs are obligate heterodimers consisting of an \( O_2 \)-labile \( \alpha \)-subunit and a stable \( \beta \)-subunit (also known as aryl hydrocarbon receptor nuclear translocator or ARNT). Both HIF alpha and beta subunits are expressed in the cytoplasm. However, whilst HIF-\( \beta \)/ARNT isoforms are constitutively expressed, HIF-\( \alpha \) subunits (HIF1\( \alpha \), HIF2\( \alpha \) and HIF3\( \alpha \)) are characterized by a very fast turnover that is post-translationally regulated by the PHD3-VHL-E3-ubiquitin ligase protein degradation complex. This mechanism is believed to maintain low basal HIF levels that can rapidly increase in order to promptly respond to decreased oxygen concentrations (Berra et al., 2001; Moroz et al., 2009; Elks et al., 2015; Köblitz et al., 2015).

Under normoxic conditions (normal oxygen levels), a set of prolyl hydroxylases (PHD1, 2 and 3) directly exploit the available molecular oxygen to hydroxylate two prolyl residues (Pro402, Pro564) within the oxygen-dependent degradation domain
(ODDD) of the HIF-α subunits. Then, the hydroxylated HIF-α isoforms are recognized and targeted by the Von Hippel Lindau protein (pVHL), which acts as the substrate recognition component of the E3-ubiquitin ligase complex. Of note, the latter is characterized by a multiprotein complex which consists of Elongin B, Elongin C, Ring-box 1 and Cullin 2 (Iwai et al., 1999). Once this complex ubiquitinates the HIF-α subunits, it directs them towards proteasomal degradation in order to avoid the aberrant stabilization and activation of the HIF pathway under normoxic conditions. In addition, HIF-α may be hydroxylated by factor-inhibiting HIF (FIH) on asparagine 803, which prevents the recruitment of the transcriptional coactivator p300/CREB-binding protein (p300/CBP) and reduces the effectiveness of HIF transcriptional activation (Shay and Simon, 2012). By contrast, the presence of reduced O₂ levels impairs both PHD and FIH enzymatic activity and leads to HIF-α stabilization. As a consequence, pVHL is no more able to recognize and target HIF-α to proteasomal degradation, when the latter is not hydroxylated. This allows the HIF-α and β subunit heterodimer formation, followed by the ARNT-mediated translocation in the nucleus.

Here, p300/CBP may interact with the HIF-αβ transcription complex to further activate the hypoxic response. This implies the upregulation of target genes which are involved in decreasing oxygen consumption and increasing oxygen and nutrient delivery (Nikolaus, Fölscn and Schreiber, 2000). In particular, this occur via HIF-αβ direct recognition and binding to hypoxia-response elements (HREs). The latter are characterized by the presence of a consensus sequence G/ACGTG located within the promoter regions of target genes such as phosphofructokinase, adrenomedullin, erythropoietin and vascular endothelial growth factor (Lando et al., 2002; Leonard et al., 2005; van Rooijen et al., 2009) (figure 1.1).
Importantly, the zebrafish (*Danio rerio*) has proved to be a very informative and genetically tractable organism for studying hypoxia and HIF pathway both in physiological and pathophysiological conditions. (C. E. Robertson *et al.*, 2014; Vettori *et al.*, 2017) In the next subchapter, I will describe the main oxygen sensing, signal transduction and transcriptional activation components that characterize the HIF signalling pathway.
Figure 1.1 Oxygen-dependent post-translational regulation of HIF-α subunit. Under normoxic conditions, the prolyl hydroxylase 3 (PHD3) hydroxylates HIF-α subunit on two specific prolyl residues within the oxygen dependent degradation domain (ODD). In turn, VHL recognizes and binds to hydroxylated HIF-α and then recruits the other components of the E3-ubiquitin ligase complex. The latter promotes the ubiquitin-mediated proteasomal degradation of HIF-α subunit. Conversely, hypoxic conditions inhibit PHDs activity and the subsequent degradation of HIF-α, which can in turn be stabilized and migrate into the nucleus after dimerization with HIF-1β subunit. Here, the HIF-αβ active complex elicits the expression of genes such as VEGF, GLUT1 and EPO involved in restoring oxygen homeostasis. (modified from Stroka and Candinas, 2010).
1.2.1. Oxygen-dependent regulation of HIF-α

The preservation of oxygen homeostasis is essential to assure the correct functioning of the cell cycle. In this regard, the ability to perceive and quickly respond to changes related to the environmental oxygen availability is initially carried out by a plethora of oxygen sensor proteins. Among them, prolyl hydroxylases (PHD) represents the first line of defence against decrease oxygen levels, by hydroxylating crucial residues in HIF-α subunits.

Within the HIF pathway, the prolyl-hydroxylase domain containing enzymes (PHDs) together with the asparagine hydroxylase factor inhibiting HIF (FIH), are the main molecular oxygen sensors (Schofield and Ratcliffe, 2004; Walmsley et al., 2011; Place et al., 2013; Ivan and Kaelin Jr, 2017). There are three different prolyl-hydroxylases (PHD1-3) which hydroxylate HIF-α subunits on two specific proline residues (Epstein et al., 2001; Kaelin and Ratcliffe, 2008). Among them, PHD2 has proven to be the main mediator of HIF-1α hydroxylation, whereas both PHD’s 1 and 3 was shown to participate more significantly to HIF-2α regulation (see also chapter 1.2.2) (Appelhoff et al., 2004). However, PHD3 proved to be more potently upregulated than PHD2 under hypoxic conditions, whilst the expression of PHD1 is insensitive to hypoxia. In addition, PHD3 itself is one the main HIF target genes, as it is required to hydroxylate and degrade the HIF-α subunit. This is because, to control its own levels and to avoid an excessive and prolonged hypoxic response, the HIF pathway is able to trigger an intracellular super short feedback loop. This is based on the upregulation of its regulators aimed to tune HIF activity itself (Aprilikova et al., 2004; Pescador et al., 2005; Henze and Acker, 2010; van Rooijen et al., 2011; Santhakumar et al., 2012).

As a consequence of hypoxia, besides the direct transcriptional regulation of the PHDs, both PHD1 and PHD3 stabilisation are tuned by the Siah1a/2 RING-finger ubiquitin
ligases, which target prolyl hydroxylases to proteasomal degradation. (Nakayama et al., 2004; Khurana et al., 2006).

As previously mentioned, an additional controller of the HIF activation is represented by FIH, which is a Fe²⁺ and oxygen dependent asparagyl hydroxylase with the ability of hydroxylating HIF1-α subunit on a specific asparagine residue (Asn803). Of note, even though this hydroxylation is essentially an oxygen dependent event, due to its high affinity with molecular oxygen, the hydroxylation performed by FIH tends to be more inhibited under severe hypoxic conditions than the PHDs’ one (Lisy and Peet, 2008). By doing so, FIH activity avoids HIF to interact with its transcriptional co-activator p300/CBP (Lando et al., 2002). At the same time, it creates a gradual hypoxic response by permitting the HIF-p300/CBP co-activation to occur only under severe hypoxic conditions. This allows the cell to further activate the HIF transcriptional response only when needed.

Another level of complexity in the regulation of the hypoxic response is represented by the fact that both FIH and PHDs are dioxygenases requiring O₂, ferrous iron (Fe²⁺), 2-oxoglutarate and ascorbate, as co-substrates, in order to hydroxylate HIF-α (Siddiq, Aminova and Ratan, 2007). In this regard, ferrous iron is essential for the enzyme to be assembled into its active conformation, since prolyl hydroxylases contain Fe²⁺ in their hydrophobic active centre. In addition, O₂ binding event requires the ascorbate-dependent maintenance of this iron molecule in its ferrous state. Moreover, HIF-α hydroxylation occurs by transferring one oxygen either to Pro402 or Pro564, while a second oxygen atom reacts with 2-oxoglutarate. This reaction yields both succinate and carbon dioxide as reaction products, making this catalytic process irreversible. Finally, during a complete reaction, Fe²⁺ is transiently oxidized to Fe⁴⁺ and reduced to the Fe²⁺ state. Interestingly, when α-ketoglutarate is converted into
succinate without hydroxylation of a peptide substrate, this Fe\(^{2+}\) is oxidized to Fe\(^{3+}\). In this process, ascorbate is also required to reduce Fe\(^{3+}\) back to Fe\(^{2+}\) in order to allow the enzyme to be recycled (Pan et al., 2007). So, HIF may also be a response to iron depletion and may explain the HIF-activation properties of iron chelators in cell culture (Bianchi, Tacchini and Cairo, 1999; Buss et al., 2004; Hatcher et al., 2009).

Cumulatively, the vertebrate O\(_2\)-sensing pathway primarily involves both prolyl and asparagyl hydroxylation of the HIF-\(\alpha\) subunits, which in normoxic conditions contribute to HIF inactivation. By contrast, in hypoxia the lack of molecular O\(_2\) avoids these oxygen sensors to act on HIF-\(\alpha\), allowing the cytoplasmic stabilization of the latter. Finally, not only O\(_2\) concentration, but also multiple mitochondrial products can coordinate PHD activity, HIF stabilization and hence the cellular responses to O\(_2\) depletion (Solaini et al., 2010).

### 1.2.2. The Hypoxia-Inducible Factor isoforms

As described in the previous subchapter, the adaptive cellular response to an adverse environment consists in the orchestration a transcriptional program based on the upregulation of HIF-dependent target genes, which code for proteins that are involved in restoring and promoting energy homeostasis (i.e. VEGF, GLUT1, EPO, PHD3 etc.) (Schofield and Ratcliffe, 2004; Semenza, 2011a; Moniz, Biddlestone and Rocha, 2014).

In this regard, HIFs are evolutionarily conserved heterodimeric proteins, related to the basic-helix loop-helix–Per-Arnt-Sim homology (class II bHLH–PAS) transcriptional regulators family, which promote metabolic changes that drive cellular adaptation to low oxygen availability (Majmundar, Wong and Simon, 2010; Nath and Szabo, 2012). In mammals, HIF proteins are obligate heterodimers composed by an
oxygen-sensitive α-subunit (HIF-1α, HIF-2α, and HIF-3α) and a constitutively stable aryl hydrocarbon receptor nuclear translocator or β-subunit (ARNT1 and ARNT2).

As also illustrated in figure 1.2, both subunits are bHLH/PAS transcription factors containing transactivation domains (TADs) (Webb, Coleman and Pugh, 2009). In particular, the initial bHLH domain is followed by a Per-Arnt-Sim (PAS) domain which acts as a molecular sensor, an oxygen-dependent degradation domain (ODD), and by an N- and C-terminal transactivation domains (TAD-N and TAD-C) (figure 1.2).

Of note, the latter is present in all the HIF-α subunits with the exception of HIF-3α, which only has the TAD-N activation domain, along with the inhibitory PAS domain protein (IPAS) (Li et al., 2006; Makino et al., 2007). On the other hand, HIF-β/ARNT being not targeted and degraded by the pVHL-E3 ligase complex lacks both the ODD and the TAD-N domains, and has a constitutively active N-terminal nuclear localization signal (NLS) (Pollenz, Sattler and Poland, 1994; Eguchi et al., 1997).

Another important difference among HIF isoforms regards their expression pattern. Indeed, if HIF-1α subunit is ubiquitously expressed, HIF-2α is selectively expressed in a tissue-specific manner and it has been observed to be present at high levels only in vascular endothelial cells, lung type-2 pneumocytes and kidney epithelial cells (Bertout, Patel and Simon, 2008). Conversely, Hif-3α highest levels in vertebrates have been observed in heart, placenta, lung, and skeletal muscle, whilst were barely detectable in the liver, brain, and kidney (Zhang et al., 2014; Duan, 2016). In addition, although HIF-1α and HIF-2α, share multiple structural similarities, their roles differ under a variety of conditions. In this respect, HIF-1α is broadly considered the major regulator of the hypoxic response in hypoxia, whereas HIF-2α is usually more associated with tumorigenesis (Loboda, Jozkowicz and Dulak, 2010).
Furthermore, *in vitro* studies highlighted that under hypoxic conditions HIF-1α tends to occupy more binding sites than HIF-2α, which instead engages a smaller subset of them (Schödel *et al.*, 2011). By contrast, HIF-3α was shown to have multiple variants in human, which can either inhibit or activate the transcription of target genes, according to variant length, the gene targeted and the abundance of HIF-β. These effects can occur via competitive binding to HIF-1α/2α at cytoplasmic level to HIF-β and also to HREs (Heikkilä *et al.*, 2011).

In addition, HIF-1α and HIF-2α usually upregulate overlapping as well as unique transcriptional targets and cooperate in reprogramming metabolic pathways to generate cellular energy especially under different hypoxic conditions (Raval *et al.*, 2005; Koh and Powis, 2012; Shay and Simon, 2012; Suzuki *et al.*, 2014). Interestingly, although both HIF-1 and HIF-2 bind to the same HRE consensus sequence in the regulatory regions of target genes, the DNA binding does not necessarily correspond to the increased transcriptional activity. This suggests that post-DNA binding mechanisms might be required for transactivation (Mole *et al.*, 2009). Moreover, the differential activation of downstream target genes has also been linked to differences among the N-terminal transactivation domains (TADs) of HIF-1α compared to HIF-2α. These findings suggested that differences in HIF-α isoforms’ coding sequences might also contribute to isoform-specific function (Hu *et al.*, 2003, 2007).

It is also important to note that previous studies highlighted the presence of HIF switches, which can directly change HIF-α isoform dependency. Indeed, it has been observed that HIF-1α is activated during short periods of severe hypoxia and upregulates genes primarily involved in glycolysis and pyruvate metabolism, whereas HIF-2α is activated under prolonged periods of mild hypoxia and mainly controls genes regulating fatty acid metabolism (Gordan *et al.*, 2007; Majmundar, Wong and Simon,
The temporal regulation of HIF-1/2α is primarily mediated by the oxygen dependent hydroxylases which differently regulate HIF-α isoforms stability and activity. In this regard, PHD2 preferentially interact with HIF-1α than HIF-2α, whereas PHD3 has relatively more influence on HIF-2α than HIF-1α (Appelhoff et al., 2004). HIF-2α is also less efficiently hydroxylated than HIF-1α by both PHDs and FIH-1, resulting in the stabilization and activation of HIF-2α in the presence of higher oxygen tension (Koivunen et al., 2004). Moreover, it has been observed that in addition to post-translational mechanisms, in lung epithelial cells, HIF-1α (but not HIF-2α) mRNA is destabilized under prolonged hypoxia, due to HIF-dependent expression of HIF-1α antisense RNA (Uchida et al., 2004).

Interestingly, the zebrafish HIF signalling pathway shows a remarkable functional conservation with humans (van Rooijen et al., 2011). Moreover, both human and zebrafish liver expresses all HIF-α family members under both physiologic and pathophysiologic conditions, suggesting that HIFs are important mediators of normal liver function and disease (Koh et al., 2012). Unfortunately, due to a genome duplication event occurred in zebrafish during evolution, there are two paralogs for each of the three Hif-α isoforms (Hif-1αa, Hif-1αb, Hif-2αa, Hif-2αb, Hif-3αa and Hif-3αb) and redundancy between the isoforms may occur. However, among these, Hif-1αb has proven to be the main zebrafish homologue in the hypoxic response (Elks et al., 2011, 2015; Köblitz et al., 2015). By contrast, as concerns HIF-1β (ARNT) paralogues, the expression of two genes encoding Arnt1 and Arnt2 proteins has been outlined in zebrafish (Wang et al., 2000; Prasch et al., 2006; Hill et al., 2009; Pelster and Egg, 2018).

In addition to the above considerations, even though microarray analysis have identified over 400 genes regulated by HIF (Mole et al., 2009; Schödel et al., 2011),
subsequent CHIPseq studies have also mapped an extremely larger number of HREs (5177) in the zebrafish genome (Greenald et al., 2015; Kim et al., 2017). Thus, HIF is likely to impact on the transcription of a much larger set of genes. These data also highlighted that the regulation of hypoxia-inducible genes may occur even via interactions with other transcription factors through still unknown mechanisms. Moreover, several studies have reported a differential regulation of specific HIF target genes performed by HIF-1α and HIF-2α (Elvert et al., 2003; Covello and Simon, 2004; Warnecke et al., 2004; Raval et al., 2005; Scortegagna et al., 2005; Holmquist-Mengelbier et al., 2006). However, the mechanism by which HIF-1α and HIF-2α distinguish their respective target genes is still unclear. Of note, it does not seem to be dependent on the core HRE sequence, but possibly on the different expression patterns of the HIFα-subunits and/or on the interaction with other transcription factors (Ruas and Poellinger, 2005; Wenger, Stiehl and Camenisch, 2005).

Finally, the universal importance of HIFs proteins has been demonstrated by the ubiquitous expression of HIF-1α and HIF-1β in almost all adult and embryonic mouse and human tissues. Furthermore, the fact that several functional knock-down studies in mice have often led to premature mortality and/or abnormal physiology with particular relevance to increased tumorigenesis confirmed this assumption (Kapitsinou and Haase, 2008).
Figure 1.2. Structural domains of HIF-1α, HIF-2α, HIF-3α and their transcriptional binding partner HIF-1β/ARNT. In humans and other vertebrate species, both HIF-1α and HIF-2α contain a basic helix-loop-helix (bHLH) domain; a Per-Arnt-Sim domain (PAS) made up of PAS-A and PAS-B; an oxygen dependent degradation (ODD) domain; an N-terminal transactivation domain (N-TAD) located in the ODD and a C-TAD localized in the C-terminal region (modified from Koh and Powis, 2012).
1.2.3. The role of von Hippel Lindau protein

As previously stated, pVHL controls HIF-α stability and consequently negatively regulates the activation of the HIF response under normoxic conditions. Since HIF activity elicits a potent transcriptional response, HIF-α stability is a process that must be finely regulated. In the cells, this is achieved by a specific multiprotein complex driven by VHL.

pVHL is the main enzyme in the pathway which regulates HIF-stability by recognizing and binding to the hydroxylated HIF-α subunit. Then, being the substrate recognition component of the E3-ubiquitin ligase complex, it is able to engage elongin B, elongin C, cullin 2, and ring-box1 to induce the poly-ubiquitination of HIF-α subunit (figure 1.1). As a result, poly-ubiquitination signals the HIF-α degradation to the 26s proteasome (Kallio et al., 1999). By doing so, cells ensure that HIF-α is constantly degraded in the cytoplasm under normoxic conditions.

pVHL is also involved in the termination of the HIF response after reoxygenation upon hypoxia. In addition, it can be present in the nucleus where it has been observed binding to HIF-α subunits in order to trigger their ubiquitination (Groulx and Lee, 2002). Of note, the VHL-ubiquitin ligase complex trafficking has been seen being more dependent on the transcription rate rather than the cellular oxygen levels (Khacho et al., 2008). It is also interesting to observe that since HIF signalling plays a pivotal role in the regulation of cellular oxygen availability by stimulating erythropoiesis, angiogenesis and metabolism through anaerobic glycolysis, it can tune and interact with several other signalling pathways in ways that are still unknown.

Moreover, even if pVHL is well-known to be the key regulator of the HIF signalling, it has been shown to have numerous additional HIF-independent roles. Although some of these may result from VHL’s role in the ubiquitination complex,
others appear to be independent of this. For instance, inhibition or loss of VHL has been demonstrated to affect microtubules stability, cilia and mitotic spindle formation and may also contribute to tumorigenesis (Kim and Kaelin, 2004; van Rooijen et al., 2011; Kim et al., 2020).

In particular, the presence of a mutation in the VHL gene is typically associated to the development of familial VHL disease and of sporadic clear cell renal cell carcinoma (ccRCC) forms (Nyhan, O’Sullivan and McKenna, 2008). In this regard, people affected by VHL disease are heterozygous for wide range of mutations at the level of this gene, which makes them more susceptible to develop cysts and highly vascularized tumors following the loss of heterozygosity. Moreover, the form and severity of the disease may vary according to both the location and the type of mutation. Finally, although VHL patients are closely monitored for kidney cancer, biallelic loss of VHL is also a major cause of sporadic kidney cancer. Frustratingly, in these cases ccRCC is usually discovered when it is in an advanced state and has become metastatic. For these patients treatment options are extremely limited (Lonser et al., 2003; Cohen and McGovern, 2005).

The role of VHL in assuring homeostasis has been demonstrated is several studies. Among those, Vhl knock-out studies performed in mice resulted in haemorrhages and defects in placental vascularisation which bring to embryonic death at around 10 embryonic days (Gnarra et al., 1997). By contrast, despite the early lethality in Vhlh knockout mice, studies performed on zebrafish vhl mutants showed that they are able to complete embryogenesis and survive up to larval stages (8-11 days post fertilization). Thus, zebrafish vhl mutants represent an exclusive vertebrate model in which VHL role can be studied during both embryonic and postembryonic stages (van Rooijen et al., 2009).
Another disease associated with VHL mutation is the Chuvash polycythaemia. Interestingly, despite other VHL disease forms characterized by a heterozygous mutation, Chuvash polycythaemia is mostly caused by a homozygous R200W mutation located at the C-terminus of the gene. Interestingly, this results in an exacerbated HIF response followed by the development of polycythaemia, peripheral thrombosis, haemangiomas and in an increased vascular tone, but not hemangioblastomas, pheochromocytoma or ccRCC (Gordeuk et al., 2004; Smith et al., 2006). This highlighted the fact that there may be additional roles played by VHL, besides negatively regulating HIF, that are essential for its role as a tumour suppressor gene (Ang et al., 2002)

In general, the VHL/HIF signalling is also highly relevant in cancer, even when there are no mutations in the HIF pathway itself. This is particularly due to the hypoxic nature of the majority of solid tumours. Since uncontrolled growth is a fundamental characteristic of tumours, oxygen is a central nutrient that quickly runs out especially in the inner part of the tumoral mass. This usually triggers the activation of the HIF pathway which makes the tumour more resistant and able to cope with a hypoxic scenario. Consequently, these series of event are often followed by the expression of growth factors that leads to hypoxic angiogenesis (Semenza, 2012).

As a consequence of the above considerations, unravelling the precise mechanism in which HIF signalling works, how it is regulated and how it affects and interacts and with other pathways may provide entry points for clinical intervention. To this end, the following subchapters will be focused on the description of the main biological pathways affected by HIF in order to mediate the hypoxic response.
1.2.4. The HIF pathway and its role in glucose metabolism

In order to reduce oxygen consumption, HIF pathway orchestrates a rapid metabolic shift from aerobic metabolism to anaerobic glycolysis, leading to upregulation of glycolytic genes (i.e. enolase 1, aldolase A, lactate dehydrogenase A, phosphoglycerate kinase 1, and phosphofructokinase L) (Semenza et al., 1996; Minchenko et al., 2002). In addition, it is able to alter the composition of the electron transport chain (Fukuda et al., 2007), to regulate mitochondrial turnover (Zhang et al., 2007, 2008) and triggers the induction of pyruvate dehydrogenase kinase in order to switch metabolism away from oxidative phosphorylation (Kim et al., 2006; Papandreou et al., 2006). Of note, this switch allows cells to strongly reduce ROS production derived from oxygen starved mitochondria, hence avoiding oxidative stress related damages. Since glycolysis is less efficient in the conversion of glucose into ATP, HIF pathway can also increase glucose trafficking into the cell by up-regulating glucose transporters gene transcription (i.e. GLUT1) (Behrooz and Ismail-Beigi, 1997).

At the same time, HIF pathway may act to increase oxygen delivery by stimulating erythrocye production via up-regulation of epo and a series of iron-absorption related genes (Haase, 2010; Zhang et al., 2012) and by increasing angiogenesis through the transcription enhancement of angiogenic factors, (i.e. VEGF), (Krieg et al., 2000; Pajusola et al., 2005; Hickey and Simon, 2006).

In the last years it has come to light that HIF is also involved in the regulation of lipid metabolism. Indeed it has been observed that mice carrying a hepatic knockout of Vhl develop severe hepatic steatosis with impaired fatty acid oxidation, increased lipid storage capacity and decreased lipogenic gene expression (Rankin et al., 2009; Greenald et al., 2015). Furthermore, the precise effect of the HIF pathway mainly depends on its activation level. For instance, low- activation level of HIF-2α in mouse
liver was shown to have a positive effect on their metabolic profile, as seen in hypomorphic \textit{phd2} mutants (Rahtu-Korpela \textit{et al.}, 2014), whilst high activation level led to hepatic steatosis (Rankin \textit{et al.}, 2009).

In particular, the activation of the hypoxic response in zebrafish can be achieved by homozygous null mutation of the \textit{vhl} gene. Previous work from my laboratory focused on the phenotypic characterization of this mutant, established a linkage among \textit{vhl} mutation, hypoxic response and cancer (Rooijen \textit{et al.}, 2009; van Rooijen \textit{et al.}, 2011). Moreover, by exploiting the highly responding target gene promoter of Prolyl Hydroxylase 3, a unique GFP readout of the VHL/HIF signalling pathway was generated to track HIF signalling \textit{in vivo}. Previous work based on the use of this reporter line also identified glucocorticoid (GC) agonists and a number of hydroxylases inhibitors as potent activators of HIF signalling, especially at the hepatic level (Santhakumar \textit{et al.}, 2012; Vettori \textit{et al.}, 2017).

\textbf{1.2.5. The HIF pathway and its role in inflammation}

The HIF signalling mediated activation of the inflammatory response is a complex mechanism, which is characterized by the simultaneous activation of both pathways in several pathological circumstances such as chronic inflammation, wounds healing and solid tumours (Safronova and Morita, 2010). Here, hypoxia triggers NF-\(\kappa\beta\) stabilisation, which acts as a master regulator of the inflammatory and anti-apoptotic response. This is achieved, as for HIF-\(\alpha\), via the oxygen dependent inhibition of prolyl hydroxylase activity. Then, this triggers the decrease in I\(\kappa\)B kinase beta (IKK\(\beta\)) hydroxylation, which leads to the activation of NF-\(\kappa\beta\) (Yamamoto, Yin and Gaynor, 2000; Cummins \textit{et al.}, 2006, 2007; Ziv \textit{et al.}, 2012). The interaction between hypoxia and inflammation has been well studied in a zebrafish model of wound healing, where
HIF-1α pathway activation was observed to delay neutrophil resolution (Elks et al., 2011). This is believed to occur as a consequence of HIF activation inside the neutrophils themselves and seems to be related to an augmented neutrophil apoptosis rate coupled to a decreased trafficking away from the comorbid site (Schild et al., 2020).

Importantly, even if hypoxia is a pro-inflammatory event, previous studies showed links between the anti-inflammatory and GC responses. In this regard, GCs have been observed, primarily in vitro, to both potentiate and inhibit HIF pathway activation (Basu et al., 2002; Kodama et al., 2003; Leonard et al., 2005; Wagner et al., 2008; Sun et al., 2010). In a similar way, hypoxia has been shown to attenuate the glucocorticoid anti-inflammatory response and to elicit corticosteroid insensitive inflammation (Charron et al., 2009; Huang et al., 2009). It is also important to note that despite hypoxia is pro-inflammatory, it can positively interact with pathways with apparently opposite effects.

From these data it has become clear that the HIF-GC crosstalk is complex and still unclear and most likely reflects a context-specific activity of the transcriptional regulators in the tissue microenvironment. In particular, it is still unknown how the crosstalk occurs in vivo, how HIF affects GC response, and vice versa. In addition, it would be interesting to know if glucocorticoid receptor (GR) is an obligatory factor for HIF response or if it is a synergistic one. Therefore, the aim of my PhD was to further the research on how precisely this crosstalk occurs in vivo, as this may have a wide physiological significance in health and disease.
1.3. Glucocorticoids

The name “glucocorticoid” is a portmanteau word (glucose + cortex + steroid), which derives from their key role in the regulation of glucose metabolism, their biosynthesis at the level of the adrenal cortex and their steroidal structure. In particular, GCs represent a well-known class of lipophilic steroid hormones synthetized, with a circadian rhythm, by the adrenal glands in humans and by the interrenal tissue in teleosts. The GC circadian production in teleosts is tuned by the hypothalamic-pituitary-interrenal (HPI) axis, which is the equivalent of the mammalian hypothalamus-pituitary-adrenal (HPA) axis. Both are essential for stress adaptation (Alsop and Vijayan, 2009; Griffiths et al., 2012; Tokarz et al., 2013; Faught and Vijayan, 2018a).

Cortisol is the main GC both in humans and teleosts and controls a series of physiological processes which includes glucose, lipid and protein metabolism, stress response and inflammation (Munck, Guyre and Holbrook, 1984; Macfarlane, Forbes and Walker, 2008; Kuo et al., 2015; Facchinello et al., 2017). Due to their potent anti-inflammatory action, synthetic GCs has been broadly used for the treatment of pathological disorders that are linked to hypoxia, including rheumatoid arthritis, inflammatory, allergic, infectious, autoimmune diseases as well as to prevent graft rejections and against immune system malignancies (Nikolaus, Fölsch and Schreiber, 2000; Neeck, Renkawitz and Eggert, 2002; Chrousos and Kino, 2005; Revollo and Cidlowski, 2009; Busillo and Cidlowski, 2013). However, due to the presence of adverse effects (Moghadam-Kia and Werth, 2010) and GC resistance (Barnes and Adcock, 2009; Barnes, 2011) their therapeutic benefits are limited in patients chronically treated with these steroids. Examples of the most common GC related side
effects include osteoporosis, glaucoma, diabetes, skin atrophy, abdominal obesity, hypertension in adults and growth retardation in children.

Cortisol exerts its functions through direct binding both to the glucocorticoid receptor (Gr) and to the mineralocorticoid receptor (Mr), which in turn bind cortisol with different affinities (Bamberger, Schulte and Chrousos, 1996; Faught and Vijayan, 2018b). Once bound together, they form an active transcription factor complex, which can function either in a genomic or in non-genomic way. For these reasons, GC and their kindred intracellular receptors, represent critical checkpoints in the endocrine control of vertebrate energy homeostasis. Indeed, even if the biological effects induced by GCs are usually adaptive, their abnormal activity may contribute to a series of acute metabolic diseases which includes insulin resistance, obesity and type 2 diabetes (Smith and Vale, 2006; de Guia et al., 2014). Thus, furthering the research on how they precisely work and interact with other pathways, such as the HIF signalling, may provide new routes to treat these diseases (i.e HIF-related ones) and to simultaneously facilitate the development of innovative GCs with a better benefits-risk ratio.

1.3.1. The Hypothalamus-Pituitary-adrenal/interrenal axis

GCs are essential hormones biosynthesized and secreted by the adrenal cortex/interrenal gland both in a circadian manner and in response to stress. The latter is generally defined as a status of real or perceived threat to homeostasis. In particular, assuring homeostasis in the presence of stressors requires the activation of an intricate series of coordinated biological responses performed by the nervous, endocrine and immune systems (Smith and Vale, 2006; Nesan and Vijayan, 2016). In this regard, the key anatomical structures that regulate the stress response are located both in the central nervous system and in peripheral tissues. The primary effectors of the stress
response are localized in the paraventricular nucleus (PVN) of the hypothalamus, in the anterior lobe of the pituitary gland and at the level of the adrenal gland. These three main structures are generally referred to as the hypothalamic-pituitary-adrenal (HPA) axis in humans and to as hypothalamic-pituitary-interrenal axis (HPI) in zebrafish (Tsigos and Chrousos, 2002; Smith and Vale, 2006; Nesan and Vijayan, 2016) (figure 1.3).

Among these, the hypothalamus is the initial stressor recognition site for both internal and external signals. In particular, in mammals, neurons localized in the paraventricular nucleus synthesize both corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) which are released into hypophyseal portal vessels that access the anterior pituitary gland. On the other hand, in teleosts there is a direct neuronal connection to endocrine cells through the hypophyseal stalk, since they lack a portal system between the hypothalamus and the pituitary gland (Schmidt and Braunbeck, 2011). Here, CRF binding to its receptor localized on pituitary corticotropes triggers the release of adrenocorticotropic hormone (ACTH) into the systemic circulation. In humans, ACTH derives by posttranslational modification of the protein encoded by the proopiomelanocortin (POMC) gene. Of note, due to genome duplication, two pomc genes named pomca and pomcb have been identified in zebrafish (Gonzalez Nunez, Gonzalez-Sarmiento and Rodriguez, 2003; Hansen et al., 2003; To et al., 2007). However, only pomca seems to be expressed in the pituitary gland and is required for the interrenal organ development. (Wagle, Mathur and Guo, 2011; Shi et al., 2020). Consequently, the main target of ACTH is the adrenal cortex in humans and the interrenal tissue in teleost, where it binds to the melanocortin 2 receptor (MC2R) on the steroidogenic cells. Here, it stimulates cortisol biosynthesis and secretion (Mommsen, Vijayan and Moon, 1999; Smith and Vale, 2006; Nesan and Vijayan, 2013,
Finally, once released into the systemic circulation, GCs can access target tissues (i.e. liver, heart and vascular tissues) to exert metabolic and cardiovascular effects and the brain itself, in order to support cognitive processes required to tackle a threatening situation (Spiga et al., 2014).

Interestingly, GCs may also tune the activity of the HPA/I axis itself and hence their own biosynthesis, in order to cease the stress response and avoid an exacerbated reaction. This is achieved via a GC-GR mediated negative feedback loop, which acts both at the hypothalamic and anterior pituitary levels, where they inhibit both CRH and ACTH biosynthesis and release (Jones, Hillhouse and Burden, 1977; Dallman et al., 1987; Alsop and Vijayan, 2008; Griffiths et al., 2012). In addition, GCs may indirectly control HPA axis activity through modulation of different brain structures, including the amygdala, the hippocampus and the prefrontal cortex, that can in turn influence the activity of the paraventricular nucleus (Ulrich-Lai and Herman, 2009; Nicolaides et al., 2010, 2015; Spiga et al., 2014).

In zebrafish, even if a functioning HPI axis-dependent stress response starts only after hatching, they already have all the steroid biosynthesis and cortisol action components before hatching. Indeed, in the zebrafish developing hypothalamus, all the CRF-synthetising neurons are fully developed by 36 hpf, and crf is quantifiable starting from egg fertilization. The anterior pituitary ACTH-synthetising corticotropes are also differentiated by 26 hpf. Moreover, despite the interrenal tissue is not completely developed until hatching, the steroidogenic cells are already developmentally mature. In addition, the essential steroidogenic enzymes, including hydroxylases and StAR, are present by 28 hpf, but cortisol biosynthesis starts only at 48 hpf (Nesan and Vijayan, 2016). For these reasons, both cortisol and GR transcripts are maternally deposited into oocytes before the spawning phase. However, a complete turnover of these
transcripts occurs just after the mid-blastula transition and the zygotically synthetised gr mRNA and protein are measurable in the embryos by 12–24 hpf. By contrast, the endogenous cortisol release in response to stressors followed by the downstream GC-GR mediated gene regulation starts only between 96 and 120 hpf (Alsop and Vijayan, 2008; Nesan et al., 2012; De Marco et al., 2013; Weger et al., 2018; van den Bos et al., 2020).

### 1.3.2. Cortisol biosynthesis and secretion

In higher vertebrates, GCs are synthesized in the zona fasciculata of the adrenal cortex starting from cholesterol, whereas in zebrafish at the level of the interrenal gland. Here, steroidogenesis is stimulated when ACTH binds to the melanocortin type-2 receptor (MC2R) which is its specific cell surface G-protein coupled receptor (Mountjoy et al., 1994). Following ACTH binding, MC2R undergoes a series of conformational changes that activate adenylyl cyclase. This leads to a raise of the intracellular cyclic adenosine monophosphate (cAMP) levels which, in turn, triggers the downstream protein kinase A (PKA) signaling pathways. The latter is able to stimulate an acute GCs biosynthesis both via genomic and non-genomic ways (de Guia et al., 2014).

The rate-limiting step in the steroid biosynthesis is the transfer of cholesterol from the outer to the inner membrane of mitochondria, which is performed by the steroidogenic acute regulatory protein (StAR) (Lin et al., 1995; Stocco and Clark, 1996). Upon delivery, cholesterol is converted to pregnenolone by cytochrome P450 cholesterol side-chain cleavage (P450scc) enzymatic activity, encoded by CYP11a1. Then, pregnenolone can be transformed either into progesterone by the 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase, encoded by 3β-HSD, or hydroxylated into 17α-hydroxyprogesterone by cytochrome P450 17α-hydroxylase,
encoded by \textit{CYP17a1}. Progesterone and 17α-hydroxyprogesterone are then metabolized via a number of steps to androgen/estrogen and aldosterone/cortisol, by enzymes such as 17β-HSD and CYP19 (Bremer and Miller, 2014).

Cortisol, which is the primary GC in teleosts and the most abundant in humans, is ultimately bound to corticosteroid-binding globulins (CBG) in order to be transported in the bloodstream from the adrenal/interrenal gland to the target tissues. Of note, CBG not only promotes cortisol distribution but also plays a key role in its release to tissues (R. Oakley, 2013). Finally, since molecular oxygen is fundamental for steroid hormones biosynthesis, recent studies have highlighted the fact that the expression of specific steroidogenic genes such as StAR, CYP17A1, and CYP19 can be controlled by hypoxia (Tan \textit{et al.}, 2017).

\textbf{Figure 1.3}. The HPA/I axis. In response to stressors, hypothalamic neurons release Corticotropin-releasing factor (CRF) and Arginine vasopressin (AVP), which in turn acts on the anterior pituitary gland to enhance the biosynthesis and release of the adrenocorticotrophic hormone (ACTH) into the blood circulation. The latter, synthetized by post-translational modification of protein encoded by proopiomelanocortin gene (POMC), upon binding to the melanocortin 2 receptor (MC2R) on the steroidogenic cells of the adrenal glands, orchestrates a signal transduction pathway that finally leads to GCs biosynthesis and secretion into the bloodstream. Then, following acute stress, the system turns itself off by way of a negative feedback loop, wherein the receipt of cortisol in the hypothalamus and pituitary gland, suppress the production of ACTH and CRF (modified from Griffiths \textit{et al.} 2012).
1.3.3. Glucocorticoid receptors and the systemic stress response

GCs can passively diffuse across the plasma membrane into the cytoplasm, thanks to their lipophilic nature. However, their biological availability within the cells is regulated by two enzymes which work in an opposite fashion: 11β-Hydroxysteroid dehydrogenase type 2 (11β-HSD2) oxidizes cortisol into its inactive form, named cortisone, whereas 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) transforms cortisone to cortisol. Once inside the cell, GCs can bind to their specific receptors GR and MR (Mangelsdorf et al., 1995; de Kloet, Joëls and Holsboer, 2005; Chrousos and Kino, 2009; Spiga et al., 2014).

Both receptors, in the absence of their ligands (unbound state) are associated in an inactive oligomeric complex with specific regulatory proteins. Among these, heat shock protein-90 kD (HSP90) which binds GR to the C-terminal domain, heat shock protein-70 kD (HSP70), p59 immunophilin, Fkbp51 and Fkbp52 and the small p23 phosphoprotein allow to maintain the correct GR's protein folding (Schoneveld, Gaemers and Lamers, 2004; R. Oakley, 2013; Spiga et al., 2014).

The GR, which belongs to the nuclear receptor transcription factor family, is composed of different conserved structural elements. These include a N-terminal variable region, required for ligand-independent gene transactivation, followed by a central zinc-finger-containing DNA-binding domain, an adjacent hinge region containing nuclear localization signals and a C-terminal ligand-binding domain. The latter also includes residues essential for dimerization and hormone-dependent gene transactivation. Of note, the interaction between GR and HSP90 is fundamental to maintain its C-terminal domain in a favourable conformational state required for ligand binding (figure 1.4).
Figure 1.4. Representative figure of the GR’s domains structure. The N-terminal domain (NTD) which is required for ligand-independent gene transactivation, includes a transcriptional activation function region (AF1). The latter, which interacts with co-regulators and with the basal transcriptional machinery, is the main posttranslational modifications site. The LBD, which is made up of 12 α-helices and 4 β-sheets, forms a hydrophobic pocket needed for GC binding and includes an AF2 domain. The latter allow to interact with coregulators in a ligand dependent way. Finally, two nuclear localization signals, named NL1 and NL2, are localized in the DBD-hinge region junction and within the LBD, respectively.

Upon GC binding, GR undergoes a conformational change within the heterocomplex that involves a FKBP51-FKBP52 exchange, which triggers the translocation of the GC-GR active complex into the nucleus. This structural modification exposes the two GR nuclear localisation signals, which allow the hormone-activated GR to dimerize with another GC-GR molecule and to migrate into the nucleus via nuclear pores (Vandevyver et al., 2013; Presman and Hager, 2017). Importantly, this transcription factor complex can also act non-genomically in the cytoplasm, where it may interact via direct protein-protein interactions with other transcriptional regulators and/or kinases (i.e. basal transcription machinery (BTM); phosphoinositide 3-kinase (PI3K); signal transducer and activator of transcription (STAT)) (Reichardt et al., 1998; Prager and Johnson, 2009; Groeneweg et al., 2011; de Guia et al., 2014) (figure 1.5).

Inside the cell nucleus, GC-GR complexes directly binds to specific GREs, as tetramers, in order to regulate the transcription of target genes. Generally, the GRE GGAACAnnnTGTTCT is an imperfect palindromic consensus sequence that consists of two 6 bp half sites. Moreover, the three nucleotides spacing in-between the two half sites is essential for the GR to tetramerize on this sequence. Previous genome-wide
studies showed that the same GRE can mediate both the GC-dependent induction of many genes (positive GRE) and the repression of others (negative GRE) (R. Oakley, 2013; Uhlenhaut et al., 2013). Interestingly, the presence of specific inverted repeats negative GREs (IRnGRE), unrelated to simple GREs has been also reported both in mice and in humans. These DNA binding sequences are palindromic sequences consisting of two inverted repeated (IR) motifs separated by 1 bp. In particular, they act on GC-GR complexes in order to promote the assembly of cis-acting GR-SMRT/NCoR repressing complexes (Guenther, Barak and Lazar, 2001; Surjit et al., 2011).

Consequently, these findings indicate that the broadly different GC effects on various tissues can be partially ascribed to cell type-specific differences in the chromatin landscape that affects the accessibility of specific GREs for GR binding (Ramamoorthy and Cidlowski, 2013; Escoter-Torres et al., 2019). Furthermore, the GCs concentration at which the GR binds to GREs change throughout the genome. Another important feature of the GC-GR complex that makes their effects even more varying is that can tune gene expression in different ways: by binding directly to DNA, by tethering itself to other transcription factors bound to DNA, or via direct binding to DNA and with neighbouring DNA-bound transcriptional regulators (composite manner) (R. Oakley, 2013) (figure 1.5).

As previously mentioned, cortisol can bind not only to GR, but also to MR. The latter is a member of the transcription factors steroid hormone receptor family, which is evolutionarily related to the GR. In particular, it has been observed that GCs, even at smaller concentrations than those required to activate the GR, bind to MRs and enhance the activity of several kinases involved in different signal transduction cascades (Nicolaides et al., 2015; Faught and Vijayan, 2018b). Like GR, MR is also
characterized by the four canonical functional domains present in all nuclear hormone receptors (figure 1.4).

In mammals, the mineralocorticoid system is essential to regulate potassium and fluid homeostasis upon aldosterone activation of MR. Even though cortisol is a high-affinity ligand for MR, this steroid is deactivated in MR-specific tissues by the 11β-hydroxysteroid dehydrogenase type 2 enzymatic activity. This allows aldosterone, a second corticosteroid present in mammals, to bind to this receptor. Importantly, teleosts do not synthesize aldosterone and cortisol have been shown to mediate the majority of the changes in iono- and osmo-regulatory functions via GR signalling (Faught and Vijayan, 2018b). Moreover, if in teleosts the Gr-mediated cortisol effects regard the stress response and the modulation of intermediary metabolism, behaviour, growth and immune function, the roles of MR is less evident.

Cumulatively, despite its conserved and ancient origin, a clear role for MR has not been defined yet in ray-finned fish. Interestingly a recent in vivo study on MR signalling shed light on a key role for this receptor in the regulation of stress axis activation and function in teleosts (Faught and Vijayan, 2018b). As a consequence of the above considerations, due to the MR involvement in controlling HPI axis and since nothing is known about its role in the HIF signalling, deepening the knowledge on MR putative contribution to the HIF pathway is warranted.
Figure 1.5. Representative picture of the canonical GR signalling pathway. After binding to GC, cytoplasmic GR undergoes a conformational change, becomes hyper-phosphorylated, dissociates from accessory proteins (chaperone complex) and translocates into the nucleus. Here, after dimerization with another GR, regulate gene expression. Interestingly, GR may enhance or repress transcription of target genes by directly binding to palindromic GC response elements (GRE), or by tethering itself to other transcription factors apart from DNA binding, or in a composite manner by both directly binding GRE and interacting with transcription factors bound to neighbouring sites.
1.3.4. GCs and their role on glucose metabolism

The regulation of systemic glucose metabolism requires the presence of a fine-tuned crosstalk between different peripheral organs and the central nervous system (Coll and Yeo, 2013; de Guia et al., 2014). Among these, the liver represents the main organ in the body involved in controlling the mammalian glucose and lipid homeostasis (van den Berghe, 1991; Vegiopoulos and Herzig, 2007). Its primary activities are gluconeogenesis and glycogenolysis during fasting, in order to provide glucose for extrahepatic tissues such as erythrocytes, renal medulla, and brain (Consoli, 1992).

Recently, genome-wide analysis of GC target gene networks highlighted that GR fine-tunes numerous aspects of hepatic energy metabolism, especially the ones related to protein and sugar homeostasis (Yu et al., 2010; Murani et al., 2019; Wang et al., 2019). In particular, GR functionally interacts with other transcription factors to control specific genetic networks (Le et al., 2005), among which only a small amount has been characterized in detail so far. In addition, GCs exert their functions by antagonizing insulin action, promoting hepatic gluconeogenesis and decreasing glucose uptake and its utilization in skeletal muscle and white adipose tissue. These result in a temporary rise in blood glucose, which is thought to be favourable under stressful conditions (Aronoff et al., 2004; Charmandari, Tsigos and Chrousos, 2005).

GCs are also able to promote gluconeogenesis mainly by transcribing specific genes encoding for enzymes involved in the gluconeogenic pathway, such as glucose-6-phosphatase (G6PC), pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase 1 (PCK1), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKB), fructose-1,6-bisphosphatase 1 (FBP1) and Solute Carrier Family 37 Member 4 (SLC37A4). The G6pc, Pck1, and Pfkb1 GREs have been defined and the mechanism of GR-regulated Pck1 transcription has been studied in great details. By contrast, the
mechanisms regulating GC-activated Pc and Fbp1 are still unknown (Kuo et al., 2015). Moreover GCs are able to exert tissue specific effects by increasing glycogen storage in the liver, whilst they promote catecholamine-induced glycogenolysis and/or suppress insulin-dependent glycogen synthesis in skeletal muscle (Kuo, Harris and Wang, 2013; Rafacho et al., 2014). Overall, the physiological GCs response that tunes glucose homeostasis has been well documented. However, the precise way by which GCs regulate glucose homeostasis is still unclear.

### 1.3.5. GCs and their role in inflammation

As described in the subchapter 1.3.3, GR can regulate gene expression in different ways (Schoneveld, Gaemers and Lamers, 2004) (figure 1.5). Among these, the negative regulation of gene transcription may occur via both genomic and non-genomic mechanisms. In particular, this inhibitory effect played by the GC-GR active complex on other transcription factors’ function constitutes the GC anti-inflammatory way of action (Stellato, 2004).

In this regard, trans-repression mainly occurs via direct binding between monomeric GC-GR complex and transcription factors (i.e. NF-κB, c-Jun, and c-Fos) activated by cytokines and other pro-inflammatory stimuli, which synergistically coordinate the expression of several proinflammatory genes (Davies, Ning and Sánchez, 2005; Nicolaides et al., 2015). As a result, the mutual antagonism between transcription factors impairs their transcriptional properties and prevents them from binding to their corresponding DNA response elements. An additional GC-mediated anti-inflammatory mechanism consists of increasing the transcription of I-κB which, in turn, may repress NF-KappaB by favouring its retention in the cytoplasm (Li, Wang and Gelehrter, 2003). Moreover, GCs have been observed to inhibit the transcriptional
activity of various pro-inflammatory genes such as cytokines, chemokines, mediator-synthesizing enzymes and adhesion molecules. Contrary to expectations, GR loss of function was speculated by Facchinello and co-workers to prevent the transcriptional activity linked to the immune response (i.e. of cytokines Il6, Il1β, Il8 and Mmp-13) (Facchinello et al., 2017), corroborating the hypothesis of a GC-GR mediated dual-action on the immune system (Busillo and Cidlowski, 2013; Duque and Munhoz, 2016). In addition, GR was shown to synergistically induce proinflammatory genes by acting on other signalling pathways (Langlais et al., 2008, 2012; Dittrich et al., 2012; Xie et al., 2019). Finally, studies also demonstrated that GCs increase the transcription of numerous anti-inflammatory molecules such as interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1RA), secretory leukocyte inhibitory protein and neutral endopeptidase (Schaaf and Cidlowski, 2002; van den Bos et al., 2020).
1.4. HIF-GC interaction insights

The modulation of the HIF pathway has the potential to be clinically exploited as therapeutic treatment for a variety of pathological conditions which includes stroke, ischemia, spinal cord injury, inflammation, cancer, wounding, chronic anaemia and bone regeneration (Bernhardt et al., 2006; Shen et al., 2009; Shi et al., 2010; Wan et al., 2008; Ratan et al., 2008; Semenza 2003, 2009, 2015).

To this end, in the last years, an unbiased chemical screen performed in my laboratory on zebrafish larvae allowed to discover that HIF associated transcriptional responses are potently activated by GC, particularly in the zebrafish liver (Vettori et al., 2017). Moreover, by translating these observations to human tissues, it has been possible to show that GCs are able to promote HIF stabilization, without the need of the GR DNA binding domain (non-genomic action), in primary human hepatocytes and intact liver slices. In this regard, since c-src inhibitor PP2 treatment was able to rescue this effect, this suggested a role for GCs in promoting c-src-mediated proteasomal degradation of pVHL, followed by stabilization of HIF-α subunit (Vettori et al., 2017).

According to these data, since the liver is an important regulator of blood glucose levels, and both GCs and HIF promote gluconeogenesis and glycogen storage in the liver, furthering the knowledge on the crosstalk between these transcriptional regulators may provide an explanation for the GC effects on glucose metabolism and may have a wider physiological significance in health and disease than previously expected.

Indeed, both GCs and hypoxic transcriptional responses are mutually involved in assuring tissue homeostasis by controlling cellular responses to various forms of stress and inflammation, especially affecting glucose metabolism. For this reason, the
interaction between these two stress-responsive pathways has become increasingly appreciated over recent years.

Synthetic GCs have been widely used for years as anti-inflammatory drugs for treating pathological conditions where hypoxia plays a role in disease progression such as rheumatoid arthritis and chronic obstructive pulmonary disease (Nikolaus, Fölschn and Schreiber, 2000; Neeck, Renkawitz and Eggert, 2002; Busillo and Cidlowski, 2013). It has also been shown that GCs release in response to atmospheric hypoxia is linked with high altitude in humans and that the prophylactic treatment with GCs has been broadly exploited to mitigate the related mountain sickness (Wright, Brearey and Imray, 2008). Additionally, it has been observed that GCs protect different organs from ischemic injury, as it has been observed in particular against experimental cerebral and hepatic ischemic/reperfusion injury (Dardzinski et al., 2000; Limbourg et al., 2002; Glanemann et al., 2004; Tokudome et al., 2009).

The presence of an interplay between hypoxia and GC dependent signalling pathways has been previously reported by different in vitro studies (Kodama et al., 2003; Leonard et al., 2005; Wagner et al., 2008; Zhang et al., 2015). However, these studies reported conflicting results on the cross-talk between GC action and hypoxia, where the latter limits GR mediated transactivation both in pulmonary endothelial and hepatic epithelial cells (Leonard et al., 2005; Wagner et al., 2008).

The first data about the interaction between HIF and GR was presented by Kodama et al. 2003 (Kodama et al., 2003). By exploiting an artificial approach using Gal4-fusion reporter assays, they found that the ligand-dependent activation of GR increases hypoxia-dependent gene expression and hypoxia response element (HRE) activity in HeLa cells. Moreover, using dexamethasone treated COS7 cells co-transfected with expression plasmids for either GR or GAL4-LBD and GFP-HIF-1α and
exposed to hypoxic conditions, they showed colocalization of the GR and HIF-1α in the nucleus. For this reason, Kodama et al. postulated the presence of a direct protein-protein interaction between the GR LBD and HIF-1α as the main mechanism for GC-dependent enhancement of HIF pathway, but failed to demonstrate it via GST pull-down assays.

Leonard et al. 2005 (Leonard et al., 2005), subsequently confirmed via microarray analysis that GR is upregulated by hypoxia in human renal proximal tubular epithelial cells. Moreover, using a cell-based GRE luciferase reporter system, they showed that hypoxic exposure can potentiate dexamethasone-stimulated GRE promoter-reporter activity.

By using AtT-20 cells, Zhang et al 2015 (Zhang et al., 2015) demonstrated that GR expression levels were enhanced by HIF-1α under hypoxic conditions. However, dexamethasone treatment was able to cause the downregulation of GR expression in a HIF-1α dependent way. Finally, even if this was confirmed by transfecting AtT-20 cells with HIF-1α siRNA and culturing them under normoxia or hypoxic conditions, the involved underlying mechanism remains unclear.

By contrast, a dexamethasone-related inhibition of HIF-1α target genes expression in hypoxic HEPG2 cells was revealed by Wagner et al. 2008 (Wagner et al., 2008). In particular, via western blot analysis they showed that dexamethasone reduces nuclear HIF-1α protein as the HIF-1α amount was higher in cytosolic cell extracts than in the nuclear extracts upon DEX treatment. This cytoplasmic retention of HIF-1α suggested a blockage of nuclear import, via a still unknown mechanism, which resulted in a reduced HIF target gene expression. Moreover, by exploiting a luciferase assay the author revealed that dexamethasone attenuates HIF-1 activity not only in a GR-dependent way, but also that this effect depends on the presence of
functional HREs. Importantly, Wagner et al., attributed these contradicting results (compared to Kodama’s ones) to the presence of Fetal Calf Serum (FCS) in all their cell-culture experiments, as many cellular processes depends on the cell cycle phase.

In the following years, Gaber et al., 2011 (Gaber et al., 2011) investigated the interaction between macrophage migration inhibitory factor (MIF), HIF and the GCs effect in human primary nontumor CD4+ Th cells and Jurkat T cells. In contrast to the previous observations, this study showed the presence of a clear dexamethasone-dose dependent inhibition of HIF-1α protein expression, which resulted in a decreased HIF-1 target gene expression. Therefore, conversely to Wagner et al. 2008 hypothesis, they proposed a model based on a rapid DEX-mediated induction HIF-1α suppressors’ activity or a quick DEX-mediated inhibition of HIF signalling.

As a result of the above considerations, the interplay between GR and hypoxia mediated responses is complex and still requires additional experimental evidence to be untangled. Importantly, these data most likely reflects a context-specific activity of the transcriptional regulators in the tissue microenvironment. Above all, it remains unsolved how GCs fine-tune the cellular pathways mediating adaptation to hypoxic environment, how HIF affects GC signalling and, in turn, whether they are acting on each other. Further studies are also warranted to uncover the underlying mechanism behind it in an in vivo animal model.
1.5. The use of zebrafish as model organism

The zebrafish (Danio rerio) has been shown to be an exemplary informative and genetically modifiable organism for studying both HIF and GC pathway not only in physiological, but also in pathophysiological conditions (M.J.M. Schaaf, Chatzopoulou and Spaink, 2009; van Rooijen et al., 2011; Santhakumar et al., 2012; Vettori et al., 2017). In particular the short generation time, transparency, anatomical simplicity, quantity of the progeny obtainable by natural mating and the ex-utero embryos development, allow biological processes to be followed in detail and in a non-invasive way. Moreover, zebrafish allow the use of medium to high throughput drug screening (A. L. Robertson et al., 2014; C. E. Robertson et al., 2014; Vettori et al., 2017) and genetic tractability, especially via the recently improved genome editing CRISPR/Cas9 based mutagenesis technology (Hruscha et al., 2013; Varshney et al., 2015; Burger et al., 2016; Wu et al., 2018).

Taking into account these undeniable advantages, this model organism has become more frequently used especially in the hypoxic and stress science field (M.J.M. Schaaf, Chatzopoulou and Spaink, 2009; Löhr and Hammerschmidt, 2011; van Rooijen et al., 2011; Griffiths et al., 2012a; Santhakumar et al., 2012; Krug Ii et al., 2014). Therefore, during my PhD I have exploited it as an in vivo model organism to study how and to what degree hypoxic signalling affects the endogenous GC’ response and vice versa. In this regard, the use of whole animals allows also to demonstrate how these signalling pathways interact with each other in a more complete way than in vitro, where interactions among different tissues and cell types cannot be easily modelled.

Importantly, zebrafish share all the components of the human HIF signalling pathway and by taking advantage of the vhl mutant line coupled to the phd3:eGFP
transgenic HIF activity reporter line, it is also possible to reliably activate and quantify the HIF response (van Rooijen et al., 2011; Santhakumar et al., 2012; Elks et al., 2015). Furthermore, analogously to humans, zebrafish are diurnal and use cortisol as the main GC hormone (Weger et al., 2016). In addition, unlike other teleosts which have additional GR paralogs, zebrafish have only a single glucocorticoid (zGr) and mineralocorticoid receptor (zMr) isoform (M.J.M. Schaaf, Chatzopoulou and Spaink, 2009; Faught and Vijayan, 2018b). Finally, zGr shares high structural and functional similarities to its human analogous, making zebrafish a reliable model for studying GC activity in vivo (Alsop and Vijayan, 2008; Chatzopoulou et al., 2015; Xie et al., 2019).
1.6. Aims

The aim of this project is to unravel the cellular mechanisms underlying the interaction between hypoxic and GC signalling \textit{in vivo} using the zebrafish as a model. In particular, the primary goal is to understand how and to what degree hypoxic signalling affects the endogenous GCs’ response and vice versa.

This is because the presence of a crosstalk between GC and hypoxia dependent signalling pathways has been previously indicated in different \textit{in vitro} studies. In addition, synthetic GCs (i.e. betamethasone and dexamethasone), which are the equivalent of naturally present steroid hormones, have been broadly used for decades as anti-inflammatory medications for treating pathological conditions associated to hypoxia (i.e. ischemic injury, asthma, rheumatoid arthritis etc.). Nowadays, thanks to the available \textit{in vitro} results, it has become evident that the HIF-GC crosstalk is an intricate mechanism and might be cell-specific. For these reasons, furthering the knowledge on how this interplay occurs \textit{in vivo} can have a vast physiological relevance in health and disease.

To this end, previous chemical screens performed in my laboratory, using unique zebrafish HIF reporter, surprisingly identified synthetic GR agonists as activators of the HIF pathway. In view of this, the main hypothesis of my project is that GC and HIF are somehow acting on each other. If so, it is likely that GR activity could be required for HIF signalling to occur.

To test these hypotheses, I planned to exploit both a genetic and pharmacological approach to modulate these two pathways during the first 120 hours post fertilisation of zebrafish embryos. I also took advantage of two different mutant lines I have generated during my PhD, named \textit{hif1βsh544} (\textit{arnt1}) and \textit{grsh543} (\textit{nr3c1})
respectively, together with an already existing \textit{vhlhu2117/+;phd3:eGFPi144/i144} hypoxia reporter line. These lines were required to investigate the effect of HIF activity on GC signalling and vice versa, via a “gain-of-function/loss-of-function” approach. Molecular and phenotypic analyses of these mutants have been coupled to optical and fluorescence microscope imaging.

This is because, by knocking out \textit{vhl} and \textit{arnt1} genes, it is possible to up-and downregulate the HIF signalling, whereas the administration of synthetic GR agonists and the generation of \textit{gr}\textsuperscript{-/-} zebrafish line allows to respectively enhance and inhibit the GC pathway. In this regard to understand whether GCs contribute to HIF phenotype, I planned to knock-out both \textit{gr} and \textit{mr} in a HIF upregulated scenario (\textit{vhl}\textsuperscript{-/-} larvae) to quantify the phenotypic and molecular outcomes, in terms of both \textit{phd3;eGFP} and target genes expression. In a similar way, to investigate the role of HIF on GC pathway, I set up to quantify both the expression of GC transcriptional activity and cortisol biosynthesis in the presence of high and suppressed HIF activity, respectively. Finally, chemical treatment with GR agonist (i.e betamethasone) will be used to check the ability of GCs to trigger the HIF response under different HIF levels.

Of note, these approaches coupled to the use of an animal model allowed me to obtain a much clearer overview about the GC/HIF crosstalk in a more global way than in cell culture and to propose a novel model of interaction between these two major signalling pathways.
2. MATERIALS AND METHODS

2.1. Zebrafish husbandry and maintenance

All zebrafish (Danio rerio) lines that were used during my research were raised and maintained under standard conditions (14 hours of light and 10 hours of dark cycle, at 28°C) in the Aquaria facility of the University of Sheffield. All zebrafish embryos used for experiments were reared in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM MgCl₂, 0.33 mM CaCl₂, pH 7.2) containing methylene blue (Sigma-Aldrich) at 0.0001% and staged according to standard method (Kimmel et al., 1995) for up to 5.2 days post fertilisation (dpf) in accordance with UK Home Office legislation (licence number PB2866ED0 and PC39B259E). PTU (1-Phenyl-2-thiourea) treatment has been performed at 24 hpf in order to prevent embryos pigmentation, when necessary, as described by Karlsson et al., 2001 (Karlsson, von Hofsten and Olsson, 2001).

2.1.1. Wild-type line

Wild-type (WT) embryos were obtained from the AB line ZDB-GENO-960809-7 and reared up to 5.2 dpf under the same aforementioned conditions (chapter 2.1).

2.1.2. vhl$hnu2117^{+/+}$;phd3:eGFP$^{i144/i144}$ mutant line

The transgenic line $Tg(phd3:eGFP)^{i144/i144}$ is a hypoxia reporter line generated via BAC transgenesis by Santhakumar et al., 2012. In particular, BAC clone CHORI73-277E22 was originally engineered in order to contain an EGFP reporter construct, with an SV40
polyadenylation site inserted at the level of prolyl hydroxylase 3 (phd3) ATG start site. This fish line was incrossed to create homozygotes. In order to create the \( vhl^{hu2117/+};phd3:eGFP^{144/144} \) line used in this project, the \( Tg(phd3:eGFP)^{144/144} \) line was originally crossed with the \( vhl^{hu2117/+} \) line. When incrossed, these fish generate \( vhl^{hu2117/hu2117};phd3:eGFP^{144/144} \) embryos (here referred to as \( vhl \) mutants) in accordance with mendelian ratio and begin to fluoresce starting from 28 hpf (Santhakumar et al., 2012). As previously described by van Rooijen et al., 2009, \( vhl \) mutants are able to complete embryogenesis and are viable up to larval stages (8-10 dpf). Therefore, unlike \( vhl \) mutant mice which die during early embryonic stages, \( vhl^{-/-} \) zebrafish larvae represent an ideal vertebrate animal model to study HIF pathway.

### 2.1.3. \( vhl^{hu2117/+};vll^{216/i216}; phd3:EGFP^{144/+} \) mutant line

The \( vhl;vll(phd3:eGFP) \) fish line used for this project was obtained by crossing \( vhl^{hu2117/+}; phd3:eGFP^{144/+} \) into the \( vll^{216/i216} \) homozygous mutant line (van Eeden, unpublished). This fish strain can be incrossed to generate \( vhl^{hu2117/hu2117};vll^{216/i216};phd3:eGFP^{144/+} \) embryos at a mendelian ratio, hereafter referred to as \( vhl;vll \) mutants. Interestingly, despite \( vhl \) larvae are not viable after 8-10 dpf, \( vll^{-/-} \) larvae develop normally and adult fish are fertile.

### 2.1.4. \( hif1\beta^{h544/+};vhl^{hu2117/+} \) mutant line

The \( hif1\beta^{h544/+};vhl^{hu2117/+} \) mutant line was created via CRISPR/Cas9-based mutagenesis method, targeting exon 5 in \( arnt1 \) gene. The induced mutation is a D125fsX1 that causes a premature stop codon (TAA) after 1 aa at amino acid 125 in the translated amino acid sequence. Homozygous mutants were obtained by incrossing heterozygous
fish and sorted according both to their phenotype at 3 dpf and their GFP fluorescence starting at 28 hpf.

2.1.5. \(gr^{sh551/+};vhl^{hu2117/+};phd3:eGFP^{i144/+}\) and \(gr^{sh543/+}\) mutant lines

Two different \(gr\) mutant lines were created via CRISPR/Cas9-based mutagenesis method for this project: \(gr^{sh551/+}\ vhl^{hu2117/+};phd3:eGFP^{i144/+}\) and \(gr^{sh543/+}\) line. The first one, originally generated by Eleanor Markham (van Eeden laboratory) using \(vhl^{hu2117/+};phd3:eGFP^{i144/+}\) embryos at the background, carried 1bp deletion in exon 2 of \(nr3c1\) gene (also known as \(gr\)), which codes for the N-terminal domain (NTD). The second one, created by me, using WT(AB) embryos at the background, carried a 11 bp deletion at the level of \(nr3c1\)’s exon 3 that codes for the DNA-binding domain. Of note, the mutation is a A377fsX12 that causes a premature stop codon (TGA) after 12 aa at aa 390 in the translated amino acid sequence. Since the GR DBD starts at 384 and ends at 463, the first zinc finger motif occurs at aa 377-480 and the second zinc finger motif occurs at aa 386-453, the mutation occurs at the very beginning of the DNA binding domain. Homozygous mutants for each allele were obtained by incrossing heterozygous fish and sorted according to their visual background adaptation (VBA) response at 5 dpf, as reported in a previous study (Griffiths et al., 2012).

2.1.6. \(gr^{sh543/+};hif1b^{h544/+};phd3:eGFP^{i144/+}, gr^{sh543/+};vhl^{hu2117/+} phd3:eGFP^{i144/+}\) and \(gr^{sh543/+};hif1b^{h544/+};vhl^{hu2117/+};phd3:eGFP^{i144/+}\) mutant lines

These fish lines were generated by crossing \(gr^{sh543/+}\) with \(hif1b^{h544/+};vhl^{hu2117/+};phd3:eGFP^{i144/+}\) fish, in order to obtain all the possible genotype combinations. Subsequently, founders were identified by adult tail-clipping and
consequently by screening the offspring for GFP fluorescence. Homozygous mutants were obtained by incrossing heterozygous fish and sorted according both to their phenotype at 3 dpf and their GFP fluorescence starting at 28 hpf.

2.1.7. $gr^{sh543/+}; mr^{sh562/+}$ mutant line

This fish line was generated by crossing the aforementioned $gr^{sh543/+}$ with the $mr^{sh562/+}$ fish line. The $mr^{sh562/+}$, created by Jack Paveley (Vincent Cunliffe/Nils Krone laboratory, Bateson Centre, TUoS), shares a 46 bp deletion at the level of $mr$ exon 3 which codes for the DNA-binding domain. The mutation is a T597fsX16 that causes a premature stop codon after 16 aa at aa 613 in the translated amino acid sequence of the DNA binding domain. The F1 generation was identified by adult tail-clipping and $gr^{+/-};mr^{+/-}$ fish were consequently selected and incrossed to get all the possible genotype combinations. Finally, F2 generation larvae were VBA sorted at day 5 post fertilisation and raised till adulthood. Homozygous mutants were then obtained by incrossing $gr^{+/-};mr^{-/-}$ fish and sorted according to their visual background adaptation response at 4 dpf.
2.2. Genotyping protocols

2.2.1. DNA extraction from whole embryos

Genomic DNA was extracted using the HotSHOT method (Wellcome Trust Sanger Institute's protocol modified by Eleanor Markham) from whole embryos, as described below. Single embryos were individually placed in sterile wells of a 96-well plate and 25 µl of 1X base solution (1.25 M KOH crystals and 10 mM EDTA in milliQ water) were added in each sample. The embryos were then incubated for 30 minutes at 95°C and cooled down for 10 minutes at 10°C. Finally, 25 µl of 1X neutralisation solution (2 M TrisHCl in milliQ water) were added to each sample and the plate was vortexed for few seconds to further dissolve the embryos. Lastly, the extract was centrifuged for 2 minutes at maximum speed (4200 rpm) and 1.5 µL of supernatant was used per PCR reaction.

2.2.2. DNA extraction from tail biopsies

Fin clipping procedure was conducted according to Home Office recommendations and tail biopsies from adult fish were transfected directly into 50 µl of 1X base solution into a 96-well plate. Clipped tissues were then incubated for 30 minutes at 95°C, followed by cooling to 10°C. for 10 minutes. To neutralise the acidic pH of the medium, 50 µl of 1X neutralisation solution were added to each sample and vortexed for 5 seconds. Finally, the dissolved tissues were centrifuged for 2 minutes at maximum speed and 1.5 µL of extracted DNA was used per PCR reaction.
2.2.3. \textit{vhl}^{hu2117/+};\textit{phd3}:\textit{eGFP}^{i144/i144} genotyping

Although \textit{vhl} mutants are clearly distinguishable from siblings by morphology and thanks to presence of a strong eGFP-related brightness overall, \textit{vhl} heterozygous are identical to WT embryos. For this reason, an already existing genotyping protocol was used as a proof of concept to confirm the genotype of \textit{vhl} embryos and to sort them for mating purposes. The selected mutation is a C/T transition (Q23X) at the level of \textit{vhl} exon1 (van Rooijen \textit{et al.}, 2009), which introduces a premature stop codon and a BciVI restriction site. A DNA region of 414 bp encompassing the mutation site was amplified via PCR and digested using BciVI restriction enzyme at 37°C, overnight. Digested PCR products were cleaved into different DNA fragments according to the corresponding genotype:

- \textit{vhl}^{+/+}: 2 fragments (222 and 192 bp)
- \textit{vhl}^{hu2117/+}: 3 fragments (414 bp, 222 and 192 bp)
- \textit{vhl}^{hu2117/ hu2117}: 1 fragment (414 bp)

However, due to the inaccuracy of the restriction enzyme activity, digestion results were often difficult to interpret in the right way. For this reason, I decided to improve this genotyping protocol by exploiting the dCAPS (derived Cleaved Amplified Polymorphic Sequence) method (Neff \textit{et al.}, 1998). dCAPS Finder 2.0 (\url{http://helix.wustl.edu/dcaps/}) and Primer3 websites were used to design 3 different primers sets, each containing a mismatch mutation, which allow to create a restriction nuclease sensitive polymorphism based on target mutation. BamHI, Accl and EcoRV - related primers set were selected among the suggested dCAPS primers due to their efficiency, survival and activity in PCR mix. In particular, BamHI-related dCAPS primers showed to work better than Accl and EcoRV's ones and was used for genotyping \textit{vhl} embryos. Primers were designed to amplify a 170 bp region enclosing the original
BciVI mutation site (primers listed on Table 2.1). PCR products were subsequently digested at 37°C for at least 4 hours and 30 minutes as recommended by New England Biolabs. The success of the reaction was assessed via gel electrophoresis on a 3% agarose gel. By design, two bands for wild-types (151 bp and an undetectable 19 bp), three bands for heterozygous (170, 151 and 19 bp) and one band for homozygous mutants (170 bp) were expected. Primers and PCR settings used for these two protocols are listed below.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Stock conc.</th>
<th>Working conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>vhl forward</td>
<td>TAAGGGCTTAGCGCATGTTC</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>vhl reverse</td>
<td>CTATCTACGCAGTTAATCG</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>Accl forward</td>
<td>GGTCTCTGATCAGCCGTATA</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>Accl reverse</td>
<td>GCATAATTTCAGCAACCCACA</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>BamHI forward</td>
<td>GGTCTCTGATCAGCCGGATC</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>BamHI reverse</td>
<td>GCATAATTTCAGCAACCCACA</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>EcoRV forward</td>
<td>AGTCTAACTCGGTGGAAGCA</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>EcoRV reverse</td>
<td>GTTACAGAACAGAAGTTGACCGG</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

*Table 2.1 List of primers sequences used for *vhl*[^2117] genotyping and relative concentrations.*

**PCR machine setting (vhl):**
1. 94°C, 4 minutes
2. 92°C, 1 minute
3. 56°C, 30 seconds
4. 72°C, 40 seconds
5. GOTO step 2, 39x
6. 72°C, 10 minutes
7. 12°C, ∞

**PCR machine setting (BamHI-dCAPS):**
1. 94°C, 4 minutes
2. 92°C, 1 minute
3. 52°C, 30 seconds
4. 72°C, 15 seconds
5. GOTO step 2, 34x
6. 72°C, 10 minutes
7. 12°C, ∞
2.2.4. \(gr^{sh551/+} vhl^{hu2117/+};phd3:eGFP^{i144/+}\) genotyping

To distinguish \(gr^{sh551/+} vhl^{hu2117/+}\) heterozygous from WT embryos, a genotyping protocol was designed in order to identify and raise only heterozygous adult fish. The mutation is an 1bp deletion at the level of exon 2 of \(nr3c1\) gene, which causes the destruction of a \(BslI\) restriction site. Primers have been designed with Primer3 to amplify a 177 bp DNA sequence surrounding the mutation site (primers listed on Table 2.2). PCR product has been subsequently digested using \(BslI\) restriction enzyme by incubating the digestion mix at 55°C overnight, as recommended by New England Biolabs. The success of the reaction was assessed via electrophoresis by running the digested samples on a 3% agarose gel. By design, one band for the homozygous mutants (177 bp), two bands for the wild-types (150 and 27 bp) and three bands for the heterozygous (177, 150 and 27bp) were expected. However, since the 27 bp DNA fragment is too small to be detected on a 3% Agarose gel, it is possible to visualize only one band for the wild-types (150 bp) and two for the heterozygous (177 and 150 bp). The following table lists the primers sequence and PCR settings used for this protocol.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Stock concentration</th>
<th>Working solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>grex2 forward</td>
<td>GCAAAATGGATCAAGGAGGA</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>grex2 reverse</td>
<td>CAGAAACTGGCTGCATCATT</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

*Table 2.2 List of primers sequences used for \(gr^{sh551}\) genotyping and relative concentrations.*

PCR machine setting:

1. 94°C, 3 minutes
2. 94°C, 30 seconds
3. 47°C, 30 seconds
4. 72°C, 15 seconds
5. GOTO step 2, 34x
6. 72°C, 5 minutes
7. 12°C, ∞
2.2.5. *grsh543* genotyping

As *grsh551/+;vhlhu2117/+* larvae, *grsh543* heterozygous were also indistinguishable from WT embryos. For this reason, I used a genotyping protocol designed to identify and raise only heterozygous fish. This method takes advantage of an 11 bp deletion occurring at the level of exon 3 of *nr3c1* gene, which introduces a premature stop codon and causes the loss of a *PvuII* restriction site. A genomic sequence of 216 bp surrounding the mutation site was amplified via PCR (primers listed on Table 2.3) and digested with *PvuII* restriction enzyme by incubating the digestion mix at 37°C for at least 4 hours as recommended by New England Biolabs.

Fish genotype was evaluated via electrophoresis by running the digested samples on a 3% agarose gel. By design, one band for the homozygous mutants (216 bp), two bands for the wild-types (127 and 89bp) and three bands for the heterozygous (216, 127 and 89 bp) were expected. The following table lists the primers sequence and PCR settings used for this protocol.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Stock concentration</th>
<th>Working solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>grex3 forward</td>
<td>CCAAACCTTCCAGGCAGCAGT</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>grex3 reverse</td>
<td>TCCGCAAGTGGAACTCCAT</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

*Table 2.3 List of primers sequences used for *grsh543* genotyping and relative concentrations.*

PCR machine setting:

1. 94°C, 3 minutes
2. 94°C, 30 seconds
3. 51°C, 30 seconds
4. 72°C, 15 seconds
5. GOTO step 2, 34x
6. 72°C, 5 minutes
7. 12°C, ∞
2.2.6. hif1β<sup>sh544/+;vhl<sup>hu2117/+;phd3:eGFP</sup></sup><sup>h144/+</sup> genotyping

As hif1β heterozygous were undistinguishable from hif1β siblings a genotyping protocol was developed to identify and raise heterozygous fish. This mutant line is characterized by a 7 bp insertion at the level of exon 5 of arnt1 gene (also known as hif1β), which introduce a premature stop codon and causes the destruction of MwoI restriction site.

Primers were designed in order to amplify a 156 bp DNA sequence surrounding the mutation site (primers listed on Table 2.4). PCR products were then digested using MwoI restriction enzyme at 60°C, overnight as recommended by manufacturer’s instructions. As expected, one band for homozygous mutants (156 bp), three bands for heterozygous (156, 134 bp and 22 bp) and two bands for wild types (134 bp and 22 bp) were detected on a 3% agarose gel. Primers and PCR setting used for this protocol are the following:

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Stock conc.</th>
<th>Working conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hif1βex5 forward</td>
<td>AGAGCTGTCGGATATGGTTCG</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>hif1βex5 reverse</td>
<td>TGGTCTGAGAAAGGATGGT</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

Table 2.4 List of primers sequences used for hif1β<sup>sh544</sup> genotyping and relative concentrations.

PCR machine setting:

1. 94°C, 3 minutes  
2. 94°C, 30 seconds  
3. 51°C, 30 seconds  
4. 72°C, 15 seconds  
5. GOTO step 2, 34x  
6. 72°C, 5 minutes  
7. 12°C, ∞
2.2.7. \textit{mr}^{sh562/+} genotyping

Since \textit{mr} heterozygous larvae are identical to \textit{mr} siblings a genotyping protocol was created to identify and raise heterozygous fish. This mutant line is characterized by a 46 bp insertion at the level of exon 3 of \textit{mr} gene (\textit{nr3c2}), which introduces a premature stop codon and causes the destruction of the DNA binding site.

Primers were designed in order to amplify a 210 bp DNA sequence surrounding the mutation site (primers listed on Table 2.5). Thanks to the mutation size, PCR was enough to appreciate a substantial difference between mutants and wildtypes in terms of DNA bands size on a 3\% agarose gel. For this reason, no restriction enzyme DNA digestion was required. As expected, one band for homozygous mutants (164 bp), two bands for heterozygous (164 bp and 210 bp) and one band for wild types (210 bp) were detected on a 3\% agarose gel. Primers and PCR setting used for this protocol are the following:

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Stock conc.</th>
<th>Working conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{mrex3} forward</td>
<td>GACCATGAGAACACCTGCAC</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>\textit{mrex3} reverse</td>
<td>TGAGTCTTACCTTCTACCGCTC</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

\textit{Table 2.5} List of primers sequences used for \textit{mr}^{sh562} line genotyping and relative concentrations.

PCR machine setting:

1. 95°C, 5 minutes
2. 95°C, 30 seconds
3. 58°C, 30 seconds
4. 72°C, 15 seconds
5. GOTO step 2, 34x
6. 72°C, 5 minutes
7. 12°C, ∞
2.3. CRISPR/Cas9-based mutagenesis method

CRISPR/Cas9-based mutagenesis method is a ground-breaking technology which allows the introduction of a mutation at the level of a specific genomic region of interest in a target gene. The system is made up of two components: an RNA-guided DNA endonuclease (Cas9) and a single guide RNA (gRNA), specifically designed against a target sequence within the genome.

It takes inspiration from the adaptive immunity mechanism discovered in *Streptococcus pyogenes* used to recognize, target and destroy exogenous DNA. In a similar manner, thanks to the use of specifically designed gRNAs, it is possible to form a ribonucleoprotein active complex with the effector Cas9 nuclease, in order to recognize, bind and cleave its complementary DNA target sequence, upstream to an NGG (PAM) site.

In this regard, to create the aforementioned stable *grh*543, *grh*551 and *hif1β*544 mutant lines, I co-injected in one-cell-stage zebrafish embryos a specific gRNA, designed to anneal to a target sequence and the Cas9 protein, as described by Hruscha et al. 2013. As a consequence of that, the activated homologous recombination (HR) and the non-homologous end joining (NHEJ) systems introduce mutations in the attempt of repairing the cleaved site. Among a plethora of mosaic mutations, I selected only the ones that were able to cause a frameshift (non-multiple of three mutations) and that were able to be transferred to the progeny. To achieve that, five different gRNA antisense oligonucleotide sequences (guide-oligos listed on Table 2.8) were obtained using CHOPCHOP website (https://chopchop.cbu.uib.no/) to target each a specific restriction site in the corresponding gene of interest (Montague et al., 2014; Labun et
al., 2016). These gRNAs were chosen according to their genomic location, GC content, efficiency and the presence of a PAM site downstream of a restriction enzyme’s cut site.

In general, it is preferable to induce a mutation as close as possible to the beginning of the open reading frame, in order to induce a frameshift that could affect the downstream domains. However, especially for GR, a drawback of this approach is related to the presence of conserved alternative start codons (AUG) that could generate progressively shorter but still functional isoforms, which could “rescue” the induced mutation (Oakley and Cidlowski, 2013). For these reasons, I decided to generate a mutation at the level of the DNA-binding domain, which is more likely to produce a non-functional protein.

To design the gRNAs used in this project, an 18 nucleotides sequence upstream to a selected PAM site (guide-oligos listed on Table 2.8) was inserted every time into a scaffold sequence (a.k.a CRISPR backbone, listed on Table 2.6) containing a promoter for the T7 Polymerase. Prior to transcription, the gRNAs were amplified via PCR and then purified from an agarose gel to increase their purity (primers listed on Table 2.7).

<table>
<thead>
<tr>
<th>Scaffold sequence</th>
<th>5’–AAAGCACCAGACTCGGTGCCACTTTTTTCAAG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TTGATAACGGACTAGCCTATTTTTAACTTGCT</td>
</tr>
<tr>
<td></td>
<td>ATTTCTAGCTCTAAACACNNNNNNNNNNNNNN</td>
</tr>
<tr>
<td></td>
<td>NNNNNNNCTATAGTGATCGTATTACG–3’</td>
</tr>
</tbody>
</table>

Table 2.6 Scaffold sequence used to insert CRISPR guide-oligos to produce a mutation in specific restriction sites.
Amplification protocol is listed below:

- Resuspend crispr guide oligo in TES buffer (Tris-HCl 10 mM pH8, EDTA 1 mM, 0.1 M NaCl) to make a 100 μM stock.
- Dilute it to 1 μM in milliQ water.
- Prepare PCR mix as follows:
  - 5x Firepol Master Mix 20 μl
  - 10μM guide-oligo primer for 2.5 μl
  - 10μM guide-oligo primer rev 2.5 μl
  - 1μM guide-oligo 2 μl
  - milliQ water up to 100 μl of final volume

- PCR machine program:
  1. 95°C, 1 minute
  2. 95°C, 15 seconds
  3. 60°C, 30 seconds
  4. 72°C, 20 seconds
  5. GOTO step 2, 39x
  6. 72°C, 5 minutes
  7. 10°C, ∞

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Stock conc.</th>
<th>Working conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>guide-oligo primer for</td>
<td>AAAGCACCGACTCGGTGCCAC</td>
<td>100 μM</td>
<td>10 μM</td>
</tr>
<tr>
<td>guide-oligo primer rev</td>
<td>GCGTAATACGACTCACTATAG</td>
<td>100 μM</td>
<td>10 μM</td>
</tr>
</tbody>
</table>

Table 2.7 List of primers with relative concentrations used to amplify and increase purity of gRNAs.

The resulting PCR product was run on a 3% agarose gel, purified using a Qiagen MinElute gel extraction kit and transcribed using a T7 megashortscript Ambion kit, using 1 μ as a template.
Transcription protocol was set up as follows:

- 10x T7 buffer 2 μl
- ATP 2 μl
- GTP 2 μl
- CTP 2 μl
- UTP 2 μl
- template DNA 2 μl
- T7 Enzyme mix 2 μl
- milliQ water up to 20 μl of final volume

The transcription mix was subsequently incubated for 2 hours at 37°C in the water bath and the following protocol was used:

- Add 1 μl of DNase and incubate it for 30 minutes at 37°C
- Make up to 100 μl volume using milliQ water
- Add 33 μl of 10 M NH4Ac and 350 μl of EtOH 100%
- Precipitate the mix at -80°C for at least 2 hours
- Centrifuge at 4°C, max speed, for 30 minutes
- Wash pellet with 70% EtOH and air-dry it
- Dissolve pellet in 15 μl of milliQ water.

The success of the transcription was determined through gel electrophoresis and the corresponding concentration was quantified using a NanoDrop ND-1000 spectrophotometer.
Finally, the gRNA was injected at 2.4 µg/µl together with Cas9 protein. As an example, I have used the following CRISPR-injection mix:

- gRNA (4.18 µg/µl) 2.87 µl
- CAS9 protein (NEB, M0386, 20µM) 0.5 µl
- Phenol red 0.5 µl
- milliQ water up to 5 µl of final volume

Each embryo was injected with 1 nl of CRISPR-injection mix and raised for 24 hours at 28°C in E3 medium. The success of the injection method was determined via whole-embryo PCR-based genotyping performed on a fraction of injected embryos at 1 dpf, which have been randomly selected.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Target sequence</th>
<th>Restriction site</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>gr^sh551</td>
<td>GTTGGAAACCATAAGATCC</td>
<td>CCNN_NNN^NNGG</td>
<td>BslI</td>
</tr>
<tr>
<td>gr^sh543</td>
<td>CAGCTGACGATGTGGCAG</td>
<td>CAG^CTG</td>
<td>PvuII</td>
</tr>
<tr>
<td>hif1bex2</td>
<td>TTTGTTGCTTTTTGACCC</td>
<td>CCNN_NNN^NNGG</td>
<td>BslI</td>
</tr>
<tr>
<td>hif1βsh544</td>
<td>TCGGTGCTGTTTCAG</td>
<td>GCNNNNN^NNGC</td>
<td>MwoI</td>
</tr>
<tr>
<td>hif1βsh544</td>
<td>TGCAGGAAGATAGATTTT</td>
<td>CCNN_NNN^NNGG</td>
<td>BslI</td>
</tr>
</tbody>
</table>

Table 2.8 List of target sequences inserted into the CRISPR scaffold to produce a mutation in specific restriction sites.
2.3.1. CRISPANT technology

Zebrafish-based genetic screenings coupled to recent advances in reverse genetic approaches (i.e. CRISPR/Cas9 method) represent powerful and efficient tools for probing molecular mechanisms governing vertebrate physiology, development and disease. However, with standard application of CRISPR/Cas9 system injection, null mutants can be obtained and analysed only at the F2 generation stage.

To increase throughput, I exploited a novel and rapid technology for directed gene knock-out created by Burger et al 2016 and implemented by Wu et al., 2018. This method, based on CRISPR/Cas9 technology, allows to generate null phenotypes in G₀ zebrafish through the simultaneous injection of four CRISPR/Cas9 ribonucleoprotein complexes, which redundantly target a single gene (Wu et al., 2018). Notably, the resulting GO nulls have a penetrance (>90% of embryos) which approaches the one of standard germline-transmitted knockouts.

To achieve that, four guide-RNAs selected from a lookup table of guide sets for most zebrafish genes, provided by the authors of the aforementioned paper to facilitate screens, were co-injected with Cas9 protein (diluted 1:10) and tracrRNA (100 µM) in one-cell stage embryos. This method was used to create G₀ CRISPANTS (CRISPR/Cas9-mediated mutants) for the following genes of interest: mineralocorticoid receptor (mr, a.k.a nr3c2), aryl hydrocarbon receptor nuclear translocator 2 (arnt2, a.k.a hif1β2) and laminin, beta 1b (lamb1b). The latter was used as CRISPR-injection control. gRNAs sequences and stock concentrations used are listed in Table 2.9.
<table>
<thead>
<tr>
<th>Transcript ID</th>
<th>gRNA name</th>
<th>Target exon</th>
<th>Target sequence</th>
<th>Stock conc.</th>
<th>Working conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENSDART00000158162</td>
<td>arnt2_Target_1</td>
<td>exon 5</td>
<td>ACGGCCTACAAACCCTCC</td>
<td>100μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>ENSDART00000158162</td>
<td>arnt2_Target_2</td>
<td>exon 6</td>
<td>GCCGATGGCCTTTTTGTGCG</td>
<td>100μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>ENSDART00000158162</td>
<td>arnt2_Target_3</td>
<td>exon 11</td>
<td>TTCACGCCACAATTCGGATG</td>
<td>100μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>ENSDART00000158162</td>
<td>arnt2_Target_4</td>
<td>exon 14</td>
<td>GTTCAGGTGCCGTTAAAAACA</td>
<td>100μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>ENSDART0000172111</td>
<td>nr3c2_Target_1</td>
<td>exon 2</td>
<td>GCATTGTGGGTCACCTCCA</td>
<td>100μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>ENSDART0000172111</td>
<td>nr3c2_Target_2</td>
<td>exon 2</td>
<td>AAGGGGATAAACAGGAAAAC</td>
<td>100μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>ENSDART0000160637</td>
<td>nr3c2_Target_3</td>
<td>exon 5</td>
<td>CAACCAGCTGCGGAAAC</td>
<td>100μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>ENSDART0000172111</td>
<td>nr3c2_Target_4</td>
<td>exon 5</td>
<td>ATATCTGACGGCGTCCGTCT</td>
<td>100μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>ENSDART00000066945</td>
<td>lamb1b_Target_1_CD95</td>
<td>5’-UTR</td>
<td>TTGTAAATAGCATAGTTAAGG</td>
<td>100μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>ENSDART00000066945</td>
<td>lamb1b_Target_2_CD96</td>
<td>exon 1</td>
<td>GAGAAGAGCTGACAAACAGTGAG</td>
<td>100μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>ENSDART00000066945</td>
<td>lamb1b_Target_3_CD107</td>
<td>5’-UTR</td>
<td>GCCGTGGTCAGGTTTTGTAG</td>
<td>100μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>ENSDART00000066945</td>
<td>lamb1b_Target_4_CD108</td>
<td>exon 2</td>
<td>TCACATGACATGTTGCG</td>
<td>100μM</td>
<td>25 μM</td>
</tr>
</tbody>
</table>

Table 2.9 List of gRNAs sequences and relative concentrations used to create rapid and consistent biallelic gene disruption in G0 embryos.
CRISPANT-injection mix was prepared as follows:

- gRNA (25 μM) 0,5 μl (each)
- Cas9 protein (NEB, M0386, 20 μM) 0.5 μl
- tracrRNA (100 μM) 1 μl
- Phenol red 0.5 μl
- milliQ water up to 5 μl of final volume

Finally, 1 nl of injection mix was injected in each embryo and they were then raised at 28°C till five days post-fertilisation in E3 medium. The criterion for success of the injection was assessed by observing the phenotype and quantifying the eGFP-related brightness of 5 dpf larvae and via genotyping.

2.3.2. lamb1b CRISPR injected embryos genotyping

To confirm the efficiency and the specificity of CRISPR/Cas9-based mutagenesis method, I chose to target a gene which is not involved in the HIF pathway. In particular, lamb1b was selected as CRISPR-injection control gene, whereas vhl

hu2117/hu2117;phd3:eGFP

i144/i144 embryos were used as CRISPR-injection control samples. lamb1b is an extracellular matrix glycoprotein involved in a variety of biological processes which include cell adhesion, differentiation and migration. Notably, since it has been reported to exhibit tissue-specific compensation, it is not able to generate any phenotype when mutated (Chris Derrick, Bateson Centre, TUsS, unpublished). For this reason, as lamb1b-injected vhl mutant embryos were undistinguishable from the uninjected ones, a genotyping protocol was designed as follows:
- Prepare PCR mix as follows:
  - 5x Firepol Master Mix 4 μl
  - [10μM] primer for 1 μl
  - [10μM] primer rev 1 μl
  - DNA 1,5 μl
  - milliQ water up to 20 μl of final volume

- PCR machine program:
  1. 94°C, 3 minutes
  2. 94°C, 30 seconds
  3. 50°C, 30 seconds
  4. 72°C, 15 seconds
  5. GOTO step 2, 34x
  6. 72°C, 5 minutes
  7. 10°C, ∞

Primers have been designed using Primer3 to amplify respectively a 262bp (CD95), 238bp (CD96), 254bp (CD107) and 256bp (CD108) DNA sequence surrounding the mutation sites (primers listed on Table 2.10). Finally, the success of the reaction was assessed via gel electrophoresis by running the PCR amplified samples on a 4% agarose gel diluted into Tris-Boric Acid-EDTA (TBE) buffer for two hours at 100 V. TBE buffer was used instead of TAE (Tris-Acetate-EDTA), because of its buffering capacity and conductivity, which make it less prone to overheating during long runs. Moreover, borate allows to resolve small DNA fragments by preserving DNA integrity better than acetate (Sanderson et al., 2014).
<table>
<thead>
<tr>
<th>gRNA name</th>
<th>Target exon</th>
<th>Primer name</th>
<th>Target sequence</th>
<th>Stock conc.</th>
<th>Working conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>lamb1b_Target_1_CD95</td>
<td>5'-UTR</td>
<td>CD97 (forward)</td>
<td>TCACACTAAGACATGGGGCA</td>
<td>100μM</td>
<td>10 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD98 (reverse)</td>
<td>TCTTGTGCAATAATCGAGGGA</td>
<td>100μM</td>
<td>10 μM</td>
</tr>
<tr>
<td>lamb1b_Target_2_CD96</td>
<td>exon 1</td>
<td>CD99 (forward)</td>
<td>TCTTCTCCCACGATCCACAC</td>
<td>100μM</td>
<td>10 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD100 (reverse)</td>
<td>ACCAAGCAACAAAAACACTGA</td>
<td>100μM</td>
<td>10 μM</td>
</tr>
<tr>
<td>lamb1b_Target_3_CD107</td>
<td>5'-UTR</td>
<td>CD119 (forward)</td>
<td>CGCACCCGGAAAAATCATATCT</td>
<td>100μM</td>
<td>10 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD120 (reverse)</td>
<td>AGAGCTGTAGTGTGGAAAAGTC</td>
<td>100μM</td>
<td>10 μM</td>
</tr>
<tr>
<td>lamb1b_Target_4_CD108</td>
<td>exon 2</td>
<td>CD121 (forward)</td>
<td>AAGTTTCATCGTGAACCTTTTC</td>
<td>100μM</td>
<td>10 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD122 (reverse)</td>
<td>TTCTTGGGCCCTACCTCTAATA</td>
<td>100μM</td>
<td>10 μM</td>
</tr>
</tbody>
</table>

*Table 2.10 List of gRNAs’ primer sequences and relative concentrations used to genotype lamb1b injected and uninjected embryos.*
2.4. Whole mount in situ hybridization

2.4.1. Embryos harvesting, treatment and fixation

Embryos to be analyzed were harvested, treated and fixed to allow the probe to penetrate the tissue, find its mRNA target and form stable, double-stranded mRNA:RNA probe hybrid RNA molecules. To this end, 24 hpf embryos were treated with 16.8 µl of 1-phenyl 2-thiourea (PTU, stock concentration 75mg/ml) diluted in 35 ml E3 medium to inhibit melanogenesis, according to Karlsson et al., 2001 protocol. Afterwards, batches of 15 embryos at 4 dpf were treated into 6-well plates with 30 µM Betamethasone 17,21-dipropionate (BME), with 1% DMSO (Sigma-Aldrich) and left untreated as control for 24 hours (Griffiths et al., 2012). Inside the 6-well plates, embryos were incubated in 3 ml total volume of E3 medium without methylene blue. Afterwards, up to 30 embryos were collected in 1,5 ml Eppendorf tubes and anaesthetized using Tricaine Solution (MS-222, Sigma-Aldrich) prior to fixation in 1 ml 4% PFA solution overnight, at 4°C. The following day, embryos were washed twice for 10 minutes in PBST and post-fixed in 1 ml 100% MeOH. Finally, samples were stored at -20°C.

2.4.2. Whole-mount in situ hybridization protocol

Whole mount in situ hybridization is a common very informative approach used to detect the location and determine the expression levels of target mRNAs in zebrafish embryos. It is a multi-step technique based on the hybridization of a synthetically produced Digoxigenin-labelled antisense-RNA probe to the complementary mRNA under investigation. Digoxigenin is an organic molecule that is recognised by a specific
monoclonal antibody that is covalently linked to Alkaline Phosphatase. In particular, the location and the expression levels of the hybridized Digoxigenin-labelled probe can be observed by treating samples with a combination of substrate and colour enhancer named BCIP and NBT, respectively. Indeed, Alkaline Phosphatase molecules that are covalently bound to the anti-Digoxigenin monoclonal antibody can convert them into a blue precipitate. The expression level of the targeted mRNA is directly proportional to the intensity of the blue staining in the area where the gene under investigation is expressed. The protocol used, described below, was adapted from Thissse and Thissse, 2008 (Thissse and Thissse, 2008). All solutions are listed on Table 2.11:

- **Day 1 (probe-target mRNA hybridisation)**
  - Rehydrate embryos by successive incubations with 60% MeOH/PBST and 30% MeOH/PBST for 10 minutes each at room temperature (RT);
  - Wash them 4 times for 5 minutes each with 100% PBST, at RT;
  - Permeabilise embryos by incubating them in Proteinase K (15 mg/ml stock, diluted 1:1000 in PBST) at RT for the appropriate amount of time according to the developmental stage used, as follows:

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Mins at RT</th>
<th>ProtK concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early somitogenesis</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Late somitogenesis</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>24 hpf</td>
<td>0</td>
<td>10µg/ml</td>
</tr>
<tr>
<td>30 hpf</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>36/48 hpf</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>3 dpf</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>4dpf</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>5 dpf</td>
<td>90-120</td>
<td>15µg/ml</td>
</tr>
</tbody>
</table>
- Wash embryos one time for 5 minutes with PBST to remove ProtK
- Refix by incubating in 4% PFA for 20 minutes at RT;
- Wash 3 times, 5 minutes each, with PBST at RT;
- Pre-incubate in hybridisation mix with tRNA and heparin (HM+) for at least 3 hours at 70°C;
- Incubate overnight at 70°C in HM+ containing Digoxigenin-labelled probe (750-1000 ng for embryos > 4 dpf)

**Day 2 (Anti-DIG antibody incubation)**

- Brief wash with 100% hybridisation mix without tRNA and heparin (HM-);
- Wash with 75%, 50% and 25% HM- in 2x SSC for 15 minutes each at 70°C;
- Wash with 2x SSC for 15 minutes at 70°C;
- Wash twice with 0.2x SSC for 30 minutes each at 70°C;
- Wash with 75%, 50% and 25% 0.2x SSC in PBST for 10 minutes each at RT;
- Wash with PBST for 10 minutes at RT;
- Incubate in blocking solution for at least 3 hours at RT;
- Incubate in blocking solution with anti-Dig antibody overnight, at 4°C, in dark.

**Day 3 (NBT/BCIP colorimetric reaction)**

- Brief wash with PBST, at RT, in the dark, to remove antiserum;
- Wash 4 times with PBST for 30 minutes each, at RT, in the dark;
- Wash with Alkaline Phosphatase buffer, without MgCl₂ for 15 minutes, at RT, in the dark;
- Wash twice with Alkaline Phosphatase (AP) buffer, with MgCl₂ for 10 minutes each, at RT, in the dark;
- Incubate in staining solution at RT, in the dark, until embryos are stained;
- Stop staining reaction by washing with PBST, for 3 times, at 4°C, in dark;
- Post-fix the stained embryos with 4% PFA for 20 minutes at RT;
- Wash embryos four times with PBST, 5 minutes each;
- Wash with 25%, 50% and 80% glycerol in PBST and store them at 4°C.

<table>
<thead>
<tr>
<th>HM*</th>
<th>AP* buffer</th>
<th>Blocking buffer</th>
<th>PBST</th>
<th>Staining solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>Tris HCl pH 9.5 5 ml</td>
<td>2% Blocking Powder 1 g in 50mls</td>
<td>PBS 1x 45 ml</td>
<td>NBT 3,4 μl/ml AP*</td>
</tr>
<tr>
<td>20x SSC 12.5 ml</td>
<td>MgCl₂ 1M 2.5 ml</td>
<td>Malate Buffer 25 ml</td>
<td>milliQ water 5 ml</td>
<td>BCIP 3,5 μl/ml AP*</td>
</tr>
<tr>
<td>Tween20 25% 200 μl</td>
<td>NaCl 5M 1 ml</td>
<td>milliQ water 25 ml</td>
<td>Tween20 25% 200 μl</td>
<td></td>
</tr>
<tr>
<td>Citric acid 1M 460 μl</td>
<td>Tween20 25% 200 μl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin 50 mg/ml 50 μl</td>
<td>milliQ water up to 50 ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tRNA 50 mg/ml 500 μl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>milliQ water up to 50 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.11 List of solutions and relative concentrations used for WISH protocol. 1: Nitro Blue Tetrazolium Chloride; 2: 5-Bromo-4-Chloro-3-Indoyl-Phosphate.
2.4.3. Antisense RNA probe design and transcription

Single-stranded antisense-RNA probes, which are perfectly complementary to the mRNA to be detected, were synthesized through \textit{in vitro} transcription using chemically modified ribonucleotide triphosphates that carry a digoxigenin side chain (Table 2.12). Prior to that, each plasmid encoding zebrafish complementary DNA (cDNA) fragments, as templates, were linearized with the appropriate restriction enzyme that cleaves on the 5’ side of the insert, such that antisense transcripts can be synthesised from the T7 or SP6 RNA polymerase promoter, located immediately downstream of the 3’ end of the cDNA clone. Afterwards, the linearized DNA fragments were purified through phenol/chloroform method and transcribed into antisense RNA probes, as follows:

- Linearized DNA 1 μg
- RNase inhibitor 0,5 μl
- T7/SP6 RNA Polymerase 2 μl
- 5x Transcription buffer 4 μl
- 10x Dig-labelled nucleotides mix 2 μl
- milliQ water up to 20 μl of final volume

The transcription mix was then incubated at 37°C for 2 hours and the following RNA precipitation protocol was used:

- Add 1 μl of DNase and incubate it for 20 minutes at 37°C;
- Add 80 μl of milliQ water and mix;
- Add 33 μl of 10 M NH₄Ac and 350 μl of 100% EtOH;
- Precipitate RNA probe by storing it at -80°C for at least 2 hours;
- Centrifuge at maximum speed for 30 minutes at 4°C;
- Wash pellet with 500 μl 70% EtOH;
- Centrifuge at maximum speed for 10 minutes at 4°C;
- Air-dry pellet for 5 minutes;
- Resuspend in 100 μl of milliQ water;
- Store it at -80°C.

The success of the reaction was evaluated by electrophoresis on a 1% agarose gel and the corresponding probe's concentration was quantified using a NanoDrop ND-1000 spectrophotometer.

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Vector</th>
<th>Restriction enzyme/Polymerase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pomca</td>
<td>pCR BluntII-TOPO</td>
<td>SP6/XhoI</td>
<td>Muthu et al., 2016</td>
</tr>
<tr>
<td>cypr17a2</td>
<td>pGEM-T Easy Vector</td>
<td>SP6/NcoI</td>
<td>Eachus et al., 2017</td>
</tr>
<tr>
<td>idha</td>
<td>pCMV-SPORT 6.1</td>
<td>T7/EcoRI</td>
<td>van Rooijen H. (van Eeden lab)</td>
</tr>
<tr>
<td>phd3</td>
<td>pCMV-SPORT 6.2</td>
<td>SmaI/T7</td>
<td>Santhakumar K. (van Eeden lab)</td>
</tr>
</tbody>
</table>

*Table 2.12 List of RNA probes used for in situ hybridization analysis.*
2.5. TaqMan Real Time-qPCR assay

For many years TaqMan has been the gold standard chemistry for real time PCR due to its unequalled specificity, sensitivity and ease of use. Similar to other types of real-time PCRs, TaqMan-based reactions require double-stranded template and two fairly standard target specific primers to work. Indeed, unlike the ones used in regular PCR reactions, TaqMan assay requires two PCR primers with a preferred product size of 50-150 bp and a third sequence-specific fluorogenic “probe”. The latter is a single stranded oligonucleotide of 20-26 nucleotides designed to bind only the DNA sequence in-between the two PCR primers. Notably, the probe cannot be extended by Taq Polymerase, as it lacks a free hydroxyl group. Moreover, TaqMan probes are covalently joined to two other molecules:

- a fluorescent FAM™ or VIC™ dye label called “reporter”, located on the probe’s 5’-end, which enables the detection of a specific PCR product, as it accumulates during PCR cycles;
- a minor groove binder (MGB) linked to a nonfluorescent quencher (NFQ) on the 3’ end, which quenches the fluorescent signal from the reporter via Fluorescent Resonance Energy Transfer (FRET), when the latter is in close proximity to the reporter.

For this reason, as long as the probe remains intact, there is no permanent increase in fluorescent signal from the reporter. Vice versa, if the reporter and the quencher are permanently separated during the polymerase reaction, the reporter does fluoresce, producing a signal that is detected by the RTqPCR machine.
At the beginning of real-time PCR, the temperature is raised at 95°C to denature the double-stranded cDNA. During this step, the fluorescent signal from the “reporter” on the 5’ end is silenced by the “quencher” on the 3’ end. In the following step, the temperature is lowered to 60°C in order to allow the primers and the probe to anneal to their specific target sequences. Finally, Taq DNA polymerase synthesizes new strands using the unlabeled primers and the cDNA template. In particular, due to its endogenous 5’-nuclease activity, when it reaches the probe, the polymerase cleaves it, separating the dye from the quencher. After each PCR cycle, more reporter molecules are released, resulting in a raise in fluorescence intensity, which is directly proportional to the amount of target amplicon synthesized. By the way, the cycle threshold (Ct) value, defined as the number of cycles required for the fluorescent signal to cross the threshold (background level) are inversely proportional to the expression level of the target gene analysed.

2.5.1. Drug treatment, RNA extraction and cDNA synthesis

Three biological replicates of 10 larvae, at 5 dpf each, were treated for 24 hours starting from 96 hpf, with 30 μM Betamethasone 17,21-dipropionate and with 1% DMSO used as control.

RNA was extracted from 5 dpf larvae derived from the following incrossed mutant lines: WT (AB), \( vh_{hu2117/hu2117;phd3:eGFP^{i144/i144}} \), \( vh_{hu2117/+;vll^{i216/i216};phd3:eGFP^{i144/+}} \), \( hif1\beta^{sh544/+;vll_{hu2117/+;phd3:eGFP^{i144/i144}};hif1\beta^{sh544/+;phd3:eGFP^{i144/i144}} \), \( gr^{sh543/+;vh_{hu2117/+;phd3:eGFP^{i144/+}};gr^{sh543/+} \), using the protocol described below:
• **Phase separation**
  - Homogenize samples in 500 µl Trizol for 5 minutes at RT;
  - Add 100 µl chloroform and shake vigorously for 15 seconds;
  - Leave at RT for 2-3 minutes;
  - Centrifuge at 12000 xg for 15 minutes at 4ºC;

• **RNA precipitation**
  - Remove clear, upper aqueous phase to new tubes;
  - Mix upper phase with 250 µl isopropanol;
  - Leave at RT for 10 minutes;
  - Centrifuge at 12000 xg for 10 minutes at 4ºC;

  - **RNA wash**
    - Discard supernatant;
    - Wash the pellet with 500 µl 75% EtOH;
    - Centrifuge at 7,500xg for 5mins at 4ºC;

  - **Re-dissolving RNA**
    - Discard supernatant;
    - Air-dry the pellet for 5 minutes;
    - Dissolve pellet in 20 µl RNase-free milliQ water

RNA extracted was then quantified using a Nanodrop ND-1000 spectrophotometer. Afterwards, cDNA was synthesized from RNA through reverse transcription using Protoscript II First Strand cDNA Synthesis Kit – easy protocol (NEB), as recommended by manufacturer's instructions. cDNA mix was set up as describe in Table 2.13:
<table>
<thead>
<tr>
<th>Components</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>template RNA</td>
<td>up to 1 µg</td>
</tr>
<tr>
<td>oligo d(T)23 VN</td>
<td>2 µl</td>
</tr>
<tr>
<td>ProtoScript II Reaction Mix (2X)</td>
<td>10 µl</td>
</tr>
<tr>
<td>ProtoScript II Enzyme Mix (10X)</td>
<td>2 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>up to 20 µl of final volume</td>
</tr>
</tbody>
</table>

*Table 2.13 List of reagent and relative concentrations used for cDNA synthesis.*

Finally, synthesized cDNA was quantified using a Nanodrop ND-1000 spectrophotometer.

### 2.5.2. TaqMan RT-qPCR protocol and settings

RTqPCR was performed using CFX96 Touch™ Real-Time PCR Detection System (BioRad) paired with CFX Maestro™ Analysis Software. Each assay contains target primers and an optimized sequence-specific probe with no extra design, optimization or lengthy melt-curve analysis necessary. RTqPCR mix was prepared as follows:

- cDNA (100ng/µl) 1 µl
- TaqMan Universal Master Mix 10 µl
- FAM™ Probe 1 µl
- milliQ water up to 20 µl of final volume

The following RTqPCR machine setting was used:

1. 95°C, 10 minutes
2. 95°C, 15 seconds
3. 60°C, 30 seconds
4. GOTO step 2, 39x
Cycle threshold (Ct) values were automatically calculated by the software with ROX as passive reference dye. Three technical replicates for each biological replicate were used for each RTqPCR reaction. Notably, due to the use of a 96 well plate format coupled to the presence of drug treated samples, only one target gene and one housekeeping gene were tested each time. Finally, 1 μl of MilliQ water, in triplicate, was used as negative control instead of cDNA, both for target and housekeeping genes.

2.5.3. Target and reference TaqMan probes used

Four hypoxia-inducible factor pathway-dependent (*egln3, pfkfb3, vegfab and slc2a1a*) and four GC pathway-dependent (*fkbp5, il6st, pck1 and lipca*) target genes expression were respectively tested against *eef1a1* and/or *rps29* housekeeping gene expression. The list of TaqMan probes used for RTqPCR analysis is described in the Table 2.14.

2.5.4. Fold change calculation

ΔΔCT method was used to calculate the fold change value (Livak and Schmittgen, 2001). Firstly, the difference between Ct values of target gene and reference gene was calculated both for control samples (ΔCt control) and for treated samples (ΔCt treatment). Then, the difference between the average ΔCt (treatment) and the average ΔCt (control), used as normalizer, was used to obtain the ΔΔCt value. Finally, due to the exponential nature of qPCR, "fold change" is calculated as $2^{\Delta \Delta Ct}$.

\[ \Delta Ct (ctrl) = Ct(target \text{ gene}) - Ct(reference \text{ gene}) \]
\[ \Delta Ct (treatment) = \text{average } Ct(target \text{ gene}) - \text{average } Ct(reference \text{ gene}) \]
\[ \Delta \Delta Ct = \Delta Ct(treatment) - \Delta Ct (ctrl) \]
\[ FC = 2^{\Delta \Delta Ct} \]
See the link below, page 57 for additional details.


<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Assay ID</th>
<th>Dye label</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egl nine homolog 3 (pdr3)</td>
<td>Dr03095294_m1</td>
<td>FAM-MGB</td>
<td>20x</td>
</tr>
<tr>
<td>pifb3</td>
<td>Dr03133423_m1</td>
<td>FAM-MGB</td>
<td>20x</td>
</tr>
<tr>
<td>vegf3</td>
<td>Dr03072613_m1</td>
<td>FAM-MGB</td>
<td>20x</td>
</tr>
<tr>
<td>slc2a1a</td>
<td>Dr03103605_m1</td>
<td>FAM-MGB</td>
<td>20x</td>
</tr>
<tr>
<td>vegf5</td>
<td>Dr03095255_m1</td>
<td>FAM-MGB</td>
<td>20x</td>
</tr>
<tr>
<td>il6st</td>
<td>Dr03095252_m1</td>
<td>FAM-MGB</td>
<td>20x</td>
</tr>
<tr>
<td>pck1</td>
<td>Dr03133423_m1</td>
<td>FAM-MGB</td>
<td>20x</td>
</tr>
<tr>
<td>lipca</td>
<td>Dr03133423_m1</td>
<td>FAM-MGB</td>
<td>20x</td>
</tr>
<tr>
<td>rps29</td>
<td>Dr03133423_m1</td>
<td>FAM-MGB</td>
<td>20x</td>
</tr>
</tbody>
</table>

Table 2.14: List of TaqMan probes used for RTqPCR analysis on HIF and GC target genes expression.
2.6. Cortisol extraction and quantification

By collaborating both with Nan Li (Nils Krone laboratory, University of Sheffield, UK) and Karl-Heinz Storbeck laboratory (Department of Biochemistry, Stellenbosch University, South Africa) I performed cortisol extraction and quantification in both hif1β<sup>sh544/+;phd3:eGFP<sup>i144/+</sup> and vhl<sup>hu2117/+;phd3:eGFP<sup>i144/+</sup> mutant lines. These lines were chosen among the available ones, due to their opposite effect on GC target genes expression, as demonstrated via RTqPCR analysis.

Three biological replicates of 150 larvae at 5 dpf each, of hif1β<sup>sh544</sup> mutants, hif1β<sup>sh544</sup> siblings, vhl<sup>hu2117</sup> mutants and vhl<sup>hu2117</sup> siblings were used for steroid hormones extraction, according to (Eachus et al., 2017) protocol, as follows:

- Transfer 150 larvae into silanized low-binding Eppendorfs and snap-freeze them on dry ice;
- Add 1 ml of phosphate-buffered saline (PBS) to the sample;
- Perform three rounds of freeze/thawing using dry ice and hot water at 55°C to lyse the cells;
- Homogenize samples with a tissue homogenizer (Power Gen 125, Fisher Scientific) equipped with blades (SN-04727-87, Disposable Plastic Tip Homogenizer Probes - LabGEN® Tip Probes, 0.25 to 50 mL, Soft Tissue, 50/pack);
- Perform a fourth freeze/thawing round;
- Store the tubes at -20°C
Once all the samples were collected, I freeze-dried them to make the material more convenient for shipping to Karl-Heinz Storbeck laboratory, which performed cortisol quantification. In particular, the dried steroids were resuspended in 150 mL 50% MeOH and were separated and quantified with an Acquity UPLC System (Waters, Milford, CT) in combination to a Xevo TQ-Standem mass spectrometer (Waters). Finally, chromatographic separation was carried out via ultra-performance liquid chromatography high-strength silica T3 column (2.1 mm, 350 mm, 1.8 mm) (Waters), as described by O'Reilly et al., 2016.
2.7. **Visual background adaptation assay**

Visual background adaptation (VBA) is a well-characterized GR-dependent neuroendocrine response that causes zebrafish skin pigment cells to shrink when exposed to bright illumination. This is because it is a natural defence strategy against predators to camouflage themselves according to the colour of the background (Kramer *et al.*, 2001; Kurrasch *et al.*, 2009). For this reason, since *grsh543/sh543* mutants were indistinguishable from siblings, a quick way to identify their genotype was to assess their VBA response. To achieve that, clutches of *grsh543/+* incrossed derived larvae were subjected to VBA assay at 5 dpf, as follows:

- keep petri dishes containing larvae in a dark environment for at least 60 minutes (covered with aluminium foil inside a Styrofoam box);
- expose them under bright, whole-field illumination, using a Leica CLS 150 Microscope Light Source Fibre Optic Lab from the top (maximum light intensity), mounted 50 cm above the petri dishes, with their lid removed, for 20 minutes.
- the plates are placed on a DVV ViewBoxes Light Box (turned on) used as white bright background. This allow to illuminate larvae with a strong bright light both from the top and the bottom.
- move the dishes on a white surface (A4 paper sheet)

Finally, larvae were sorted according to their VBA response by eye. The success of the experiment was assessed via genotyping, as described in section 2.2.5. These conditions are able to reliably trigger VBA response in zebrafish larvae at 5 dpf (Muto *et al.*, 2005; Hatamoto and Shingyoji, 2008).
2.8. Microscopy

2.8.1. Fluorescent stereo microscope

Live zebrafish embryos and *in situ* experiments related pictures were captured using a Leica M165FC fluorescent stereo microscope, equipped with a digital colour camera (DFC310FX) and a Leica external fluorescent light source (EL6000).

2.8.2. Phenotypic analysis of live embryos

Embryos were anesthetized via incubation in Tricaine methanesulfonate (MS-222), diluted 1:25 in the petri dish, and placed on a 2% methylcellulose (Sigma) drop, located on the inner part of a petri dish lid. All embryos were oriented with their anterior to the left side up, so that the lateral left sagittal plane was visible.

2.8.3. Analysis of whole-mount embryos

*In situ* hybridization-processed whole-mount embryos were mounted in 80% glycerol in a glass embryo-dish. Embryos were oriented either with their anterior to the left or dorsal side up, so that either the lateral left sagittal plane or the pituitary gland region were visible.
2.9. Image analysis

2.9.1. Quantifying \textit{phd3:eGFP}-related rightness

Pictures were acquired using Leica Application Suite version 4.9, which allowed to capture both bright-field and GFP-fluorescent images. To quantify the \textit{phd3:eGFP}-related brightness of live embryos derived from each incrossed mutant line used in this project, I used Fiji (Image J) software v.2.0.0. This software allows to measure the mean grey value of an image, by converting it in a 8-bit format and subsequently by summing the grey values of all the pixels in the selected area, divided by the number of pixels. By default, since value = 0 is assigned to black and value = 255 to white, the quantified mean grey values are proportional to the intensity of the \textit{eGFP}-related brightness present in the embryos. In particular, head, liver and tail (from the anus to the caudal peduncle) (\textbf{figure 2.1}) related brightness were selected and measured in all the mutant lines used in this project.

\textbf{Figure 2.1} Representative picture of the head, liver and tail areas selected in each embryo to quantify the \textit{phd3:eGFP}-related brightness via mean grey value quantification. The embryo shown in the picture above is 5 dpf \textit{vhl} mutant.
2.10. Statistical analysis

GraphPad Prism version 8.4.0 for MacOS (GraphPad Software, La Jolla, California, USA, [www.graphpad.com](http://www.graphpad.com)) was used to perform statistical analysis on all the samples analysed. D’Agostino and Pearson’s normality test was initially run on all datasets prior to analysis. Unpaired t test was used to check the presence of significant differences between two normally distributed sample groups (i.e. for cortisol quantification). Vice versa, not normally distributed populations were analysed using the non-parametric Kruskal-Wallis test. For the comparison of means of more than two sample groups, One- or Two-way ANOVA method followed by Sidak’s post hoc test was used. In particular one-way ANOVA was used for assessing mean grey values data quantification, whereas two-way ANOVA was used to evaluate RTqPCR data. As post-hoc correction tests, Sidak’s method for multiple comparisons was used on normally distributed populations, whereas Dunn’s correction was used for not normally distributed populations.
3. GENERATION AND CHARACTERIZATION OF ZEBRAFISH MUTANT LINES

3.1. Introduction

In the last decades in vitro studies have highlighted the presence of a crosstalk between hypoxia inducible factor- and GC- signalling pathways (Kodama et al., 2003; Leonard et al., 2005; Wagner et al., 2008; Zhang et al., 2015, 2016). However, how this interplay precisely works in vivo and which is the molecular mechanism behind it are questions that still remain unanswered.

In this project the comprehension of the molecular mechanisms underlying the interaction between GC and HIF signalling has been initially tackled in vivo through the modulation of these two pathways via a “gain-of-function/loss-of-function” approach.

In particular, both a genetic and pharmacological method have been utilised to alter these two pathways during the first 120 hours post fertilisation of zebrafish embryos. On the one hand, CRISPR/Cas9-based mutagenesis has been used as gene editing tool to generate both hif1β<sup>sh544</sup> (arnt1) and gr<sup>sh543</sup> (nr3c1) mutant lines into an already existing vhl<sup>h2117</sup>+;phd3:eGFP<sup>i144/i144</sup> hypoxia reporter line (Santhakumar et al., 2012). On the other hand, Betamethasone 17,21-dipropionate (BME, Sigma Aldrich), a synthetic GR agonist, has been used as pharmacological tool to upregulate HIF signalling pathway in zebrafish larvae (Vettori et al., 2017).

The zebrafish is an exceptional genetic vertebrate model system for endocrine studies, because analogously to humans, they are diurnal and use cortisol as the main GC hormone (Weger et al., 2016). Additionally, both the human GC and HIF pathways
components are also present in zebrafish. Consequently, they are informative and genetically modifiable organisms to study both hypoxia and HIF signalling *in vivo* (van Rooijen *et al.*, 2011; Santhakumar *et al.*, 2012; Elks *et al.*, 2015).

In this chapter, the generation and characterisation of the mutant lines used in the present study will be described in detail. Finally, thanks to the presence of the aforementioned *phd3:eGFP*<sup>i144/i144</sup> hypoxia reporter, it has been possible to outline not only the differences between mutants and wild types, but most importantly to better understand how the interplay between HIF and GC signalling occurs *in vivo*. 
3.2. Generation and characterization of \textit{arnt1} and \textit{arnt1;vhl} knockout in zebrafish

The first questions I was keen to answer were the following: how does crosstalk between hypoxia-dependent signals and GC-mediated regulation of gene expression occur? If Hif and GCs are able to act on each other, how do they fine-tune the cellular pathways mediating adaptation to hypoxic environment? Are GCs contributing to Hif functions? Is Hif contributing to GC functions as well? Is GR an obligatory factor for the Hif response or vice versa?

I believed that answers to these questions could be provided through the creation and the analysis of \textit{gr} and \textit{hif1β} mutant lines. To this purpose, to enable an effective downregulation of the HIF pathway I chose to create a \textit{hif1β} mutant to study the effect of HIF on GC signalling. In this regard, knocking out HIF-α was considered impracticable due the presence of 6 isoforms (\textit{hif-1A,B}, \textit{hif-2A,B} and \textit{hif-3A,B}) (Köblitz \textit{et al.}, 2015).

Hif-1β (hypoxia-inducible factor 1 beta, or Arnt1) is a basic helix-loop-helix-PAS protein which translocates from the cytosol to the nucleus after ligand binding to the Hif-α subunit, following the stabilization of the latter in the cytoplasm. It represents the most downstream protein in the HIF pathway and for this reason it is the most suitable target to efficiently repress it. In particular, zebrafish have two Arnt1 homologues named Arnt1a and Arnt1b, with the shorter one (Arnt1a) apparently non-functional as demonstrated in a previous \textit{in vitro} study (Prasch \textit{et al.}, 2006).

For this reason, I chose to target the \textit{arnt1b} gene at the level of the exon 5, because of its favourable length, its genomic location and the presence of ideal restriction sites for the gRNA make-up. An additional important advantage is that
alternative start codons cannot rescue that mutated allele. Furthermore, since it codes for the DNA binding domain (DBD), a mutation in that region was predicted to block the ability of the protein to act as a transcription factor. In particular, a 7 bp insertion (figure 3.1) was obtained using CRISPR/Cas9 based mutagenesis in vhl;phd3:eGFP heterozygote embryos. The resulting frameshift mutation (allele name sh544) was expected to cause a premature stop codon at the level of the DNA-binding domain, which would result in a severely truncated protein. Consequently, I predicted to observe a strong downregulation of the HIF reporter related brightness in 5 dpf mutant larvae.

The phd3:eGFP hypoxia reporter line was previously generated in my laboratory via BAC transgenesis. The phd3 locus plus >30 kb 3’ and a 5’ flanking sequence was inserted into a Bacterial Artificial Chromosome (BAC) and recombinereed to replace the translation initiation codon (ATG) of phd3 itself with a eGFP reporter construct (Santhakumar et al., 2012). The already existing vhl^hu2117/+ line (van Rooijen et al., 2009) was crossed into the Tg(phd3:eGFP)^i144/i144 line in order to create the aforementioned vhl^hu2117/+;phd3:eGFP^i144/i144 fish. In this work, the resulting hif1β^sh544/+;vhl^hu2117/+;phd3:eGFP^i144/i144 line will be called arnt1+/-;vhl+/-, whereas the vhl^hu2117/+;phd3:eGFP^i144/i144 line will be called vhl+/- hereafter.

Figure 3.1. Schematic representation of zebrafish hif1β (arnt1) gene. Exons are shown as black boxes, whereas introns as lines. The red arrowhead shows the position of a 7 bp insertion in exon 5 (encoding the bHLH DNA binding domain). In the arnt1 wt and mutant sequence: CRISPR target site: bold. Protospacer-adjacent-motif (PAM) sequence: red. Underlined nucleotide sequence: bp inserted.
Initial analysis performed on arnt1+/−;vhl+/− incross-derived 5 dpf larvae (F1 generation) confirmed the suppressive effect that arnt1 mutation was expected to have on the HIF signalling in vhl mutants. As predicted, arnt1+/−;vhl−/− larvae showed a substantially attenuated vhl phenotype, characterized by a reduced phd3:eGFP related brightness, especially at the hepatic level, coupled to the absence of pericardial oedema, excessive caudal vasculature and improved yolk usage compared to vhl−/− larvae (figure 3.2A). In particular, this was quantified as a 39% downregulation (P<0.0017) at the level of the head, a 75% downregulation (P<0.0001) in liver and a 58% downregulation (P<0.0001) in the rest of the body (from the anus to the caudal peduncle), in terms of phd3:eGFP-related brightness, compared to vhl−/− larvae (figure 3.2B). Of note, the HIF-reporter brightness observed in the double mutants was still slightly higher than the wildtype’s one (figure 4.7B compared to D). Finally, arnt1+/− ;vhl+/− larvae (siblings) were identical to wildtypes in terms of both phenotype and hypoxia reporter expression levels.
**Figure 3.2.** A. Magnified pictures of a representative 5 dpf vhl\(^{-}\) larva compared to 5 dpf arnt1\(^{-}\);vhl\(^{-}\). Among the 120 GFP\(^{+}\) embryos derived from arnt1\(^{-}\);vhl\(^{-}\) (phd3:eGFP) x arnt1\(^{-}\);vhl\(^{-}\) (phd3:eGFP), 15 larvae were characterized by the absence of pericardial oedema, no ectopic extra vasculature at the level of the tail, no bright liver and a reduced brightness in the rest of the body (black and white arrowheads). Genotyping post phenotypic analysis on sorted larvae confirmed genotype-phenotype correlation. Fluorescence, exposure = 2 seconds. Scale bar 200 µm. B. Statistical analysis performed on mean gray value quantification (fluorescence, exposure = 991.4 seconds) at the level of the head, liver and tail, after phenotypic analysis on the aforementioned larvae (n=245). vhl\(^{-}\) n=10 larvae: head 93.10 ± 2.33 (mean ± s.e.m); liver 99.64 ± 3.49 (mean ± s.e.m); tail 29.57 ± 0.73 (mean ± s.e.m). arnt1\(^{-}\);vhl\(^{-}\) n=10 larvae: head 56.49 ± 3.36 (mean ± s.e.m); liver 24.69 ± 2.35 (mean ± s.e.m); tail 12.39 ± 0.75 (mean ± s.e.m). Unpaired t-test (*P < 0.05; **P < 0.01; ***P<0.001; ****P < 0.0001).
Homozygous vhl mutants are lethal by 8-10 dpf (van Rooijen et al., 2009). To analyse the efficacy of arnt1 mutation in rescuing the vhl phenotype over time, I attempted to raise arnt1\(^{+/−}\);vhl\(^{−/−}\) after day 5 post fertilization. Notably, double mutants survived beyond 15 dpf, but failed to grow and thrive when compared to wildtypes, which led us to euthanise them due to health concerns at 26 dpf (Figure 3.3). Arnt1 homozygote mutants, both in a vhl\(^{−/−}\) or wild-type background, are instead morphologically indistinct from wildtypes, and adults are viable and fertile. In contrast, the previously published arnt2\(^{−/−}\) zebrafish larvae were embryonic lethal around 216 hpf (Hill et al., 2009).

Figure 3.3. Kaplan-Meier survival curves of the zebrafish arnt1\(^{+/−}\); vhl\(^{−/−}\)(phd3:eGFP) line analysed in this study. Time is shown in days. Siblings n = 30; arnt1\(^{+/−}\); vhl\(^{−/−}\)(phd3:eGFP) n = 8. The Log-rank (Mantel-Cox) test was used for statistical analysis. arnt1\(^{+/−}\); vhl\(^{−/−}\)(phd3:eGFP) vs. siblings: **P < 0.0027.
Phenotypic analysis performed both on 26 dpf $\text{arnt}1^{+/+};\text{vhl}^{+/+}$ and wildtype siblings showed the presence of substantial morphological differences between these two groups (figure 3.4). A common strategy to determine fish maturation is the measurement of the standard body length (SL), assessed from the tip of the snout to the base of the tail (caudal peduncle). Moreover, since fish size may be influenced by genetics and also environmental factors, the combination of standard body length with the evaluation of the maturation of external traits is currently the most reliable measure of fish maturation (Singleman and Holtzman, 2014).

Double mutant body length (SL = 5.05 mm) was comparable to the one of a 13-15 dpf wild-type larva, as reported by Parichy et al. 2009 work (Parichy et al., 2009), but not to its corresponding developmental stage traits. In particular, 26 dpf $\text{arnt}1^{+/+};\text{vhl}^{+/+}$ larvae showed a clear body compared to a fully pigmented wild-type one. In addition, it was characterized by a larval-like anteriorly protruding open mouth and a clearly visible gut beneath the swim bladder, which are typical of a 4.9 mm total body length larva (13-15 dpf) (Kimmel et al., 1995). An early larval pigment pattern with melanophores in stripes over the dorsal and ventral myotomes (no longer dispersed over the yolk sac) was also observable. Interestingly, even if the presence of food in the final tract of the intestine is a clear evidence of active feeding, the reduced body length and the absence of properly developed traits, are all distinct signs of a considerable developmental delay.

In addition, the head did not exhibit the dorsal indentation at the level of the pineal gland, which normally appear in 5.7 mm SL larvae. An increasingly pronounced caudal fin condensation, coupled to the presence of the first fin rays, which typically appear by ~4.9 mm SL were visible. On the other hand, the slight bulge in the dorsal fin fold (where the dorsal fin condensation usually arises), as well as the one on the
ventral side were not yet apparent. Finally, the distal end of the larval fin fold was still relatively rounded over the developing caudal fin which resembled, once again, the traits of a 13-15 dpf larva. Vice versa, the body length of the 26 dpf arnt1+/--;vhl−/− sibling (SL = 12.22 mm) matched the standards of the corresponding wild-type larvae and no particular phenotypic traits were observed in them (Parichy et al., 2009) (figure 3.4).

Consequently, these results suggest that arnt1 mutation is able to partially rescue the Vhl phenotype and to triple the lifespan of vhl mutant larvae. However, since arnt1+/--;vhl−/− did not show fully rescued traits, I speculate that this could be due to the presence of other Hif1β isoforms (mainly arnt2) that could still interact with HIF-α subunits. In support of this, arnt1+/--;vhl−/− showed a slightly higher phd3:eGFP expression than the wildtypes.

Since double mutants’ head was the brightest region in the body (in terms of phd3:eGFP-related brightness), both in 5 dpf and 26 dpf double mutant larvae (figure 3.2A & 3.4), this strengthened the hypothesis that mainly Arnt2 compensates for the lack of Arnt1 within the CNS to mediate the hypoxic response (Michaud et al., 2000; Hill et al., 2009). On the other hand, arnt1+/--;vhl−/− liver was the organ with the lowest phd3:eGFP related brightness observed. This phenotypic analysis confirmed that zArnt1 is particularly expressed in organs outside the central nervous system and predominantly in the liver of zebrafish larvae (Hill et al., 2009). This is also broadly consistent with mouse data (Jain et al., 1998).

As a consequence of the above considerations, since my data show that HIF pathway could still be partially activated in 5 dpf arnt1+/--;vhl−/− larvae, I set up to test whether this was due to the presence of other arnt isoforms. To this end, I took advantage of a novel and rapid CRISPR/Cas9-based technology for directed gene
knock-out, initially developed by Burger et al., 2016 and improved by Wu et al. 2018, as described in the chapter below.

Figure 3.4. Representative picture of a 26 dpf zebrafish arnt1+/; vhl+/ (phd3:eGFP) (top image, SL= 12.22 mm) and a arnt1−/−; vhl−/− (phd3:eGFP) (middle and bottom image, SL= 5.05 mm) larva analysed in this study. Scale bar: 1 mm. Fluorescence, exposure 2 sec.
3.3. **Arnt1 and Arnt2 are mutually involved in assuring HIF response in zebrafish**

As arnt1;vhl double mutants still moderately activate the phd3:eGFP HIF reporter, this chapter will be mainly focused on clarifying the differential expression pattern of Arnt1 and Arnt2 isoforms in the zebrafish and their relative importance in the HIF signalling.

The aryl hydrocarbon receptor nuclear translocator (ARNT) is a member of the bHLH/PAS protein super-family. It can dimerize with several PAS superfamily members, which mainly includes members of the hypoxia inducible factors family to assure adaptation to hypoxic conditions.

Both in mammals and in zebrafish ARNT is coded by arnt1 and arnt2 (Li, Dong and Whitlock, 1994; Drutel et al., 1996; Hirose et al., 1996; Prasch et al., 2006). However, even if both genes are expressed in almost all the tissues during zebrafish embryonic development, tissue distribution of arnt2 seems to be more limited than arnt1 (Hill et al., 2009).

Arnt2 has been the first Arnt isoform to be identified (Tanguay et al., 2000; Wang et al., 2000; Prasch et al., 2006) and its mutation has been shown to be embryonic lethal in zebrafish larvae around 216 hpf (Hill et al., 2009). Previous work also showed that Arnt2 isoform is predominantly expressed at the level of the brain, heart, liver and vascular endothelium in mice (Andreasen et al., 2002). However, the function of Arnt2 still remains poorly understood. On the other hand, Arnt1 is primarily expressed in organ outside the central nervous system and is thought to be required for normal liver development in mice (Jain et al., 1998; Walisser, Bunger, Glover and Bradfield, 2004; Walisser, Bunger, Glover, Harstad, et al., 2004). In addition, there are no indications in
the literature about the spatial and temporal expression patterns of the zebrafish *arnt1* isoform.

To provide further insights about it, I compared the gene expression of both isoforms by knocking out one of them and by observing the corresponding phenotypic outcomes and the relative *phd3:eGFP*-related brightness of the other one. To this aim, I exploited the novel and rapid Wu et al 2018 mutagenesis protocol to generate G0 CRISPants. Phenotypic analysis of Arnt2 CRISPant larvae created both in a *vhl*<sup>+/−</sup> and *arnt1*<sup>+/−;vhl</sup> background, was carried out on 5 dpf larvae using a Leica M165FC fluorescent stereo microscope.

By comparing the phenotype of *arnt1*<sup>+/−;vhl</sup> with the one of 4x *arnt2* gRNAs CRISPR-injected *vhl*<sup>−/−</sup> (that will be called *arnt2*<sup>−/−;vhl</sup> hereafter), it was possible to show the presence of similarities between these two lines. In particular, the absence of both pericardial oedema and of excessive caudal vasculature, coupled to improved yolk usage and the presence of an inflated swim bladder were all noticeable traits in both double mutants at 5 dpf. Furthermore, *arnt1*<sup>−/−;arnt2*<sup>−/−;vhl</sup> CRISPants (triple mutants) exhibited an even more rescued *vhl* phenotype, which strongly resembled wild-type larvae (figure 3.5A).

Interestingly, by analysing and quantifying the expression of the *phd3:eGFP* transgene, it was possible to appreciate that *arnt2* CRISPR injected *vhl* mutants were characterized by a significant downregulation of the HIF reporter-related brightness at the level of the head (equals to 53%, P<0.0001), in the liver (equals to 54%, P<0.0001) and in the rest of the body (equals to 46%, P<0.0001), compared to uninjected *vhl* mutant larvae (figure 3.5A & B). On the other hand, *arnt1*<sup>+/−;vhl</sup> showed a weaker *phd3:eGFP* downregulation in the head (equals to 39%, P<0.0017), coupled to a stronger one both at the hepatic level (equal to 75%, P<0.0001) and in the
rest of the body (equals to 58% downregulation, P<0.0001), compared to vhl−/− larvae. Furthermore, when both arnt1 and arnt2 isoforms were simultaneously knocked-out, the downregulation of the hypoxia reporter was even stronger at the level of the head (equals to 74%, P<0.0001), liver (equals to 86%, P<0.0001) and in the rest of the body (equals to 83%, P<0.0001) compared to vhl−/− larvae (figure 3.5A & B). Of note, the phd3:eGFP-related brightness in the triple mutants (arnt2 CRISPR injected arnt1−/−; vhl−/−) was similar to the one observed in wildtypes, at the level of the liver, the gut and the tail (figure 3.5A, white arrowheads).

Overall, these data allow me to better understand the partial redundancy of these two isoforms in zebrafish larvae. My data allowed also to confirm that even if Arnt1 is not fundamental for survival, it is the main Arnt isoform expressed at the hepatic level, whereas Arnt2 is more expressed in the developing central nervous system (CNS), as supported by (Hill et al., 2009). Finally, since both isoforms can form a functional complex with HIF-α and both appear to function in the same organs, this demonstrates that they have partially overlapping functions in vivo and they synergistically contribute to the HIF signalling pathway.
Figure 3.5. A Representative picture of 5 dpf CRISPANT mutants created by redundantly targeting arnt2 gene via co-injection of 4x gRNAs in arnt1+/vhl+/+ (phd3:eGFP) x arnt1+/vhl+/+ (phd3:eGFP) derived embryos (n=300). Uninjected embryos were used as control (n=120). Fluorescence, exposure = 991.4 ms. Scale bar 500 µm. B. Statistical analysis performed on mean grey values quantification (at the level of the head, liver and tail), after phenotypic analysis on 5 dpf arnt2 4x gRNAs injected and uninjected larvae. vhl+/+ uninjected n = 8 larvae: head 93.1 ± 2.33 (mean ± s.e.m); liver 99.65 ± 3.49 (mean ± s.e.m); tail 29.58 ± 0.73 (mean ± s.e.m). arnt1+/vhl+/+ uninjected n = 10 larvae: head 56.49 ± 3.36 (mean ± s.e.m); liver 24.7 ± 2.36 (mean ± s.e.m); tail 12.39 ± 0.75 (mean ± s.e.m). vhl+/+ injected n = 12 larvae: head 43.69 ± 3.25 (mean ± s.e.m); liver 45.54 ± 4.57 (mean ± s.e.m); tail 16.09 ± 1.37 (mean ± s.e.m). arnt1+/vhl+/+ injected n = 11 larvae: head 24.66 ± 1.63 (mean ± s.e.m); liver 13.88 ± 0.66 (mean ± s.e.m); tail 5.16 ± 0.33 (mean ± s.e.m). Ordinary One-way ANOVA followed by Sidak’s multiple comparison test (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).
3.4. Generation and characterisation of \textit{gr} and \textit{gr;vhl} knockout

in zebrafish

3.4.1. Generation and characterisation of zebrafish \textit{grsh551/v;vhlhu2117/+} line

Previous chemical screens carried out in my laboratory allowed the identification of GCs as potent activators of the HIF pathway (Santhakumar \textit{et al.}, 2012; Vettori \textit{et al.}, 2017). However, it still remains uncertain how GCs contribute to the HIF functions, if the GR is an obligatory factor for assuring the HIF response or vice versa, and how the interaction between these two major pathways occurs \textit{in vivo}. For these reasons, a novel zebrafish \textit{grsh551} mutant line was initially created in my laboratory.

The \textit{grsh551/v;vhlhu2117/+} line was generated by Eleanor Markham (van Eeden lab, Bateson Centre, TUoS) in a \textit{phd3:eGFP;vhl+/−} background, using CRISPR/Cas9-based mutagenesis. It was characterized by a 1bp deletion located at the beginning of exon 2 (first coding exon) which codes for the N-terminal domain (NTD). The injected embryos were partly genotyped to test CRISPR efficiency and partly raised till adulthood. Once fertile, this fish line was outcrossed with a wildtype AB fish, to test the presence of carriers for the mutated \textit{gr} gene. To this end, I initially performed PCR-based genotyping on live adult zebrafish tail biopsies. The identified cluster of carriers (10 males and 9 females) was then incrossed and the resulting progeny was genotyped at 120 hpf, in order to raise the F1 generation. Finally, phenotypic analysis was carried out on F1 incrossed-derived embryos by using a fluorescent stereo microscope both on 3 dpf and 5 dpf larvae. Results showed that despite the presence of the \textit{phd3:eGFP;vhl+/−} background, no significant \textit{gr}-related phenotypes were detected in those larvae.
Since previous experiments performed in my lab showed that the strongest GC effect in terms of hypoxia reporter activation occurred at the hepatic level, I tried to sort larvae according to the phd3:eGFP related brightness in the whole body and, most of all, in the liver. To this end, I predicted to observe reduced phd3:eGFP expression in gr<sup>esh551/sh551</sup>;vhl<sup>hu2117/2117</sup> compared to vhl<sup>hu2117/2117</sup> larvae. However, genotyping results showed that there was no clear correlation between reduction in phd3:eGFP expression levels and having a mutant phenotype for gr<sup>esh551</sup>.

To better test this mutant line, I performed a whole-mount Gr antibody staining on gr<sup>esh551/+</sup> incrossed 100 μM Betamethasone 17,21-dipropionate (BME, Sigma Aldrich) treated and untreated (DMSO, vehicle compound) embryos at 1 dpf. A clutch of both BME treated and untreated samples, with no primary antibody added, were also used as negative controls. BME treatment was used because, being a GR agonist, it might enhance the difference between gr<sup>-/-</sup> and wild-types. As a consequence of that, I would have expected to observe 25% of antibody stained embryos (putative gr mutants) with a loss of staining. However, no differences were detectable in all the clutches.

Whole-embryo genotyping analysis was also repeated on 5 dpf larvae sorted according to their brightness at the level of the liver. These larvae came from the same clutch of embryos that were antibody stained. I chose this developmental stage in order to exclude any possible maternal cortisol and gr transcripts contribution. Once again, no genotype-phenotype correlation was present (figure 3.6).
Figure 3.6. A. Whole-embryo genotyping analysis on gr^{ab551} (phd3:eGFP;vhl^{+/-}) larvae sorted according to their phd3:eGFP brightness at 5 dpf, deriving from the same clutch of embryos that were antibody-stained (n = 15). Samples A1-A6 showed weak phd3:eGFP brightness; samples A8-B4 showed strong phd3:eGFP brightness.

B. Genotyping results on GFP sorted gr^{ab551} (phd3:eGFP;vhl^{+/-}) 5 dpf larvae (n = 40). A1-B7: weak phd3:eGFP brightness; B8-C12 showed strong phd3:eGFP brightness. I have selected larvae which were not vhl mutants, sorting the ones that were brighter and the ones that were less bright among the weak ones. I did that in the expectation that if I had gr loss-of-function mutants, they might end up in the least bright group. Indeed, since previous work showed that GCs are able to activate the phd3:eGFP hypoxia reporter, I could expect that phd3:eGFP levels will be reduced in gr mutants.

C. Whole-embryo genotyping analysis on GR antibody stained 1dpf embryos. Embryos have been sorted according to their phd3:eGFP related brightness (n = 9). A1-A4: weak phd3:eGFP. A5-A9: strong phd3:eGFP.

In all cases shown here (A, B, C) the results did not show a clear genotype-phenotype correlation.
To further check whether this fish line carried a true null allele for \textit{gr}, I also performed whole mount \textit{in situ} hybridization (WISH) on DMSO and BME [30 μM] treated 5 dpf \textit{gr}^{h551/+} incross derived larvae, using pro-opiomelanocortin a (\textit{pomca}) as probe. I chose it because it plays a pivotal role at the level of the pituitary gland in the regulation of the GC-mediated stress response. For this reason, it provides a reliable readout of Gr function in zebrafish larvae (Griffiths \textit{et al.}, 2012; Ziv \textit{et al.}, 2012).

The administration of a synthetic \textit{gr} agonist is supposed to elicit a strong GC response, which in turn triggers the so called “GC-GR mediated negative feedback loop” aimed to shut down their own biosynthesis. Notably, this mainly occurs at the level of the pituitary gland by downregulating the expression of \textit{pomca} gene. This is because the stress-mediated activation of the HPI axis helps the organism to quickly cope with stressful situations and to allow adaptation aimed to restore homeostasis (de Kloet, Joëls and Holsboer, 2005). On the other hand, chronic HPI axis/\textit{pomca} activation would generate constantly elevated GC levels, which would be detrimental to the organism (Laryea \textit{et al.}, 2015).

In this regard, if Gr is not functional, the GC-GR negative feedback loop cannot occur and \textit{pomca} expression cannot be downregulated (Griffiths \textit{et al.}, 2012), resulting in an uncontrolled cortisol biosynthesis. Importantly, \textit{in situ} hybridization analysis on 5 dpf \textit{gr}^{h551/+} incross derived larvae revealed that both BME-treated \textit{gr} wildtypes and mutants had reduced levels of \textit{pomca} transcripts at the level of the anterior part of the pituitary gland (black arrowheads). This allowed to show that the existing \textit{gr}^{h551} was not a strong loss of function allele, as true null mutants should not be able to respond to BME treatment and should show upregulated \textit{pomca} expression at the level of the anterior part of the pituitary gland (\textbf{figure 3.7}).
Figure 3.7. Representative pictures of WISH performed on DMSO and BME [30 µM] treated gr<sup>sh551</sup>-;<sup>vhl</sup><sup>+</sup>/-(phd3:eGFP) mutant line, at 5 dpf, using pomca as probe. gr siblings DMSO treated (n= 20/20 larvae) showed normal pomca expression; gr siblings BME treated (n= 20/20 larvae) showed downregulated pomca expression after BME treatment. On the other hand, DMSO treated (n= 18/20) gr<sup>−/−</sup> larvae showed upregulated pomca expression, whereas BME treated (n= 20/20) gr<sup>−/−</sup> larvae showed downregulated expression. As a consequence of that, since gr<sup>sh551</sup> mutants were still able to activate the GC-GR negative feedback loop and to downregulate the pomca expression at the level of the anterior part of the pituitary gland, I showed that this mutant line was not a true null. Anterior part of the pituitary gland (black arrowhead). Scale bar 50 µm.

Finally, although the N-terminal transactivation domain (NTD) is conserved, literature reviews and sequence alignments of human, monkey, rat and mouse GRs revealed that there are other 8 conserved AUG start codons in the exon 2. In human, these were shown to produce various GR isoforms with progressively shorter N-terminal transactivation domain (NTD) (figure 3.8). These are formed due to the presence of alternative Kozak translation initiation sequences which can cause either ribosomal shunting or ribosomal leaky scanning mechanisms. This allow the generation of different GR subtypes with truncated N-termini (Yudt and Cidlowski,
2001, 2002; Oakley and Cidlowski, 2011; Merkulov and Merkulova, 2012; Rafacho et al., 2014). As a consequence of the above considerations, even if the alternative Kozak translation initiation sequences have not been identified in zebrafish, it is plausible to speculate that these isoforms are also present in teleosts (Kino et al., 2017). For this reason, I speculate that these are still functional in gr<sup>rh551</sup> mutant larvae and are able to compensate for the induced mutation. As a consequence of the above considerations, it was clear that a mutation in a different exon of gr gene was warranted.

Figure 3.8 Representative picture of the hGR translational isoforms. Eight GR isoforms with progressively shorter N-terminal domains are produced as a consequence of the presence of eight different AUG start codons at the level of exon 2 in the GRα mRNA (location of the 8 AUGs is indicated by asterisks). The initiator methionine in each isoform (indicated by numbers) and the AF1 region (located at 77-262aa) are for the human GRα protein. (modified from R. Oakley, 2013)
3.4.2. Generation and characterisation of zebrafish $gr^{sh543/+}$ line

Since the $gr^{sh551}$ mutant line proved to be not a null and the already existing $gr^{s357}$ allele may still retain some activity via non-genomic pathways or tethering, promoting HIF activation upon GC treatment (Griffiths et al., 2012; Ziv et al., 2012; Vettori et al., 2017), I generated a novel $gr$ mutant line, as described below.

Of note, true null $gr$ mutants has been proven to be hypercortisolaemic (Facchinello et al., 2017; Faught and Vijayan, 2018b), due to the inability of GC to bind to their functional receptor (Gr). As a result, they fail to provide a negative feedback at the pituitary level and to consequently shut down their own biosynthesis (Facchinello et al., 2017; Faught and Vijayan, 2018b).

In this regard, by exploiting the CRISPR/Cas9 method, I generated a zebrafish line characterized by an 11 bp deletion at the level of $gr$ exon 3. The latter was predicted to have a truncated DNA binding domain, to lack the C-terminal ligand binding domain and for these reasons to be a true null (figure 3.9).

![Figure 3.9](image_url)

**Figure 3.9. Schematic representation of zebrafish $gr$ (nr3c1) gene.** Exons are shown as boxes, introns as lines. The red arrowhead shows the position of a -11 bp deletion in exon 3 (encoding the DNA binding domain). In the $gr$ wt and mutant sequence the CRISPR target site is bolded and the protospacer-adjacent-motif (PAM) sequence is denoted in red. Dotted line: bp deleted.
To confirm the efficiency of this mutation, I initially incrossed *gr* heterozygous fish and I treated the resulting clutch at 4 dpf for 24 hours with DMSO and BME [30 \( \mu \text{M} \)], respectively. Phenotypic analysis carried out on 5 dpf larvae revealed that vehicle treated *gr* mutants were morphologically similar to wild types, except for the presence of a reduced yolk usage and a flattened swim bladder (figure 3.10A, right column). As expected, synthetic GC treatment was ineffective on *gr*\(-/-\) larvae which showed only a mild pericardial oedema (figure 3.10B, right column). On the other hand, DMSO treated *gr*\(+/-\) and *gr*\(+/+\) larvae looked normal (figure 3.10A, left column), whereas betamethasone treatment was able to cause a more severe phenotype to them. In particular, it was possible to observe both an acute pericardial and periorbital oedema, coupled to a reduction of yolk usage and a not completely inflated swim bladder, compared to DMSO treated siblings (figure 3.10B, left column).
Figure 3.10 A. Representative pictures of DMSO treated 5 dpf gr<sup>sh543/+</sup> incross derived larvae (n = 120). Phenotypic analysis carried out at 5 dpf allowed to show the presence of a reduced yolk usage and a flattened swim bladder in DMSO treated gr mutants compared to wild types.

B. Representative pictures of BME [30 µM] treated 5 dpf gr<sup>sh543/+</sup> incross derived larvae (n = 120). Phenotypic analysis carried out at 5 dpf showed the presence of severe pericardial and periorbital oedemas in gr siblings as a consequence of BME treatment, which was instead negligible in gr mutants (black arrowheads: yolk usage).
To further confirm the gr loss-of-function, I repeated the same fish cross and I subjected gr larvae to visual background adaptation (VBA) test. Since VBA is a Gr-dependent neuroendocrine response, it can be exploited to check the presence of impairments related to GC biosynthesis and action (Griffiths et al., 2012; Muto et al., 2013).

Larvae derived from gr+/− incrossed fish were pre-sorted via VBA test according to dorsal melanophores size at 4 dpf, and finally sorted again and photographed at 5 dpf. As illustrated in the figure below, statistical analysis performed on melanophores size quantification at the level of the head, highlighted the presence of statistically significant differences between wild types (VBA+, 0.0009 mm², P=0.0123), and heterozygous (VBA+, 0.0015 mm², P<0.0001) and mutants’ melanophores (VBA−, 0.0028 mm², P<0.0001) (figure 3.11A). In particular, only melanophores on the dorsal side of the head were used for the measurements and this region was always kept constant in order to improve the reliability of measurements. This is because the head is the main region containing the biggest melanophores and where VBA-induced changes are more evident. Moreover, since the head is wider than the tail, it is possible to precisely count all the melanocytes (even the partially overlapped ones) by observing both the cell membrane and the nucleus inside each melanophore, thanks to their darker colour.

To further demonstrate the presence of a true null allele, complementation test was also performed by crossing two gr heterozygous fish, which carried a different mutation at the level of the same exon (11 bp deletion and 1 bp insertion, respectively) and by analysing the resulting progeny via VBA test at 5 dpf. Importantly, a similar result was obtained for the trans-heterozygous incross-derived larvae, compared to the previously mentioned one. Indeed, a statistically significant difference was
quantified between wild-types (0.0008 mm$^2$), heterozygous (0.0013 mm$^2$) and mutant melanophores size (0.0021 mm$^2$) (figure 3.11B).

In both cases, PCR-based genotyping on negative VBA-response sorted samples revealed that most larvae were homozygous for the gr allele, whereas positive VBA-response sorted larvae were always gr siblings. Finally, since the same VBA responses were observed and quantified in trans-heterozygotes at 5 dpf, it confirms that the phenotype is due to a defect in GR and not due to mutation of a nearby gene.
Figure 3.11 A. VBA test performed on gr<sup>+/+</sup> incross-derived 5dpf larvae (n=240), followed by genotyping and statistical analysis. Statistical analysis was performed on melanocytes average size (expressed in mm<sup>2</sup>) of 5 dpf larvae post VBA test. Wildtype melanocytes size 0.0009 ± 0.0001 mm<sup>2</sup> (mean ± s.e.m, *P= 0.0123, n = 6 larvae), heterozygous melanocytes size 0.0015 ± 0.0001 mm<sup>2</sup> (mean ± s.e.m, ****P<0.0001, n=8 larvae) and mutant melanocytes size 0.0028 ± 0.0002 mm<sup>2</sup> (mean ± s.e.m, ****P<0.0001, n=5 larvae). One-way ANOVA followed by Sidak’s multiple comparisons test has been used for calculating significance (**P<0.05; **P<0.01; ***P<0.001; ****P<0.0001). Scale bar 200 µM.

B. VBA test performed on gr<sup>+/−</sup> (1 bp deletion) x gr<sup>+/−</sup> (11 bp insertion) derived 5 dpf larvae (n = 130), followed by genotyping and statistical analysis. Statistical analysis was performed on melanocytes average size (expressed in mm<sup>2</sup>) of 5 dpf larvae post VBA test. Wildtype melanocytes size 0.0008 ± 0.0001 mm<sup>2</sup> (mean ± s.e.m, *P= 0.0494, n=5 larvae), heterozygous melanocytes size 0.0013 ± 0.0001 mm<sup>2</sup> (mean ± s.e.m, ****P<0.0001, n=6 larvae) and mutant melanocytes size 0.0021 ± 0.0001 mm<sup>2</sup>(mean ± s.e.m, **P= 0.0017, n=5 larvae). One-way ANOVA followed by Sidak’s multiple comparisons test has been used for calculating significance (**P<0.05; **P<0.01; ***P<0.001; ****P<0.0001). Scale bar 200 µM.
To test the effectiveness of the mutation at a transcriptional level, I performed whole mount *in situ* hybridisation (WISH) on 5 dpf DMSO and BME treated *gr<sup>eh543/+</sup>* incross derived larvae, using *pomca* as probe.

As expected, results showed the presence of upregulated *pomca* expression in DMSO treated *gr<sup>-/-</sup>* larvae at the level of the anterior part of the pituitary gland, compared to DMSO treated *gr* wild-types (*figure 3.12, top and bottom left, black asterisks*). This is because the absence of a functional GR avoids the GC-GR negative feedback loop to occur. For this reason, it was also possible to observe the presence of unvaried (still upregulated) pituitary *pomca* levels in BME treated *gr<sup>-/-</sup>* larvae, compared to DMSO treated *gr<sup>-/-</sup>* ones.

Vice versa, in BME treated *gr* wild types thanks to the presence of functional Gr, the synthetic GC treatment was able to trigger the negative feedback loop. Consequently, it was possible to observe a strong downregulation of *pomca* in BME treated *gr* siblings, compared to BME treated *gr* mutant larvae (*figure 3.12, top and bottom left, black arrowheads*). As a consequence of the above considerations, the fact that *gr<sup>-/-</sup>* larvae were insensitive to GC and showed an unvaried upregulated *pomca* expression after synthetic GC treatment confirmed the presence of a true null *gr* allele in my *gr<sup>eh543</sup>* mutant line.
Figure 3.12. Representative pictures of WISH performed on DMSO and BME [30 µM] treated gr mutant line, at 5 dpf, using pomca as probe. Scale bar 100 µm. gr siblings DMSO treated (n = 30/30 larvae) showed normal expression (top left); BME treated gr siblings (n= 29/30 larvae) showed downregulated pomca levels (top right). On the other hand, both DMSO treated (n= 30/30) (bottom left) and BME treated (n= 30/30) (bottom right) gr−/− larvae showed upregulated pomca expression. Of note, the fact that BME treatment was not able to downregulate pomca expression in gr−/− allowed to confirm that these mutants were true nulls.
Lastly, Gr loss of function was also confirmed via RTqPCR analysis carried out in 5 dpf gr mutants. Once again, as expected, a strong downregulation of fkbp5 mRNA levels quantified in gr-/- larvae both in the presence (BME treated, fold change = 0.01; P<0.0001) and in the absence of synthetic GC treatment (DMSO treated, fold change=0.01; P<0.0001) (figure 3.13) corroborated this. On the other hand, both in the presence and in the absence of synthetic GR agonists no significant alterations were quantified in the expression levels of the other three GC target genes selected (pck1, il6st and lipca). This could be due to the fact that fkbp5 is a well-established readout of Gr activity (M. J M Schaaf, Chatzopoulou and Spaink, 2009), whereas the other aforementioned genes by not directly taking part of the GC-GR negative feedback loop, might have other inputs and developmental regulation as well.

In this regard, I set up to assess the expression levels of Fkbp5 (FK506 binding protein 51) as it plays a key role in the multiprotein Hsp90/Hsp70-based chaperone machinery which governs GR’s sensitivity. It is also critical for assuring GR’s correct folding, maturation, trafficking to the nucleus and DNA binding. Finally, both Fkbp5 mRNA and protein expression can be induced by Gr activation via intronic hormone response elements, which provides a super-short feedback loop for Gr-sensitivity (Binder, 2009). Consequently, this makes it the most reliable target gene to assess GR activity.

By contrast, the other aforementioned genes have been selected from previous zebrafish and mice studies (Le et al., 2005; Facchinello et al., 2017; Vettori et al., 2017) as reliable readouts for hepatic lipid metabolism (lipca), inflammation (il6st) and glucose metabolism (pck1). So, they were supposed to provide a general overview of the changes that take place in an organism to adapt to hypoxic conditions. However, unlike fkbp5 is expressed at early developmental stages, the endogenous cortisol-GR
mediated transcriptional regulation in response to stressors starts only at 96 hpf in zebrafish (Alsop and Vijayan, 2008; Nesan et al., 2012; De Marco et al., 2013; Weger et al., 2018). For this reason, since these developmental changes occur during the synthetic GC treatment step (at 4 dpf), it is plausible to infer that the cortisol-induced expression of these specific target genes was not yet responsive.

Figure 3.13. RTqPCR analysis performed on four GC target genes expression (fkbp5, il6st, pck1 and lipca) has been carried out on both DMSO and BME [30 µM] treated gr−/− and siblings at 5 dpf, (n = 10 larvae, per group, in triplicate). Statistical analysis, based on ΔΔCt values, was performed using Ordinary Two-way ANOVA followed by Dunnnett’s multiple comparison test. Data in the graph are instead shown as fold change values to facilitate understanding (*P < 0.05; **P < 0.01; ***P <0.001; ****P < 0.0001). Blue bars: wild-types and/or heterozygotes (siblings); red bars: mutants.
Observational analysis carried out during pair-mating on \( gr^- \) adult fish also revealed the absence of distinctive chromatic and sexual traits which make them difficult to identify by gender. However, the typical body shape of a WT male zebrafish characterized by a slender body with its characteristic pinkish cast was not discernible in adult \( gr^- \) fish, which looked more feminine and with a darker blue tint compared to wild-types.

Finally, due to the absence both of a functional glucocorticoid receptor and of a \( gr \) mediated negative feedback loop, \( gr^- \) adult fish were not able to properly cope with stress, as previously described in the literature (Griffiths et al., 2012; Ziv et al., 2012; Facchinello et al., 2017).
3.5. Discussion

The main focus of this chapter has been the generation and characterisation of novel zebrafish mutant lines aimed to investigate how the interaction between hypoxic and GC signalling occurs \textit{in vivo}. To date, this scientific question has been primarily tackled through the use of \textit{in vitro} studies, where interactions between different tissues and cell types are not easily modelled (Kodama \textit{et al.}, 2003; Leonard \textit{et al.}, 2005; Wagner \textit{et al.}, 2008; Zhang \textit{et al.}, 2015, 2016). Therefore, many questions regarding how this interplay precisely occurs in a whole organism and which is the molecular mechanism behind it still remain unanswered.

Previous research in my laboratory based on the use of an unbiased chemical screen identified synthetic GCs as activators of hypoxia-inducible factors (HIF) pathway, especially in the liver of zebrafish embryos. Importantly, a model for GCs to stabilize HIF via Von Hippel Lindau degradation has also been proposed (Vettori \textit{et al.}, 2017). In addition, in a counterintuitive way, GR loss of function was described by Facchinello and colleagues to hamper the transcriptional activity linked to immune response (i.e. of cytokines Il1β, Il8 and Il6 and of the metalloproteinase Mmp-13) (Facchinello \textit{et al.}, 2017). Finally, GR has been also found to synergistically activate proinflammatory genes by interacting with other signalling pathways (Langlais \textit{et al.}, 2008, 2012; Dittrich \textit{et al.}, 2012; Xie \textit{et al.}, 2019).

However, there are currently still controversial \textit{in vitro} data and no clear indications about how the crosstalk between these two major signalling pathways precisely occur \textit{in vivo}. In this regard, the elucidation of this intricated interplay may represent an important stepping-stone to reduce GC-related side effects and to treat
pathological conditions that are linked to hypoxia (i.e., asthma, rheumatoid arthritis, COPD, cancer, inflammation, VHL disease, High altitude sickness etc.).

To this end, I used both a genetic and pharmacological approach to alter these two pathways during the first 5 days post fertilisation of zebrafish embryos. In particular, I took advantage of two different mutant lines I have generated: hif1βsh544 (arnt1) and grεh543 (nr3c1) respectively, coupled to the already existing vhlhu2117/+;phd3:eGFPi144/i144 hypoxia reporter line (Santhakumar et al., 2012).

Initial analysis carried out on the arnt1;vhl mutant line showed that arnt1 loss of function substantially attenuated vhl phenotype. Of note, double mutants were characterized by a reduced phd3:eGFP related brightness, improved yolk usage, properly developed and air-filled swim bladder as well as by the absence of pericardial oedema and excessive caudal vasculature, compared to vhl−/− larvae. However, beyond 5 days, the double mutants exhibited only partial recovery from the vhl phenotype, they developed well till 15 dpf, but subsequently failed to grow and thrive when compared to their siblings. Of note arnt1 homozygous mutants were found to be viable and fertile, in contrast to both vhl and arnt2 homozygous mutants, which are embryonic lethal by 8-10 dpf (Hill et al., 2009; van Rooijen et al., 2009).

Given these data, I set out to understand which was the difference in terms of spatial expression pattern of the two ARNT isoforms in zebrafish larvae. So far, the temporal and spatial expression of arnt1 has been described only in developing mice (Jain et al., 1998; Walisser, Bunger, Glover and Bradfield, 2004; Walisser, Bunger, Glover, Harstad, et al., 2004) and there are just few information about arnt2 expression pattern in zebrafish (Andreasen et al., 2002; Hill et al., 2009). Furthermore, in both cases, there are no information in the literature about their expression levels under upregulated HIF conditions. To examine this aspect, I exploited the rapid state-of-the-
art CRISPR method to generate G0 mutants (Burger et al., 2016; Wu et al., 2018) to create arnt2 mutants both in a vhl and in a vhl;arnt1 background.

My data allowed to show that Arnt1, even if not fundamental for survival, is predominantly expressed in the liver and in organs outside the central nervous system of zebrafish larvae. Conversely, Arnt2 is mainly expressed in the developing central nervous system (CNS), as also reported by Hill et al. in 2009. However, the similarities observed in terms of phd3:eGFP-related brightness in both arnt1−/−;vhl−/− and arnt2 CRISPR injected vhl mutants suggest there is no strong functional separation. Finally, when both isoforms were simultaneously knocked out in a vhl−/− background, triple mutant larvae exhibited an even more rescued Vhl phenotype, which strongly resembled the wild-type’s one. Importantly, an even weaker phd3:eGFP-related brightness overall coupled to normal yolk usage, properly developed and air-filled swim bladder and the absence of both pericardial oedema and excessive caudal vasculature were observed in arnt2-injected arnt1;vhl double mutants, compared to the uninjected ones. As a consequence of the above considerations, since both Arnt1 and Arnt2 can form a functional complex with HIF-α subunits and are expressed in the same organs, I speculate that both Arnt2 and Arnt1 have partially overlapping functions in vivo and are synergistically involved in assuring HIF response.

To further investigate the reverse role of GCs on HIF pathway, I initially tested the already existing gr mutant line, grsh551, for true null mutation. This line has been created in Van Eeden’s laboratory because the existing grs357 allele may still act non-genomically or via tethering method, promoting HIF activation upon GC treatment (Griffiths et al., 2012; Ziv et al., 2012; Vettori et al., 2017). Of note, due to the inability of GCs to bind to a functional receptor (GR), it has been proved that gr mutants are
hypercortisolaemic (Facchinello et al., 2017; Faught and Vijayan, 2018). This is because GCs, by failing to provide negative feedback cannot shut down their own biosynthesis (Griffiths et al., 2012; Facchinello et al., 2017; Faught and Vijayan, 2018a). Interestingly, this was translated into the presence of reduce yolk usage, coupled to a flattened swim bladder in gr mutants compared to wt siblings. In this regard, due to the importance of gr both in physiology and development, I speculate that gr mutants viability and phenotype could also be due to an overstated MR response induced by the presence of high cortisol levels. Of note, this aspect will be discussed in detail in the following chapter.

To confirm loss-of-function, I initially subjected gr<sup>eh551</sup> larvae to a series of molecular and pharmacological analysis. Importantly, all the tests carried out on this mutant line showed no differences between wild-types and gr mutants, proving that the existing gr<sup>eh551</sup> line does not carry a strong loss of function allele. In particular, the fact that gr<sup>eh551</sup> mutants were able to respond to betamethasone treatment and to consequently downregulate pomca expression, as wild-type larvae did, confirmed the presence of a still functional Gr. Finally, detailed literature review unveiled the presence of 8 conserved start codons in the first coding exon (exon 2), which were shown to produce various GR isoforms with progressively shorter N-terminal transactivation domain (NTD) (Oakley and Cidlowski, 2011).

Given the need for a true null mutant line, I set out to generate a novel glucocorticoid receptor mutant line, named gr<sup>eh543</sup>, which was predicted to have a truncated DNA binding domain and, therefore, no C-terminal ligand binding domain too. The absence of shrunk dorsal melanophores observed in gr<sup>eh543</sup> mutants at 5 dpf, typical of a positive visual background adaptation response, represented the first evidence that the gr-dependent negative feedback was missing in mutants.
Furthermore, the failure of gr<sup>sh543/sh543</sup> to respond to betamethasone treatment by inhibiting pomca expression, as observed in wt larvae, via WISH analysis was the main evidence of gr loss of function. Finally, decreased baseline levels of fkbp5 both in the presence and in the absence of BME treatment, as quantified via RTqPCR analysis, also supported this assumption.

Having fully tested these two fish lines (gr<sup>sh543</sup> and arnt1<sup>sh544</sup>) for true null mutations, I then attempted to answer to the questions: how does the inactivation of one of the two pathways affects the other one, and vice versa?
4. EFFECTS OF GC SIGNALLING ON HIF PATHWAY ACTIVATION

4.1. Introduction

GCs constitute a well-characterized class of lipophilic steroid hormones, which act as peripheral effectors of the hypothalamic-pituitary-adrenal/interrenal axis, playing a pivotal role in the stress response and the regulation of carbohydrate, lipid and protein metabolism (Alsop and Vijayan, 2009; Griffiths et al., 2012; Tokarz et al., 2013; Faught and Vijayan, 2018). GCs exert their function via direct binding to the intracellular GR (Bamberger, Schulte and Chrousos, 1996), and together act as a transcription factor, which can function either in a genomic or in non-genomic way (Stahn and Buttgereit, 2008; Mitre-Aguilar, Cabrera-Quintero and Zentella-Dehesa, 2015; Facchinello et al., 2017; Panettieri et al., 2019).

Synthetic GCs (i.e. betamethasone and dexamethasone), which are analogous to naturally occurring steroid hormones, have been extensively used for decades as anti-inflammatory drugs for treating pathological conditions which are very often related to hypoxia (i.e. acute mountain sickness, rheumatoid arthritis, ischemic injury, asthma, etc.) (Nikolaus, Fölschn and Schreiber, 2000; Neeck, Renkawitz and Eggert, 2002; Busillo and Cidlowski, 2013).

However, conflicting results have been reported in the last decade about the crosstalk between GC action and hypoxia (Kodama et al., 2003; Wagner et al., 2008). Moreover, due to the presence of adverse effects (Moghadam-Kia and Werth, 2010) and GC resistance (Barnes and Adcock, 2009; Barnes, 2011), their use has been
restricted. Therefore, extending the research on how precisely this interplay occurs *in vivo*, may have a wide physiological significance in health and disease.

In this chapter, the effect of GC on HIF signalling will be furthered via both a genetic and a pharmacological approach aimed to alter these two pathways during the first 5 days post fertilisation of zebrafish embryos. To this end, I took advantage of a GR mutant line (*gr*<sup>h543</sup>) I have generated, coupled to the aforementioned *vhl*<sup>hu2117/+;phd3:eGFP<sup>i144/i144</sup> hypoxia reporter line (Santhakumar et al., 2012).

Of note, since cortisol has high affinity not only for GR but also for MR and they have been recently shown to be differentially involved in the regulation of stress axis activation and function in zebrafish (Faught and Vijayan, 2018), I also analysed the role of *mr* in the HIF response. To achieve this, I knocked-out *mr* in *gr<sup>+/+</sup>;vhl<sup>+/+</sup>;phd3:eGFP incrossed derived embryos using the aforementioned G<sub>0</sub> mutagenesis CRISPant technology (Wu et al., 2018). Both phenotypic and molecular analysis of these mutant lines were carried out in combination with fluorescence and optical microscope imaging.
4.2. *gr* mutation partially rescues Vhl phenotype

To extend the knowledge on the role of GC on HIF signalling I started to analyse the effect of *gr* loss of function on *vhl* phenotype. To this purpose, I have initially crossed the glucocorticoid receptor mutant line (*gr*<sup>sh543/+</sup>) I generated with the aforementioned *vhl*<sup>huz2117/+;phd3:eGFP<sup>i144/i144</sup> HIF-reporter line. Genotyping analysis following fin-clipping procedure on 3 months old fish allowed to select carriers for both mutations.

Phenotypic analysis carried out on 5 dpf larvae, derived from a *gr<sup>+/−;vhl<sup>+/−</sup>;phd3:eGFP* incross revealed that *nr3c1* knockout may cause an efficient, but not complete rescue of *vhl* phenotype, in a way that resembled *arnt1* mutation (figure 3.2A). In particular, in accordance with the Mendelian ratio, one sixteenth of GFP positive larvae showed a 43% downregulation at the level of the head (P<0.0005), a 66% downregulation in the liver (P<0.0005) and a 51% downregulation in the tail (from the anus to the caudal peduncle) (P=0.0020), in terms of *phd3:eGFP*-related brightness, compared to 5 dpf *vhl<sup>/−</sup>* larvae (figure 4.1B). PCR-based genotyping carried out on these larvae revealed that they were *gr<sup>/−;vhl<sup>/−</sup>*; which allowed me to confirm the presence of a genotype-phenotype correlation.

Of note, rescue was evident by morphology as well. Indeed, even if *gr<sup>/−;vhl<sup>/−</sup>* displayed reduced yolk usage, they showed no pericardial oedema, a reduction in the ectopic vessel formation in the dorsal tail fin and a developed air-filled swim bladder (figure 4.1A). On the other hand, once again, *gr* homozgyote mutant embryos in a *vhl<sup>/−</sup>* or wild-type background, were morphologically similar to wild-types, except for the presence of reduced yolk usage, and adult *gr<sup>/−</sup>* fish were viable and fertile.
Figure 4.1. A. Magnified pictures of a representative 5 dpf vhl+/ larva compared to 5 dpf gr+/vhl+. Among the 450 GFP+ embryos derived from gr+/vhl+ (phd3:eGFP) x gr+/vhl+ (phd3:eGFP), 27 larvae were characterized by the absence of pericardial oedema, no ectopic extra vasculature at the level of the tail, no bright liver and a reduced brightness in the rest of the body (black and white arrowheads). Genotyping post phenotypic analysis on sorted larvae confirmed genotype-phenotype correlation. Fluorescence, exposure = 2 seconds. Scale bar 200 µm. B. Statistical analysis performed on mean gray value quantification (fluorescence, exposure = 2 seconds) at the level of the head, liver and tail, after phenotypic analysis on 5dpf gr+/vhl+ (phd3:eGFP) incross derived larvae. vhl/+ n= 9 larvae: head 186 ± 15.12 (mean ± s.e.m); liver 177.01 ± 20.85 (mean ± s.e.m); tail 62.34 ± 7.27 (mean ± s.e.m). gr+/vhl+ n= 7 larvae: head 106.96 ± 3.21 (mean ± s.e.m); liver 60.75 ± 2.56 (mean ± s.e.m); tail 30.67 ± 1.27 (mean ± s.e.m). Unpaired t-test (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).
In addition, by exploiting the \textit{phd3:}\textit{eGFP} transgene at the background, it was possible to check the effect of \textit{gr} loss of function on the HIF-signalling pathway also in \textit{gr} mutants larvae, in the presence or absence of synthetic GC treatment.

Interestingly, phenotypic analysis carried out at 5 dpf on the aforementioned clutch of larvae revealed that DMSO treated \textit{gr} mutants were characterized, not only by a reduced yolk usage, but also by a significant downregulation of the \textit{phd3:}\textit{eGFP}-related brightness at the level of the liver, where HIF is mainly expressed, (equals to 27\%, $P=0.0406$), compared to DMSO treated \textit{gr} siblings (figure 4.2 and 4.3). This result strengthened the hypothesis that the presence of functional GR is fundamental to assure the HIF response at the hepatic level and to demonstrate that its function is detectable even in the presence of normoxic HIF levels.

Moreover, as expected, BME treated \textit{gr}$^{-/-}$ larvae did not show any significant change in terms of \textit{phd3:}\textit{eGFP} expression levels, compared to DMSO treated \textit{gr} siblings at the level of the head, liver and tail (figure 4.3, red bars). Vice versa, as expected, BME treated \textit{gr} siblings were able to respond to synthetic GC administration as indicated by the upregulation of the hypoxia reporter both at the level of the head (equals to +41\%, $P=0.0077$) and the liver (equals to +123\%, $P<0.0001$), compared to DMSO treated \textit{gr} siblings (figure 4.2 and 4.3, blue bars). These data confirmed once again that the \textit{gr}$^{sh543}$ mutation is a true null, and that BME is able to act as an activator of the HIF signalling pathway in zebrafish larvae.
Figure 4.2 Representative pictures of DMSO and BME [30 µM] treated 5 dpf $gr^{h543/+} (phd3:eGFP)$ and $gr^{h543/h543} (phd3:eGFP)$ larvae (n=120). Phenotypic analysis carried out at 5dpf allowed to show the presence of a reduced yolk usage and a flattened swim bladder in DMSO treated $gr$ mutants, coupled to a reduced $phd3:eGFP$-related brightness quantified at the hepatic level, compared to wild-types. As expected, BME treated $gr$ mutants were not able to respond to the administration of synthetic GCs. In this regard, they only showed a mild pericardial oedema coupled to a still downregulated $phd3:eGFP$-related brightness, especially at the hepatic level. On the other hand, as a consequence of BME treatment, $gr$ siblings were characterized by a severe pericardial and periorbital oedema, coupled to an increased hypoxia reporter expression in the liver (black arrowheads: yolk; white arrowhead: liver's GFP brightness; white asterisks: oedema).
Figure 4.3 Statistical analysis performed on mean grey values quantification (at the level of the head, liver and tail), after phenotypic analysis on 5 dpf DMSO and BME [30 µM] treated gr ^545/+(phd3:eGFP) incross-derived larvae. DMSO treated gr siblings n = 15 larvae: head 11.39 ± 0.68 (mean ± s.e.m); liver 35.08 ± 2.24 (mean ± s.e.m); tail 7.41 ± 0.27 (mean ± s.e.m). BME treated gr siblings n = 9 larvae: head 16.08 ± 1.37 (mean ± s.e.m); liver 78.33 ± 3.35 (mean ± s.e.m); tail 7.97 ± 0.43 (mean ± s.e.m). DMSO treated gr mutants n = 8 larvae: head 11.14 ± 0.86 (mean ± s.e.m); liver 25.73 ± 1.96 (mean ± s.e.m); tail 8.49 ± 0.56 (mean ± s.e.m). BME treated gr mutants n = 8 larvae: head 12.46 ± 1.29 (mean ± s.e.m); liver 23.95 ± 0.93 (mean ± s.e.m); tail 7.17 ± 0.42 (mean ± s.e.m). Ordinary One-way ANOVA followed by Sidak’s multiple comparison test (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).
Finally, as previously done with the arnt1+/−;vhl+/− mutant line, I also tested the efficacy of nr3c1 mutation in rescuing the vhl phenotype. To this end, I attempted to raise gr+/−;vhl−/− larvae after day 5 post fertilization. Three different tanks with the same size and the same amount of larvae (n = 20) have been used in this experiment: one for gr+/−;vhl−/−, one for gr+/−;vhl+/− and one for wild-type AB larvae. Interestingly, whereas vhl mutants are inevitably deceased by 8-10 dpf (van Rooijen et al., 2009), it has been possible to raise gr+/−;vhl−/− larvae beyond 15 dpf. Then, similarly to the arnt1+/−;vhl−/− larvae scenario, they failed to grow and thrive when compared both to their heterozygous siblings and wild-type (AB). This led me to euthanise them due to welfare concerns at 21 dpf (figure 4.4).

Figure 4.4 Kaplan-Meier survival curves of the zebrafish gr+/−;vhl+/− (phd3:eGFP) line analysed in this study. Time is shown in days. Wildtypes n = 20; gr+/−;vhl+/− n = 20; gr+/−;vhl−/− (phd3:eGFP) n = 5. The Log-rank (Mantel-Cox) test was used for statistical analysis. gr+/−;vhl−/− (phd3:eGFP) vs. gr+/−;vhl+/−, ****P < 0.0001; gr+/−;vhl+/− (phd3:eGFP) vs. wt, ****P < 0.0001.
Phenotypic analysis performed on $gr^{-/-};vhl^{-/-}$ larvae at 21 dpf showed the presence of substantial morphological differences compared to both with wild-types and $gr;vhl$ siblings (figure 4.5). Double mutant body length (standard length (SL) = 4.66 mm) was comparable to a 13 dpf wild-type larva, according to Parichy et al. 2009 study (Parichy et al., 2009), but not to its corresponding developmental stage. Vice versa, the body length of 21 dpf $gr^{+/-};vhl^{+/-}$ siblings (SL = 10.94 mm) matched the standard length of wild-type larvae (SL = 10.74 mm) and no particular phenotypic traits have been observed in them.

Interestingly, 21 dpf $gr^{-/-};vhl^{-/-}$ larvae showed a still clear body, a larval-like anteriorly protruding open mouth and a visible gut beneath the swim bladder, which are typical traits of a 11-13 dpf wildtype larva (Kimmel et al., 1995). An early larval pigment pattern with small melanophores in stripes was also observable over the dorsal and ventral myotomes (not dispersed over the yolk sac). As previously observed in the $arnt{1}^{-/-};vhl{-/-}$ larvae, the head of 21 dpf $gr^{-/-};vhl^{-/-}$ did not exhibit the expected dorsal indentation at the level of the pineal gland, which normally appear in 5.7 mm SL larvae. In addition, an increasingly pronounced caudal fin condensation, coupled to the appearance of the first fin rays, typical of a ~4.9 mm SL larva, were not yet visible. Moreover, the slight bulge in the dorsal fin fold as well as the one on the ventral side were not yet apparent. Finally, the distal end of the larval fin fold was still relatively rounded over the caudal fin (figure 4.5). Consequently, despite the increased lifespan of these double mutants, the absence of properly developed traits coupled to a reduced body length are all distinct signs of a substantial developmental delay.

As a consequence of the above considerations, these results confirmed that $gr$ mutation is able to partially rescue the Vhl phenotype and to double the lifespan of $vhl$ mutant larvae. However, since $gr^{-/-};vhl^{-/-}$ did not show fully rescued traits, I speculate
that this could be due to the presence of the mineralocorticoid receptor (MR) that could still act on the HIF signalling pathway.

Since both double mutant embryos and wild-type siblings have been sorted according to their phd3:eGFP-related brightness at 5 dpf, a further confirmation of their genotype was provided by the presence of a still moderately upregulated brightness in 21 dpf gr\textsuperscript{-/-};vhl\textsuperscript{-/-} larvae, compared to the low basal levels observed in gr\textsuperscript{+/-};vhl\textsuperscript{+/-} siblings (figure 4.5).

Altogether, these data indicate for the first time in an in vivo animal model, that Gr function is essential to assure a proper HIF response in zebrafish larvae. Furthermore, the fact that Gr loss of function was able to partially ameliorate the phenotype of vhl mutant and to double the lifespan of vhl\textsuperscript{-/-} larvae, may shed light on important clinical applications. Finally, the fact that only a partial rescue was observed, highlighted the possibility that also MR might have a key role in assuring the HIF signalling.
Figure 4.5 Representative picture of a 21 dpf zebrafish WT (top image, SL = 10.74 mm), a 21 dpf gr+/−; vhl+/−(phd3:eGFP) (middle image, SL = 10.94 mm) and a 21 dpf gr+/−; vhl−/−(phd3:eGFP) (bottom images, SL = 4.66 mm) larva analysed in this study. Scale bar: 1 mm. Fluorescence, exposure 2 sec.
4.3. *gr* loss of function can further reduce HIF signalling in 

*arnt1*/;*vhl*/- larvae

The similarities between *arnt1* and *gr* mutations could mean that they work in a single linear “pathway”. If this assumption was true, mutation of both should not lead to a further attenuation of the reporter expression. To test this, I bred the *gr<sup>sh543</sup>* mutant line with the *arnt1;vhl* double mutant line and then I crossed *gr*/+;*arnt1*/+;*vhl*/- triple carrier fish, once adults.

Phenotypic analysis performed on 5 dpf *phd3:eGFP* positive larvae (n = 488) showed a small class of larvae with an even more rescued phenotype and a stronger downregulation of the *phd3:eGFP* related brightness, compared to both *gr*/-;*vhl*/- and *arnt1*/-;*vhl*/- double mutants (figure 4.6). Moreover, these larvae were characterized by the absence of pericardial oedema (black arrowheads, left) and the lack of ectopic extra vasculature at the level of the tail (black arrowheads, right). Of note, they were also characterized by an even more reduced expression of the hypoxia reported in the head and in the rest of the body (white arrowheads, right) coupled to the absence of a visible *phd3:eGFP* expression in the liver (white arrowheads, left). These 7 putative very weak eGFP<sup>+</sup> larvae were selected and genotypic analysis confirmed that 5 out of 7 were indeed *gr*/-; *arnt1*/-;*vhl*/- larvae.

Of note, these triple mutants showed a 54% downregulation at the level of the head, a 71% downregulation in the liver and a 72% downregulation in the tail region, in terms of *phd3:eGFP*-related brightness compared to *vhl*/- (figure 4.6). As a consequence of the above considerations, these data suggest that GCs are likely to act on both Arnt1 and Arnt2 mediated HIF signalling pathway.
Figure 4.6A. gr loss of function effect is stronger when HIF-signalling is moderately upregulated. A-E. Representative picture of the main differences between vhl<sup>-/-</sup>, arnt1<sup>-/-</sup>;vhl<sup>-/-</sup>, gr<sup>-/-</sup>;vhl<sup>-/-</sup> and triple gr<sup>-/-</sup>;arnt1<sup>-/-</sup>;vhl<sup>-/-</sup> larvae at 5 dpf. Among the 488 phd3:eGFP positive larvae analysed, 7 larvae were characterized by the absence of pericardial oedema (black arrowheads, left), no ectopic extra vasculature at the level of the tail (black arrowheads, right), no visible phd3:eGFP HIF reporter in the liver (white arrowheads, left) and even more reduced levels of this marker in the head and in the rest of the body (white arrowheads, right). Genotypic analysis allowed to confirm the presence of a genotype-phenotype correlation in 5 out 7 samples and to prove that they were triple mutants. Fluorescence, exposure = 2 seconds. Scale bar 200 μm.
Figure 4.6B. Statistical analysis performed on mean grey values quantification (at the level of the head, liver and tail), after phenotypic analysis on 5 dpf gr+/-;arnt1+/-;vhl+/- (phd3:eGFP) incross-derived GFP+ larvae (n = 488). vhl+/- n = 5 larvae: head 125.82 ± 13.05 (mean ± s.e.m); liver 98.52 ± 3.8 (mean ± s.e.m); tail 37.43 ± 2.45 (mean ± s.e.m). gr+/-;arnt1+/-;vhl+/- n = 5 larvae: head 40.24 ± 2.46 (mean ± s.e.m); liver 26.07 ± 1.31 (mean ± s.e.m); tail 11.22 ± 0.47 (mean ± s.e.m); unpaired t-test (**P = 0.0002; ****P < 0.0001).
4.4. Betamethasone-induced HIF response is Arnt1 dependent

To further study the effect of GCs on the HIF signalling pathway, I performed BME [30 µM] treatment on all the available mutant lines. Of note, unlike cortisol, betamethasone has a really high affinity for GR, but has an insignificant affinity for MR (Montgomery et al., 1990; Fromage, 2012). Moreover, its effect has been mainly appreciated in the zebrafish liver.

In particular, I observed in 5 dpf gr wildtypes larvae the presence of a mild upregulation of the HIF reporter-related brightness at the hepatic level, compared to untreated controls (figure 4.2, gr siblings: DMSO vs BME treated). Interestingly, betamethasone treatment was also able to further increase the phd3:eGFP-related brightness at the level of the head and the liver of 5 dpf vhl−/− larvae, compared to the DMSO treated counterpart. (figure 4.7A-A’). Of note, this was quantified as a 20% upregulation at the level of the head (P=0.017) and as a 61% upregulation in the liver (P<0.0001) of the hypoxia reporter expression, compared to DMSO treated vhl−/− larvae (figure 4.7E).

This data was also confirmed by whole-mount in situ hybridisation carried out on vhl−/− incrossed-derived larvae at 5 dpf analysis using both lactate dehydrogenase A (ldha) (figure 4.8B–B’, black arrowheads) and prolyl hydroxylase 3 (phd3) as probes (figure 4.8D–D’, black arrowheads). These two genes were chosen as antisense RNA probes, as they provide a reliable readout of HIF signalling pathway in zebrafish larvae. Indeed, both are upregulated by HIF itself under hypoxic conditions or in a vhl-deficient background. In particular, phenotypic analysis performed post in situ hybridisation, showed for the first time in vivo that both ldha and phd3 expression are upregulated in DMSO treated vhl−/−(phd3:eGFP) mutants compared to vhl siblings, (figure 4.8B-A, and
4.8D-C), and that their expression can be further enhanced as a consequence of BME treatment (figure 4.8B'-B, and 4.8D'-D).

Figure 4.7 A-D’. Representative picture of phenotypic analysis performed on DMSO and BME [30 µM] treated 5 dpf larvae, derived from arnt1+/+;vhl+/+(phd3:eGFP) x arnt1+/+;vhl+/+(phd3:eGFP) (n=540). All the genotype combinations observed are represented in the figure. Among the 405 GFP+ larvae, all the 25 arnt1+/+;vhl−/− showed the aforementioned partially rescued vhl phenotype. Moreover, all the vhl−/− larvae analysed showed an upregulated expression of the hypoxia reporter both in the head and the liver at 5 dpf. Fluorescence, exposure = 2 seconds. Scale bar 500 µm.

E. Statistical analysis performed on mean gray value quantification (at the level of the head, liver and tail), after phenotypic analysis on 5dpf DMSO and BME [30 µM] treated arnt1+/+;vhl+/+(phd3:eGFP) x arnt1+/+;vhl+/+(phd3:eGFP) derived larvae (n=540). vhl−/− DMSO treated n=17 larvae: head 166.67 ± 9.63 (mean ± s.e.m); liver 138.61 ± 12.05 (mean ± s.e.m); tail 50.31 ± 4.51 (mean ± s.e.m). arnt1+/+;vhl−/− DMSO treated n = 13 larvae: head 121.05 ± 6.99 (mean ± s.e.m); liver 49.61 ± 3.88 (mean ± s.e.m); tail 21.75 ± 1.12 (mean ± s.e.m). vhl−/− BME treated n = 18 larvae: head 199.88 ± 7.71 (mean ± s.e.m); liver 222.57 ± 8.72 (mean ± s.e.m); tail 57.57 ± 4.11 (mean ± s.e.m). arnt1+/+;vhl−/− BME treated n = 12 larvae: head 153.71 ± 8.66 (mean ± s.e.m); liver 62.58 ± 5.16 (mean ± s.e.m); tail 25.82 ± 1.54 (mean ± s.e.m). Ordinary One-way ANOVA followed by Sidak's multiple comparison test (⁎P < 0.05; ⁎⁎P < 0.01; ⁎⁎⁎P < 0.001; ⁎⁎⁎⁎P < 0.0001).
Figure 4.8 A-B’. Representative pictures of WISH performed on DMSO (A-B) and BME [30 µM] (A’-B’) treated vhl+/− incross derived larvae, at 5 dpf, using ldha as probe. DMSO treated vhl siblings showed basal ldha expression (34/35 larvae), which showed to be upregulated after BME treatment (33/35 larvae). On the other hand, DMSO treated vhl+/− showed upregulated ldha expression (32/35 larvae), which was further upregulated after BME treatment (34/35 larvae) (black arrowhead: head and liver) Chi-square test (***P < 0.0001). Scale bar 200 µm.

C-D’. Representative pictures of WISH performed on DMSO (C-D) and BME [30 µM] (C’-D’) treated vhl+/− incross derived larvae, at 5 dpf, using phd3 (egln3) as probe. As expected, vhl siblings DMSO treated (n= 30/30 larvae) showed basal phd3 expression, which was mildly increased after BME treatment (n= 27/30 larvae). Vhl+/− DMSO treated (n= 28/30 larvae) showed upregulated phd3 expression, which was further increased after BME treatment (n= 26/30 larvae) (black arrowhead: head and liver) Chi-square test (****P < 0.0001). Scale bar 200 µm.
To further the analysis, I also treated arnt1<−/−;vhl<−/−(phd3:eGFP) incrossed-derived larvae with BME [30 μM] at 4 dpf and I observed the phenotypic outcomes at 5 dpf. Once again, these data confirmed that BME was able to upregulate the hypoxia reporter expression in the liver and the head of vhl<−/− larvae and to mildly do that only at the hepatic level of wild-type larvae (figure 4.7A′-A and D′-D).

Vice versa, as expected, the phd3:eGFP-related brightness both of arnt1<−/− and arnt1<−/−;vhl<−/− larvae was unaffected after BME treatment (figure 4.7B-B′ and 4.7C-C′). In the same way, gr<−/− and gr<−/−;vhl<−/− mutants were unaffected due to the absence of functional Gr and showed no significant differences overall in terms of hypoxia reporter expression, following Betamethasone treatment (figure 4.9B′-B and C′-C). Of note, RTqPCR analysis carried out on both double mutant larvae on HIF (vegfab and egln3) and GC target genes (fkbp5) expression confirmed these data (figure 4.10).

Interestingly, since the head was the brightest region observed in these double mutants, I speculate that mineralocorticoid receptor could compensate for the absence of Gr and maintain moderate levels of HIF, particularly within the CNS. Since recent study has shown that both Gr and Mr are differentially involved in the regulation of the stress axis activation and function in zebrafish (Faught and Vijayan, 2018b), I set up to test this hypothesis via WISH and CRISPa Pant method, as it will be thoroughly described in the next subchapters 4.5 and 4.6.

Altogether, these data indicate that in vhl<−/− larvae BME treatment can upregulate HIF signalling in a Vhl independent way. This is because, as described in a previous study from my lab (Vettori et al., 2017) the activation of GR signalling can negatively regulate VHL protein in human liver cells. However, my analysis shows that in zebrafish larvae there must be an additional point of interaction between these two pathways.
To further test why BME can still activate HIF pathway, even in the absence of functional Vhl, I have repeated the same BME treatment on the already existing \( vhl^{+/−};vll^{−/−}(phd3:eGFP) \) fish line using incross-derived 4 dpf larvae, followed by phenotypic analysis at 5 dpf. This is because, in zebrafish, the function of human VHL is split over two genes, named \( vhl \) and \( vhl-like \) (\( vll \)). Interestingly, unlike pVhl, VII seems to play minor, non-essential HIF-negative regulation functions in zebrafish. Here, I speculate that this is due to the fact that VII functions can be compensated by Vhl activity, but not vice versa. Consequently, \( vll^{−/−} \) larvae develop normally and adult fish are viable and fertile.

In this scenario, phenotypic analysis performed at 5 dpf on DMSO and BME treated \( vhl^{+/−};vll^{−/−} \) and \( vhl^{−/−};vll^{−/−} \) larvae showed that betamethasone is able to potently activate HIF pathway also in a VII-independent way (figure 4.11A). This was quantified as a 34% upregulation at the level of the head (\( P<0.0001 \)), a 16% upregulation in the liver (\( P=0.0007 \)) and a 72% upregulation in the tail (\( P<0.0001 \)) of the HIF-reporter expression in BME treated \( vhl^{+/−};vll^{−/−} \) larvae, compared to the DMSO treated counterpart (figure 4.11B). Moreover, also \( vhl^{+/−};vll^{−/−} \) showed and increased HIF-reporter expression in the head and the liver, as usually occurs in wild types (figure 4.11A).

Importantly, these data reveal for the first time that VII is also involved in counteracting HIF pathway overactivation induced by Betamethasone in zebrafish.
Figure 4.9 A-D’. Representative picture of phenotypic analysis performed on DMSO and BME [30 \(\mu\)M] treated gr\(^{+/+}\);vhl\(^{+/-}\) (phd3:eGFP) incross-derived 5 dpf larvae (\(n = 600\)). All the genotype combinations observed are represented in the figure. Among the 450 GFP\(^{+}\) larvae analysed, 28 showed a partially rescued vhl phenotype which resembled the arnt1’s one. Three experimental repeats. In all panels: *\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\); ****\(P < 0.0001\). Fluorescence, exposure = 2 seconds. Scale bar 500 \(\mu\)m.

E. Statistical analysis performed on mean gray value quantification (at the level of the head, liver and tail), after phenotypic analysis on 5 dpf DMSO and BME [30 \(\mu\)M] treated gr\(^{+/+}\);vhl\(^{+/-}\) (phd3:eGFP) \(\times\) gr\(^{+/+}\);vhl\(^{+/-}\) (phd3:eGFP) derived larvae. vhl\(^{+/+}\) DMSO treated \(n = 9\) larvae: head 186 ± 15.12 (mean ± s.e.m); liver 177.01 ± 20.85 (mean ± s.e.m); tail 62.34 ± 7.27 (mean ± s.e.m). gr\(^{+/+}\);vhl\(^{+/-}\) DMSO treated \(n = 7\) larvae: head 106.96 ± 3.21 (mean ± s.e.m); liver 60.75 ± 2.56 (mean ± s.e.m); tail 30.67 ± 1.27 (mean ± s.e.m). vhl\(^{+/+}\) BME treated \(n = 14\) larvae: head 224.32 ± 6.83 (mean ± s.e.m); liver 244.07 ± 5.31 (mean ± s.e.m); tail 80.51 ± 5.49 (mean ± s.e.m). gr\(^{+/+}\);vhl\(^{+/-}\) BME treated \(n = 9\) larvae: head 125.85 ± 3.6 (mean ± s.e.m); liver 63.56 ± 2.91 (mean ± s.e.m); tail 33.67 ± 1.02 (mean ± s.e.m).

Ordinary One-way ANOVA followed by Sidak’s multiple comparison test (*\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\); ****\(P < 0.0001\)).
Figure 4.10 RTqPCR analysis carried out both on HIF and GC target genes expression on *gr*<sup>−/−</sup> *vhl*<sup>−/−</sup> and sibling at 5 dpf, (n = 10 larvae, per group, in triplicate) compared to *arnt1*<sup>−/−</sup> *vhl*<sup>−/−</sup> larvae and siblings, at 5dpd (n = 10 larvae, per group, in triplicate). Both *vegfab* and *egln3* are HIF target genes, whereas *fkbp5* is a GC target gene. Statistical analysis was performed on ΔΔCt values, whereas data are shown as fold change values, Ordinary Two-way ANOVA followed by Dunnett’s multiple comparison test.
Figure 4.11. A. Representative pictures of phenotypic analysis performed on DMSO and BME [30 µM] treated vhl+/−vll+/− (phd3:eGFP) incross-derived 5dpf larvae (n=140). Statistical analysis allowed to show that betamethasone is able to significantly activate the Tg(phd3:eGFP) expression in a Vhl and Vll-independent way. B. Statistical analysis performed on mean gray value quantification (at the level of the head, liver and tail), after phenotypic analysis on DMSO and BME [30µM] treated vhl+/−vll+/− (phd3:eGFP) larvae. vhl+/−vll+/− DMSO treated n = 17 larvae: head 168.42 ± 7.18 (mean ± s.e.m); liver 212.91 ± 7.95 (mean ± s.e.m); tail 55.76 ± 3.50 (mean ± s.e.m). vhl+/−vll+/− BME treated n = 17 larvae: head 225.32 ± 7.32 (mean ± s.e.m); liver 245.73 ± 4.14 (mean ± s.e.m); tail 95.98 ± 5.05 (mean ± s.e.m). Ordinary One-way ANOVA followed by Sidak’s multiple comparison test (**P < 0.001; ****P < 0.0001).
4.5. *gr* loss of function overrides HIF-mediated *pomca* downregulation in a *vhl* mutant scenario

To deepen the knowledge about the effect of GCs on the HIF signalling I examined the effect of *gr* loss of function on steroidogenesis in a *vhl* mutant scenario. To this purpose, I performed a whole mount *in situ* hybridisation on 5 dpf *gr*+/−;*vhl*+/− incross-derived larvae using proopiomelanocortin a (*pomca*) as probe. Interestingly, unlike normal expression of *pomca* observed at the level of the anterior part of the pituitary gland in the *gr;vhl* wild-type siblings (figure 4.12A), *vhl* mutants displayed a downregulated expression (figure 4.12B). In contrast, in line with previous observations, *gr* mutants showed upregulated *pomca* (figure 4.12C). The latter phenotype is known to be induced by the absence of a functional GC-GR mediated negative feedback loop, which triggers the upregulation of *pomca* gene at the level of the pituitary gland. This has been shown to be followed by an overproduction of cortisol at the level of the interrenal gland, which makes zebrafish hypercortisolaemic (Facchinello et al., 2017; Faught and Vijayan, 2018b). Since *vhl* mutants, which are characterized by an upregulated HIF signalling pathway, displayed a downregulated *pomca* expression, I speculated that this might occur via HIF-mediated activity at the pituitary level, aimed to downregulate the cortisol-mediated stress response.

Since GCs control a broad range of physiological processes, act on almost every tissue and organ in the body to maintain homeostasis and are characterized by a robust immunosuppressive and anti-inflammatory actions, their secretion must be finely tuned at the level of the HPA/I axis. In addition, since previous data from my laboratory identified GCs as HIF activators (Santhakumar et al., 2012; Vettori et al., 2017), I inferred that, being HIF particularly expressed at the level of the head, it might in turn
regulates GC levels by acting on *pomca* expression. If this was true, it would enable HIF signalling not only to tune its own levels, but also to ensure homeostasis. Moreover, being HIF signalling a master regulator of pro-inflammatory responses to hypoxia, it would counteract the anti-inflammatory activity played by GCs. To this end, I speculate that the concomitant expression of both upregulated HIF and GC pathway would be detrimental to homeostasis. In particular, the following chapter will be focused on testing and confirming these hypotheses.

Additionally, WISH analysis carried out on the aforementioned mutant line, showed that a strong *pomca* upregulation was present in *gr*/*;vhl*/* larvae (figure 4.12D), compared to both *vhl*/* and wild types. Then, PCR analysis carried out post *in situ* hybridisation allowed to confirm the presence of genotype-phenotype correlation. In this regard, if my hypothesis was true, it would suggest that *gr* loss of function mutation overrides the HIF-mediated *proopiomelanocortin* a inhibition.
Figure 4.12 A–D'. Representative pictures of whole-mount *in situ* hybridisation carried out on *gr*+/−; *vhl*+/− incross derived larvae at 5 dpf, using *pomca* as probe. Of note, *gr*+/−; *vhl*+/− larvae showed upregulated *pomca* expression (20/20 larvae), as observed in *gr*−/− (20/20 larvae); *vhl* mutants showed downregulated *pomca* expression (20/20 larvae), whereas wild types showed normal *pomca* expression (19/20). Chi-square test (P < 0.0001). Scale bar 50 μm.
4.6. Both Gr and Mr are directly required for properly assuring HIF response

Endogenous cortisol has high affinity more for Mr than Gr and both have been lately shown to be differentially involved in the control of the stress axis activation and function in zebrafish larvae (Faught and Vijayan, 2018b).

For these reasons, I have speculated that also the GC-MR complex might contribute to trigger the HIF response in zebrafish larvae. To test this, I knocked-out mr gene in the gr+/−;vhl+/−;phd3:eGFP incrossed derived embryos using the aforementioned CRISPant technology (Burger et al., 2016; Wu et al., 2018).

Interestingly, phenotypic analysis performed on 5 dpf injected and uninjected larvae revealed that mr CRISPR injected vhl mutants were characterized by a decreased phd3:eGFP-related brightness at the level of the head (equals to 49%, P<0.0001), in the liver (equals to 56%, P<0.0001) and in the rest of the body (equals to 47%, P<0.0001), compared to vhl mutant uninjected larvae (figure 4.13D compared to 4.13A). Furthermore, the downregulation was even stronger at the level of the head (equals to 62%, P<0.0001), the liver (equals to 77%, P<0.0001) and the rest of the body (equals to 63%, P<0.0001) when mr was knocked-out in gr+/−;vhl+/− larvae, compared to uninjected vhl mutants (figure 4.13E compared to 4.13A). Notably, mr injection in vhl+/− larvae was also more efficient in downregulating the HIF-reporter expression only at the level of the head (equals to 31%, P = 0.0087) compared to uninjected gr+/−;vhl+/− larvae (figure 4.13D compared to 4.13B). This led me to hypothesise that Mr could play a key role at the level of the CNS in terms of HIF pathway regulation, compared to Gr.
At this point, to confirm the specificity of Wu et al., 2018 method, I chose to target a gene which was not involved in the HIF pathway. Laminin, beta 1b (*lamb1b*), which codes for an extracellular matrix glycoprotein, was injected as CRISPR-injection control in *vhl*+/− incross-derived embryos at 1 cell stage. Genotypic analysis carried out on these larvae confirmed that these guides were effective.

As expected, quantification of *phd3:eGFP*-related brightness performed on 5 dpf injected and uninjected larvae, showed no significant differences between these two groups (figure 4.14A-C and 4.14E). Overall, these data further supported my hypothesis that not only GR but also MR play an essential role in assuring a proper HIF response in zebrafish.

In light of these facts, understanding the precise role of MR and mineralocorticoid in the HIF signalling is warranted and will require further testing.
Figure 4.13 A-F. Representative pictures of 5 dpf CRISPANT mutants created by redundantly targeting *nr3c2* (*mr*) gene via co-injection of 4x gRNAs in *gr^+/-;vhl^+/- (phd3:eGFP) × gr^-/-; phd3:eGFP* derived embryos (n=344). Uninjected embryos were used as control (n=170). Fluorescence, exposure = 991,4 ms. Scale bar 500 μm. G. Statistical analysis performed on mean grey value quantification (at the level of the head, liver and tail), after phenotypic analysis, on 5 dpf *mr* 4x gRNAs injected and uninjected larvae. *vhl^+/−* uninjected n = 17 larvae: head 48.28 ± 2.99 (mean ± s.e.m); liver 46.47 ± 3.55 (mean ± s.e.m); tail 16.15 ± 1.06 (mean ± s.e.m). *gr^-/-;vhl^+/−* uninjected n = 8 larvae: head 35.48 ± 2.03 (mean ± s.e.m); liver 23.56 ± 1.72 (mean ± s.e.m); tail 10.98 ± 0.75 (mean ± s.e.m). *vhl^-/-* injected n = 15 larvae: head 24.62 ± 0.97 (mean ± s.e.m); liver 20.67 ± 1.10 (mean ± s.e.m); tail 8.57 ± 0.39 (mean ± s.e.m). *gr^-/-;vhl^-/-* injected n = 16 larvae: head 18.33 ± 0.46 (mean ± s.e.m); liver 10.71 ± 0.56 (mean ± s.e.m); tail 6.07 ± 0.26 (mean ± s.e.m); ordinary One-way ANOVA followed by Sidak’s multiple comparison test.
Figure 4.14 A-D. Representative pictures of 5 dpf CRISPANT mutants created by redundantly targeting lamb1b gene via co-injection of 4x gRNAs in vhl+/− (phd3:eGFP) incross-derived embryos (n=400). Uninjected embryos were used as control (n=470). Fluorescence, exposure = 991.4 ms. Scale bar 500 µm. E. Statistical analysis performed on mean grey values quantification (at the level of the head, liver and tail), after phenotypic analysis on 5 dpf lamb1b 4x gRNAs injected and uninjected vhl+/− (phd3:eGFP) incross-derived larvae. vhl+/− un.injected n = 24 larvae: head 54.83 ± 3.68 (mean ± s.e.m); liver 77.86 ± 6.46 (mean ± s.e.m); tail 19.56 ± 1.43 (mean ± s.e.m). vhl+/− injected n = 25 larvae: head 59.74 ± 4.05 (mean ± s.e.m); liver 83.23 ± 5.92 (mean ± s.e.m); tail 19.9 ± 1.38 (mean ± s.e.m); unpaired t-test (all panels: *P < 0.05; **P < 0.01; ***P <0.001; ****P < 0.0001).
4.6.1. Generation and characterisation of zebrafish gr<sup>sh543/+;mr<sup>sh562/+</sup> line

To further the knowledge on the MR role on the HIF signalling when the latter is normally expressed, I initially created the gr<sup>sh543/+;mr<sup>sh562/+</sup> mutant line as mentioned in the subchapter 2.1.7 of the Material and Methods. Then, fin-clipping procedure followed by genotyping analysis on 3 months old fish allowed to select carriers for both mutations. As previously described in chapter 4.2, gr mutation is able to partially rescue Vhl phenotype, gr<sup>-/-</sup> larvae can be sorted via visual background adaptation test and have a visible phenotype. On the other hand, mr mutation is known not to cause any phenotype, mr<sup>-/-</sup> larvae cannot be VBA sorted and its role in the HIF pathway is still unknown (Cruz et al., 2013; Facchinello et al., 2017; Faught and Vijayan, 2018b). As a consequence of the above considerations, since my data showed that Mr mutation is able to further downregulate the HIF signalling in zebrafish larvae, I decided to check whether the simultaneous knockout of both mr and gr was able to modulate the phenotype and/or the viability of gr<sup>-/-</sup> mutant larvae.

Phenotypic analysis carried out on VBA sorted gr<sup>+/+;mr<sup>-/-</sup> incross-derived larvae at 5 dpf, revealed that gr<sup>+/+;mr<sup>-/-</sup> mutants are characterized by a slightly improved yolk usage compared to the previously described gr<sup>-/-</sup> larvae (figure 4.15B compared to 4.15A, white asterisk). At the same time, double mutants display a still rounded head compared to wild types and a VBA negative response as observed in gr<sup>-/-</sup> larvae (figure 4.15B compared to 4.15A, black arrowhead). Vice versa, mr<sup>-/-</sup> didn’t show any particular phenotype and they looked like wild-type larvae, as expected (figure 4.15C). Genotyping post phenotypic analysis on these VBA sorted larvae confirmed the presence of a genotype-phenotype correlation. Consequently, I inferred that since cortisol cannot bind neither to GR nor to MR anymore, it is unable to elicit any effect, other than the one caused by a cortisol resistance-like state.
Figure 4.15 A-C. Representative pictures of 5 dpf gr+/-;mr+/- incross-derived larvae (n=600), after being VBA tested and sorted according to their phenotype in three different dishes (n=60 each). In the first dish, there were VBA negative gr+/- larvae derived by a gr+/- fish incross used as control. The second dish (B) contained VBA negative larvae with a bigger yolk than the wild-types one, but smaller than gr+/- larvae (white asterisk), coupled to the presence of a still rounded head (back arrowhead). Finally, the third dish (C) included VBA positive larvae with a normal phenotype. Of note, PCR-based genotyping carried out on 12 larvae per group, allowed to confirm the presence of a genotype-phenotype correlation. White asterisk: yolk/gut; black arrowhead: head.
Since it is well known that in zebrafish GCs regulate a variety of physiological processes and are essential in the stress response, hatching and swim activity at early developmental stages (Oakley and Cidlowski, 2011; Wilson et al., 2013; Timmermans, Souffriau and Libert, 2019), I inferred that these double mutants were unable to cope with stress and were not viable or fertile at adult stages.

To test this, I set up to raise \( gr^+/mr^- \) incrossed derived larvae at 5 dpf till adulthood. Surprisingly, PCR-based genotyping on adult zebrafish tail biopsies, revealed that double mutants were viable and adult males showed feminised secondary sex characteristics, including a slightly bigger belly and bluish stripes. Moreover, both sexes showed an increased average size.

To better examine this aspect, I collaborated with Jack Paveley (PhD student in Vincent Cunliffe lab, The University of Sheffield) to weigh 58 nine-month old fish derived from the same aforementioned cross together with wild type AB fish with the same age, used as control. Each fish was anesthetised with tricaine and gently dried on a tissue to remove the excess of water. Then, it was quickly weighed on an analytical balance and was put back in a new tank. Weighing results allowed to confirm that both wild types and \( mr^-/\) , which share the same phenotype, have also the same weight (wt = 0.383g compared to \( mr^- = 0.396g \)). On the other hand, as expected, \( gr^-/\) showed an increased weight compared to both wild-types AB (equals to 0.505, \( P<0.0038 \)) and \( mr^/- (P<0.0001) \). Finally, \( gr^/-; mr^-/\) fish surprisingly weighed more than double the wild types (equals to 0.933, \( P<0.0001 \)) and almost twice as much as \( gr^-/\) (\( P<0.0001 \)) (figure 4.16). Of note, the sex ratio was kept the same among all the groups analysed and a similar amount of fish was raised in each tank according to their genotype.

Consequently, we wanted to check whether the knockout of both \( gr \) and \( mr \) was able to affect the ability of these fish to perform the typical male breeding behaviours.
Of note, \( gr^-/\) adult fish are viable and can be mated without any issue like wild types and \( mr^-/\) fish. However, unlike the latter, they are unable to properly cope with stress and show freezing behaviour when put in a new environment. For this reason, I wanted to test the ability of \( gr^-;/mr^-/\) adult fish to perform breeding. Notably, preliminary behavioural analysis carried out in collaboration with Jack Paveley, showed that these double mutants were less motile, failed to show chasing behaviour (the typical mating ritual) and they did not lay eggs every time we pair-mated them (> 4 times). As a consequence of the above consideration, since reproductive events are also regulated by hypothalamic-pituitary-adrenal axis (Whirledge and Cidlowski, 2013) and double mutants cannot sense cortisol at all, I speculated that this could be the main reason behind this behaviour. Consequently, understanding whether the concomitant presence of both mutations is able to cause defects at the level of the reproductive system in zebrafish is warranted and will require further testing.

![Graph showing weight comparison](Figure 4.16. Statistical analysis carried out on weighing results of 60 nine-month-old fish derived from the aforementioned \( gr^-;/mr^-/\) incross. Wild types fish \((n=12): 0.383 \pm 0.018 \text{ (mean \pm s.e.m).} \) \( gr^-/\) fish \((n=17): 0.505 \pm 0.027 \text{ (mean \pm s.e.m);} \) \( mr^-/\) fish \((n=17): 0.396 \pm 0.017 \text{ (mean \pm s.e.m);} \) \( gr^-;/mr^-/\) fish \((n=12): 0.933 \pm 0.030 \text{ (mean \pm s.e.m).} \) Ordinary One-way ANOVA followed by Sidak’s multiple comparison test (all panels: \(*P < 0.05; **P < 0.01; ***P <0.001; ****P < 0.0001). \)
4.7. Discussion

This chapter is intended to provide further insights regarding the role of GCs on the HIF signalling. Since previous work from my lab showed that synthetic GC can activate the phd3:eGFP hypoxia reporter particularly in the zebrafish liver (Vettori et al., 2017), however the question whether GR activity is essential for proper HIF pathway activation was yet unanswered.

The best way to answer this question was to analyse the effect of gr loss of function in a HIF upregulated scenario (vhl−/−). Here, phenotypic analysis performed on 5 dpf larvae showed that gr mutation is able to cause an efficient, but not complete rescue of the vhl phenotype. This is interesting because the phenotype induced by gr mutation resembled the one generated by knocking down arnt1 gene in the vhl−/− background. Indeed, in both cases it was possible to observe a downregulation of the hypoxia reporter equals to 39% in arnt1;vhl double mutants and to 43% in gr−/−;vhl−/− larvae at the level of the head, compared to 5 dpf vhl mutants larvae. Moreover, a 75% downregulation in the liver of arnt1−/−;vhl−/− larvae and a 66% downregulation was instead observed in the same organ in gr;vhl double mutants. Finally, a 58% and a 51% downregulation of the phd3:eGFP-related brightness were quantified from the anus to the caudal peduncle both in arnt1−/−;vhl−/− and gr−/−;vhl−/− larvae, respectively.

In line with these observations, gr−/−;vhl−/− larvae survived much longer than the vhl−/− (≥21 dpf compared to max. 10 dpf) but then, similarly to the arnt1−/−;vhl−/−, failed to grow and thrive when compared both to their siblings and wild types. Moreover, both double mutants showed lots of morphological similarities with each other, in terms of development stage and anatomical traits, compared to normally developed wild-type siblings.
Cumulatively, the analogies appreciated between arnt1 and gr mutations in a Vhl mutant scenario could suggest that they work in a single linear "pathway". If this assumption was true, the mutation of both genes should not lead to a further attenuation of the hypoxia reporter expression.

Importantly phenotypic analysis carried out on gr\(^{+/}\);arnt1\(^{+/}\);vhl\(^{+/}\) triple carrier fish’s progeny showed that the addition of gr mutation in a arnt1;vhl double mutant scenario generates a stronger downregulation of the phd3:eGFP expression and a more rescued Vhl phenotype than the one induced by single mutations. This allowed me to strengthen the hypothesis that these two major signalling pathways are not acting in a linear way.

To then study the effect of gr loss of function on the HIF signalling when the latter is not constantly overactivated (i.e. in wild type-like scenario), I analysed the hypoxia reporter gene expression in gr\(^{+/}\);phd3:eGFP incrossed derived larvae at 5 dpf. Quantification data based on the HIF-reporter expression in the aforementioned progeny showed that gr loss of function is able to cause a reduction of the phd3:eGFP-related brightness even in the absence of a HIF upregulated pathway. As a consequence, these data indicate for the first time in an in vivo animal model that Gr is essential to ensure high HIF signalling levels. In addition, the fact that Gr loss of function was able to partially rescue the Vhl phenotype and to double the lifespan of vhl\(^{+/}\) larvae, may have important clinical applications. In this regard, I speculate that the inhibition of glucocorticoid receptors might be a potential avenue to downregulate HIF for clinical purposes.

Furthermore, WISH data derived from gr\(^{+/}\);vhl\(^{+/}\) incross-derived larvae suggested that in gr\(^{+/}\);vhl\(^{+/}\) larvae the upregulation of pomca induced by the lack of a functional Gr, cannot be inhibited with the same efficiency by the HIF signalling activity
at the level of the pituitary gland. For these reasons, I speculate that the resulting upregulated endogenous cortisol can interact only with Mr to stimulate the HIF pathway. This would also explain the presence of a moderately upregulated phd3:eGFP reporter expression in gr−/−;vhl−/− larvae (figure 4.9B), whose levels are in-between the ones observed in vhl mutants and wild-type larvae (figure 4.9A and D).

If this was true, I predicted to obtain an even more rescued phenotype by knocking-out mr in a gr−/−;vhl−/− background. This is important because nothing is known so far about mineralocorticoid receptor contribution to the HIF signalling. Moreover, a recent work published by Faught and Vijayan, 2018 showed that both Gr and Mr are differentially involved in the regulation of zebrafish stress axis activation and function.

Interestingly, my data showed that both mr injected- vhl−/− and gr−/−;vhl−/− 5 dpf larvae displayed a significant reduction of phd3:eGFP-related brightness. This suggests that, in fish, not only the GR, but also the MR is involved in promoting HIF pathway activation, as a consequence of cortisol stimulation. In addition to that, phenotypic analysis performed on VBA sorted gr+/−;mr−/− incross-derived larvae revealed the presence of an intermediate phenotype in gr+/−;mr−/− that was in-between the on of gr+/− and wild types (figure 4.15). However, once adults, these double mutants were characterized by an increased size compared to both wild types and gr−/− fish. Consequently, I speculate that the additional presence of mr mutation in a gr−/− scenario makes larvae resistant to cortisol and promote a normal yolk usage, by protecting the body from an overexposure of GC-MR activity. However, although the absence of both functional genes is surprisingly not critical for survival in zebrafish, it causes a significant increase of weigh compared to both wild types and gr−/− fish once adults (figure 4.16). Since both Gr and Mr are involved in adipose tissue differentiation,
expansion and proinflammatory capacity (Marzolla et al., 2012; Geer, Islam and Buettner, 2014), this could explain the phenotype observed in these double mutants.

Moreover, in contrast to mammals, teleosts lack aldosterone and cortisol is the primary GC hormone which can interact with both Gr and Mr to assure the correct HPI axis functioning (Cruz et al., 2013; Tokarz et al., 2013; Baker and Katsu, 2017; Faught and Vijayan, 2018b). Of note, my hypothesis is also supported by Faught and Vijayan, 2018 elegant work, showing that both Gr and Mr signalling is involved in the negative feedback regulation of cortisol biosynthesis during stress. In conclusion, although Mr contribution to HIF response in other organisms remains unclear, my work suggests research into its function is warranted.

To finally examine the role of GCs on the HIF signalling, I next analysed the effect of betamethasone treatment on gr−/−;vhl−/−, arnt1−/−;vhl−/−, arnt1−/− and vhl−/− mutants. Importantly, my data besides confirming that BME acts as a potent HIF activator, indicate a key role for Arnt1 in regulating the BME-induced HIF response. In particular, the fact that BME did not increase phd3:eGFP-related brightness both in arnt1−/− and in arnt1−/−;vhl−/− larvae, even if other functional Arnt isoforms were present, confirmed that. Indeed, if BME activity was not Arnt1 dependent, it should have been be able to enhance the HIF signalling in both mutants. These data are surprising and would be best explained by assuming that a BME-Gr complex would preferentially interact with a HIFα/ARNT1 complex, instead of with a HIFα/ARNT2 complex. Finally, whether this holds up in mammalian cells would be interesting to address and requires further testing.

Additionally, unlike previous work from my lab showed that activation of GR signalling negatively regulates VHL protein, via c-src upregulation, in human hepatic
cells (Vettori et al., 2017), my current research demonstrates that there must be an additional point of interaction in zebrafish larvae between these two pathways. Indeed, a further activation of the hypoxia reporter was observable after BME treatment even in the absence both of functional Vhl and Vll. For this reason, it is plausible to infer that in BME treated vhl/- larvae, not all the Vhl proteins are broken down, allowing Vll to partially degrade HIF-α subunits and to counteract BME-induced HIF activation. Consequently, I speculate that GC must act downstream, rather than at the level of Vhl and Vll. Indeed, as it will be confirmed in the following chapter, betamethasone can act not only downstream of Vhl and Vll, but also of the HPI axis itself to upregulate the HIF response (figure 4.7A-A’, 4.8B-B’ and D-D’, 4.97A-A’).

These data highlighted for the first time in vivo, that the absence of functional Gr is able to cause an efficient inhibition of the HIF signalling, both under normoxic and hypoxic-like (vhl/-) scenario. Indeed, although GC-GR activity is expected to be basal under non-stressful conditions (i.e. normoxia), its function is detectable with respect to assuring proper HIF activity. By contrast, the BME-mediated HIF activation can occur in a Vhl/Vll independent way in zebrafish and requires Arnt1 function to occur.

Cumulatively, this current study permitted to further the knowledge on the importance of the GCs and their receptors in the hypoxia-inducible factor pathway.
5. ROLE OF HIF SIGNALLING ON GC PATHWAY

5.1. Introduction

Hypoxia is a prevalent pathophysiological condition to which cells have to quickly reply in order to avoid metabolic shutdown and consequent death. Oxygen levels are continuously monitored via the activity of Hypoxia-Inducible Factors (HIFs) family, which is made up of key oxygen sensors that manage the ability of the cell to cope with lowered oxygen levels. In particular, this is tackled by orchestrating a metabolic shift from aerobic (oxidative phosphorylation) to anaerobic metabolism (glycolysis) aimed to reduce oxygen consumption.

Albeit the HIF response aims to reinstate both tissue oxygenation and perfusion, it can in some cases be inappropriate, and might contribute to a variety of pathological conditions such as: inflammation, stroke, tissue ischemia and the growth of solid tumours (Cummins and Taylor, 2005; Murdoch, Muthana and Lewis, 2005; Cummins et al., 2007; Elks et al., 2015). In this respect, synthetic GCs have been exploited for decades, being the equivalent of naturally occurring steroid hormones, due to their potent anti-inflammatory action, in order to treat pathological conditions that are linked to hypoxia (i.e. asthma, rheumatoid arthritis, ischemic injury). Interestingly, previous in vitro studies have point out the presence of a crosstalk between HIF and GC signalling (Kodama et al., 2003; Leonard et al., 2005; Wagner et al., 2008; Zhang et al., 2015, 2016). Of note, it has been recently reported that under hypoxic conditions HIF-1 is also able to negatively regulate steroidogenic acute regulatory (StAR) protein expression and steroidogenesis in mouse granulosa cells (Kowalewski, Gram and Boos, 2015). Moreover, both hypoxia and HIF-1α overexpression were shown to
downregulate the expression of StAR also in zebrafish embryos, but to simultaneously upregulate the expression both of CYP11a and 3β-HSD gene. Since the expression of these three genes were reversed in zHIF-1α knockdown embryos under normoxic conditions, this fact suggested that these genes were likely regulated by Hif-1 (Tan et al., 2017).

However, the existence of two paralogs for each of the three zHIF-α isoforms (hif-1A,B, hif-2A,B and hif-3A,B) (Köblitz et al., 2015), coupled to the lack of a proper reverse genetics approach used in the above-mentioned studies, highlighted the need to further the research on this topic. In particular, it still remains unclear how HIF contributes to GC functions, how the interplay between hypoxia-dependent signals and GC-mediated regulation of gene expression precisely occurs in an organism and what the molecular mechanism is behind it.

In this chapter a thorough molecular analysis on the role of hypoxia-inducible factor signalling on GC pathway will be provided. To tackle this, I took advantage mainly of the vhl+/-(phd3:eGFP) line together with the aforementioned arnt1+/-(phd3:eGFP) mutant line I created. Of note, all the data I collected from RTqPCR analysis, whole mount in situ hybridisation (WISH) and cortisol quantification converged towards the same direction and allowed me to propose a logical model of interaction between HIF and GC signalling, in vivo. Once again, both phenotypic and molecular tests performed on these mutant lines were followed by optical and fluorescence microscope imaging.
5.2. Modulation of the HIF signalling affects GC response

To investigate the role of HIF signalling on GC pathway, I initially set up to test the expression of four potential GC target genes from mammalian studies (*fkbp5, il6st, pck1 and lipca*) in a HIF upregulated (*vhl*−/−) and downregulated scenario (*arnt1*−/−), compared to wild types, via RTqPCR analysis on 5 dpf larvae. The aim was indeed to check how the GC response varies according to different HIF activation levels.

As previously stated in chapter 3.4, the aforementioned target genes have been selected as potential GC response reporters, however it is important to note that they may have both other inputs and developmental regulation. In this regard, my data confirmed that in 5 dpf zebrafish larvae *fkbp5* is the most well-established and sensitive readout of Gr activity, whereas the other aforementioned genes (*il6st, pck1 and lipca*) were observed to be less reliable ([figure 5.1A-H](#)). For this reason, I focused this analysis on *fkbp5* expression ([figure 5.1I-L](#)).

Importantly, qPCR data obtained from my analysis shows that *fkbp5* expression is downregulated (fold change = 0.1; P = 0.0035) in the presence of an upregulated HIF pathway (*vhl*−/− larvae) compared to DMSO treated *vhl* siblings ([figure 5.1I](#)). On the other hand, when the HIF pathway is downregulated (*arnt1*−/− larvae), *fkbp5* expression is upregulated (fold change = 24.1; P<0.0001), compared to DMSO treated wild-type levels ([figure 5.1L](#)). Of note, these data highlighted the presence of a 240 fold difference in the *fkbp5* expression between *vhl*−/− (HIF overexpressed) and *arnt1*−/− larvae (HIF suppressed).

To further investigate the role of HIF signalling on GC response, I also performed betamethasone treatment [30 μM] on the aforementioned mutant lines, followed by RTqPCR analysis. Notably, the administration of synthetic GCs was able to increase
fkbp5 expression in vhl siblings but was only able to weakly do that in vhl mutants. In particular, its induction levels were not only lower in BME treated vhl mutants (fold change = 2.1) than in BME treated wild-type siblings (fold change = 7, P = 0.0286), but also fkbp5 expression was not substantially different from DMSO treated wild types (figure 5.1I). Vice versa, when the HIF pathway was suppressed (arnt1−/− larvae), betamethasone treatment was able to further upregulate the expression of fkbp5 (fold change = 107.5; P = 0.0031), compared to DMSO treated arnt1 mutant larvae (figure 5.1L).

Cumulatively, these results indicate that upregulated HIF levels are somehow able to repress GR activity and can blunt or abolish its sensitivity to an exogenous GR agonist (BME treatment). On the other hand, it is interesting to note that although HIF activity is expected to be low in wild-type larvae in a normoxic environment, its function is also detectable with respect to suppression of GR activity. Indeed, if arnt1 gene is knocked-out (arnt1−/−) an increased GR responsiveness is observed (figure 5.1L).

Consequently, since these data shed lights on a novel route mediated by HIF to control the GC response, I asked whether this was due to any potential upstream effect of HIF signalling on steroidogenesis. To this end, in order to further analyse whether this had repercussions either on steroidogenesis and/or cortisol levels, I tested them both in a HIF upregulated (vhl−/−) and downregulated scenario (arnt1−/−). In this regard, the following section will focus on a series of experiments performed to verify this hypothesis.
Figure 5.1. Schematic view of RTqPCR analysis on il6st, pck1, lipca and flbp5 (GC target genes) expression carried out both on vhl+/-(phd3:eGFP) and arnt1+/-(phd3:eGFP). The aim was to quantify GC target genes expression in the presence of upregulated and downregulated HIF signalling pathway. Statistical analysis performed on ΔΔCt values; data are shown as fold change values for RTqPCR analysed samples; ordinary Two-way ANOVA followed by Dunnett’s multiple comparison test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001). Blue bars: wid-types and/or heterozygotes (siblings); red bars: mutants.
5.3. HIF signalling acts as negative regulator of steroidogenesis

5.3.1. arnt1 loss of function derepressed GC responsiveness and upregulates cyp17a2 expression

To better examine the interplay between HIF signalling and steroidogenesis, I initially tested the expression of pro-opiomelanocortin (pomca) and Cytochrome P450 family 17 polypeptide 2 (cyp17a2), via whole mount *in situ* hybridisation, on DMSO and BME [30 µM] treated 5 dpf arnt1 sibling and mutant larvae.

I have chosen these two probes because they are reliable and well-known readouts of HPI axis functioning and steroidogenesis. In particular, Pomca is the precursor molecule of the adrenocorticotropic hormone (ACTH), which is transported via the peripheral circulation to its effector organ, the interrenal tissue, where it stimulates the synthesis and secretion of GCs. Furthermore, it is mainly synthetized by the pituitary anterior lobe corticotropes and the intermediate lobe melanotrophs, in response to stressor recognition and corticotropin-releasing factor (CRF) release. Moreover, pomca is also negatively regulated by increased blood cortisol levels via GC-GR mediated negative feedback loop, as part of the HPI axis regulation (Griffiths *et al*., 2012; Ziv *et al*., 2012). For these reasons, its expression is a well-established readout of the HPI axis functioning in zebrafish larvae. Finally, previous study carried out in mice suggested that HIF promotes POMC activity in the pituitary gland (Zhang *et al*., 2011).

By contrast, Cyp17a2 plays a pivotal role in steroidogenesis, as it is one of the steroid-oxidizing cytochromes in the interrenal gland which catalyses the two-step oxidation reaction of progesterone and pregnenolone into 17-OH-progesterone and
17-OH-pregnenolone. For this reason, it is one of the main enzymes required for the conversion of cholesterol into cortisol in zebrafish, upon ACTH stimulation (Ramamoorthy and Cidlowski, 2016; Eachus et al., 2017; Weger et al., 2018).

As expected, data derived from whole mount in situ hybridisation analysis on 5 dpf larvae showed that arnt1 siblings displayed normally expressed cyp17a2 (figure 5.2C-C') and pomca expression (figure 5.2A), which were observed to be suppressed only as a result of BME treatment (figure 5.2D-D' and 5.2A'). Interestingly, on the other hand, 5 dpf arnt1−/− larvae displayed an upregulated cyp17a2 expression (figure 5.2E-E') coupled to downregulated pomca (figure 5.2B), compared to arnt1 siblings.

For this reason, since arnt1−/− larvae were characterized by an upregulated GC responsiveness (figure 5.1L), I hypothesize that in a HIF suppressed scenario, in the absence of functional arnt1, the observed pomca downregulation is most likely to occur as a result of the GC-GR induced negative feedback loop, triggered by the presence of putatively high cortisol levels (figure 5.5). Of note, if this was true, this would also explain the presence of high cyp17a2 expression levels observed in DMSO treated mutant larvae (figure 5.2E-E'), compared to arnt1 siblings (figure 5.2C-C')

Finally, in accordance with this hypothesis, I also speculate that the administration of an exogenous source of synthetic GCs, by signalling for the presence of upregulated pomca and high cortisol levels, triggers the downregulation of pomca at the level of the pituitary gland and consequently of the interrenal cyp17a2 gene even further (figure 5.2F-F' and 5.2B').
Figure 5.2 Representative pictures of WISH performed on DMSO and BME [30 µM] treated arnt1 mutant line at 5 dpf, using pomca as probe. arnt1 wt DMSO treated (n= 30/30 larvae) showed normal pomca expression; arnt1 wt BME treated (n= 29/30 larvae) showed downregulated pomca expression. In contrast, arnt1−/− DMSO treated (n= 28/30) and arnt1−/− BME treated (n= 30/30) larvae showed downregulated pomca expression. Chi-square test (****P < 0.0001). Scale bar 50 µm. Representative pictures of WISH performed on DMSO and BME [30 µM] treated arnt1 mutant line at 5 dpf, using cyp17a2 as probe. arnt1 wt DMSO treated larvae (n= 26/28) showed normal cyp17a2 expression, whereas 2/28 larvae showed a weaker one; arnt1 wt BME treated larvae (n= 28/30) showed downregulated cyp17a2 expression, whereas 2/30 larvae showed a normal one. In contrast, arnt1−/− DMSO treated larvae (n= 24/28) showed upregulated cyp17a2 expression, whereas 4/28 larvae showed a weaker one. arnt1−/− BME treated larvae (n= 25/29) showed downregulated cyp17a2 expression, whereas 4/29, showed a normal one. Chi-square test (****P < 0.0001). Scale bar 200 µm.
5.3.2. Overexpressed HIF signalling represses GC responsiveness and downregulates cyp17a2 expression

To further study the effect of HIF pathway on GC signalling, I subsequently investigated both pomca and cyp17a2 expression in the opposite - HIF upregulated - scenario, by carrying out in situ hybridization on the vhl mutant line. As expected, vhl siblings displayed normally expressed pomca (figure 5.3A) and cyp17a2 (figure 5.3C-C’), which were observed to be downregulated only as a consequence of BME treatment (figure 5.3A’ and 5.3D-D’).

Interestingly, on the other hand, 5 dpf vhl-/- larvae which were characterized by a suppressed GR activity (figure 5.11), showed downregulated cyp17a2 (figure 5.3E-E’), and downregulated pomca expression (figure 5.3B) compared to vhl siblings (figure 5.3C-C’ and 5.3A). Here, as previously monitored in the arnt1 mutant line, betamethasone treatment was observed to decrease cyp17a2 expression at the level of the interrenal gland even further.

Therefore, I speculate that in the absence of functional vhl (HIF upregulated scenario), proopiomelanocortin-a downregulation is most likely to occur as a consequence of a HIF-mediated downregulation of pomca expression (figure 5.5).

As a consequence of the above considerations, if this was true, I predicted to observe reduced levels of endogenous cortisol in vhl-/- larvae and normal or even increased cortisol levels in arnt1-/- larvae at 5 dpf.
Figure 5.3. Representative pictures of WISH performed on DMSO and BME [30 µM] treated vhl mutant line, at 5 dpf, using *pomca* as probe. DMSO treated *vhl* siblings (n= 26/28) showed normal *pomca* expression; BME treated *vhl* siblings (n= 28/30) showed downregulated *pomca* expression. In contrast, *vhl*−/− DMSO (n= 28/29) and BME (n= 28/28) treated larvae showed downregulated *pomca* expression. Chi-square test (****P < 0.0001). Scale bar 50 µm. Representative pictures of WISH performed on DMSO and BME [30 µM] treated *vhl* mutant line, at 5 dpf, using *cyp17a2* as probe. DMSO treated *vhl* siblings (n= 18/21) showed normal *cyp17a2* expression, whereas 3/21 larvae showed a weaker one; BME treated *vhl* siblings (n= 28/30) showed downregulated *cyp17a2* expression, whereas 2/30 larvae showed a normal one. On the other hand, *vhl*−/− DMSO treated larvae (n= 27/28) showed weak *cyp17a2* expression, whereas 1/28 larvae showed a normal one. *vhl*−/− BME treated larvae (n= 30/30) showed downregulated *cyp17a2* expression. Chi-square test (****P < 0.0001). Scale bar 200 µm.
5.3.3. Steroidogenesis is impaired in vhl mutant and is enhanced in arnt1 mutant zebrafish larvae

To confirm the hypothesis that overexpressed HIF levels negatively act not only on GC responsiveness but also on steroidogenesis, whereas arnt1 loss of function derepresses them, I quantified cortisol levels on the aforementioned vhl and arnt1 mutant lines, respectively. Three biological replicates of 150 larvae each for hif1ßh544 mutants, hif1ßh544 siblings, vhlu2117 mutants and vhlu2117 siblings at 5dpf were respectively used for steroid hormone extraction and quantification, as thoroughly described in the subchapter 2.6 of Material and methods. Interestingly, quantification analysis revealed that cortisol concentration was significantly reduced (P value <0.0028) in vhlu larvae (92,7 fg/larva), compared to vhlsiblings (321 fg/larva) (figure 5.4A). On the other hand, cortisol was significantly increased (P value <0.0001) in arnt1 mutants (487.5 fg/larva), compared to arnt1 siblings (325 fg/larva) (figure 5.4B).

Taken together, these data confirmed my hypothesis and showed for the first time that in zebrafish larvae the HIF signalling can act as negative regulator both of GR transcriptional activity and of steroidogenesis. Indeed, if only GR transcriptional activity was blocked by HIF, cortisol levels would be expected to be higher in vhl mutants. This is because by blocking GR (i.e. as occurs in gr−/− larvae), the GC-GR mediated negative feedback cannot occur anymore and this makes larvae hypercortisolaemic (Faught, Best and Vijayan, 2016; Facchinello et al., 2017). Importantly, since vhlu larvae are characterized both by downregulated GR transcriptional activity and decreased cortisol levels, this strongly indicates that HIF
signalling can act both at the level of the pituitary gland (to inhibit pomca expression) and intracellularly to block GR transcriptional activity itself.

Figure 5.4 A. Steroid quantification results showed a significantly reduced cortisol concentration (P value <0.0028) in vhl mutants (92.7 fg/larva, in triplicate), compared to vhl siblings (321 fg/larva, in triplicate) at 5 dpf. B. Furthermore, a significantly increased cortisol concentration (P value <0.0001) was quantified in arnt1 mutants (487.5 fg/larva, in triplicate), compared to arnt1 wild-types larvae (325 fg/larva, in triplicate) at 5 dpf; unpaired t-test (**P < 0.01; ****P < 0.001).
5.4. Discussion

Previous *in vitro* studies highlighted the potential for crosstalk between HIF and GC pathways. However, insights about how the interplay between these two major signalling pathways precisely occurs *in vivo* are still fragmentary and scarce. To this end, I was particularly keen to answer to the following question: is HIF pathway contributing to GC functions? If so, how is this occurring?

In this regard, I initially investigated how HIF signalling was able to affect the expression of selected GC target genes. To do so, I set up to perform RTqPCR analysis on two opposite mutant lines, characterised by an overexpressed (*vhl* `-/-`) and a downregulated (*arnt1* `+/-`) HIF pathway, respectively.

Collectively, my data show that the strong activation of the HIF signalling pathway, induced by *vhl* mutation, is able to blunt the GR transcriptional regulation, as judged by *fkbp5* expression. Vice versa, the inhibition of the HIF pathway, induced by *arnt1* loss of function, derepressed it. Furthermore, betamethasone treatment performed on the aforementioned mutant lines, followed by RTqPCR analysis, confirmed these data. Indeed, by triggering a further upregulation of the HIF signalling in *vhl* `-/-` larvae (as observed via hypoxia reporter expression), BME strengthens the HIF-mediated inhibition of the GC response, which in turn cannot be upregulated as strongly as occur in wild-types. In particular, this occur because the administration of synthetic GCs, by directly acting downstream of the HPI axis, is able to overcome the HIFmediate negative regulation of the GC signalling.

By contrast, in the *arnt1* loss of function scenario, since HIF signalling cannot blunt GC responsiveness anymore, BME can further upregulate GC reporters. Furthermore, since these experiments were carried out under normal atmospheric
oxygen conditions, it is plausible to infer that even the low normoxic HIF activity nonetheless suffices to attenuate GR transcriptional regulation.

Then, in order to check whether this was due to any potential effect played by HIF signalling on steroidogenesis (Tan et al., 2017) I quantified the expression of steroidogenesis-related genes (pomca and cyp17a2) both in vhl−/− and in arnt1−/− larvae, via whole-mount in situ hybridization analysis. Unexpectedly, both mutants showed downregulation of pomca. However, arnt1−/− larvae, which were characterized by an upregulated GC responsiveness, showed also an upregulated cyp17a2 expression. Vice versa, vhl−/− larvae which have a downregulated GC response, displayed a downregulated cyp17a2 expression.

As a consequence of the above considerations, taking into account the qPCR data on fkbp5 expression in both mutant lines, I assume that in an arnt1 knock-out scenario, the downregulation of pomca occurs as a consequence of the GC induced negative feedback loop, aimed to tune cortisol biosynthesis.

This assumption is also coherent with the upregulated basal cortisol levels quantified in these mutants. On the other hand, when HIF signalling is upregulated (in vhl−/−), I infer that the downregulation both of pomca and cyp17a2 may occur via HIF-mediated activity, which leads to the observed low cortisol levels and suppressed GR activity. Indeed, GCs control a plethora of physiological processes, act on almost every tissue and organ in the body to preserve homeostasis and have a strong anti-inflammatory and immunosuppressive activities. For this reason, the HPA/I axis must finely control their secretion (Oakley and Cidlowski, 2013).

Since previous studies in my laboratory demonstrated that GCs also act as HIF activators (Santhakumar et al., 2012; Vettori et al., 2017), I speculate that HIF pathway can in turn control cortisol levels by acting on pomca. This would allow HIF signalling
not only to monitor its own levels, but also to ensure homeostasis. Of note, the fact that HIF is particularly expressed in the brain, where it was found to directly act on POMC transcription to mediate glucose sensing in mice, (Sharp, Bergeron and Bernaudin, 2001; Baranova et al., 2007; Fan et al., 2009; Zhang et al., 2011), would allow to strengthen my hypothesis about the negative regulatory function played by HIF on pomca expression. Moreover, since the GC circadian production in teleost is tuned at the level of the pituitary gland and several studies described the presence of links between HIF signalling and circadian rhythms (Egg et al., 2013, 2014; Pelster and Egg, 2015; Peek et al., 2017), this further enhances the strict relationship observed between GC and HIF signalling pathways.

Finally, as HIF signalling is a primary controller of cellular pro-inflammatory responses to hypoxia (Imtiyaz and Simon, 2010; Eltzschig and Carmeliet, 2011; Palazon et al., 2014), which would counteract the anti-inflammatory activity played by GC, I infer that the concurrent expression of both upregulated HIF and GC pathways would be detrimental to homeostasis. In addition, since chronically elevated HIF level may have deleterious effects on an organism (i.e. on energy balance, immune system, blood circulation etc.), the need of HIF signalling to tune the stress response and avoid an uncontrolled systemic overexpression of the HIF pathway itself is warranted.

Importantly, my data would also be consistent with previous work which showed that exposure to hypoxia leads to downregulation of steroidogenic genes (StAR, cyp11c1, cyp19a, cyp19b, hmgcr and hsd17b2) in 72 hpf larvae, whilst zHIF-α loss of function triggered the upregulation especially of StAR, cyp17a1 and cyp11b2 (Tan et al., 2017).

In this regard, to further test my hypothesis and confirm my assumptions, I set up to extract and quantify cortisol levels both in a HIF upregulated (vht+/) and HIF
downregulated scenario (*arnt1*⁺⁻) in 5 dpf larvae. In particular, I predicted to observe reduced levels of endogenous GCs in *vhl*⁺⁻ and normal or even increased levels in *arnt1*⁺⁻ larvae. Importantly, the fact that cortisol levels were reduced in *vhl* mutant larvae and were upregulated in *arnt1* mutants, is coherent with my hypothesis.

Cumulatively, the HIF-mediated negative regulation of *pomca* seems to be a logic homeostatic interaction: cortisol increases HIF, which then reduces GR activity, that in the end should lead to less HIF signalling. For all these reasons, my data allow to fill the gaps and to provide a logic explanation on how this crosstalk may occur *in vivo* (figure 5.5).
Figure 5.5 Speculative schemes of how the putative HIF-GC crosstalk occurs in wildtypes and how it is affected both in arnt1/− and in vhl/− larvae at 5 dpf, after BME [30 μM] treatment. In all the cases, *since* betamethasone acts downstream of the HPI axis, by directly binding to Gr, it is able to upregulate GC target genes expression. Consequently, since GCs are able to stimulate the HIF signalling, as expected, I observed an increased *phd3:eGFP*-related brightness both in wildtypes and in vhl/−. However, the fact that I did not observe any HIF upregulation both in arnt1/− and in arnt1/−;vhl/− larvae, highlighted the fact that the BME induced HIF signalling activation is an Arnt1 dependent mechanism.
6. GENERAL DISCUSSION AND FUTURE WORK

Homeostasis is a state of optimal functioning in an organism, based on internal, physical and chemical conditions, which is maintained by several regulatory mechanisms aimed to assure both survival and correct functioning of all vertebrate species’ body (Biddlestone, Bandarra and Rocha, 2015).

In particular, one of the main stressors that perturbs homeostasis is represented by hypoxia. It is a common stressful condition to which cells must rapidly respond, which is characterized by the lack of appropriate oxygen supply to meet metabolic requirements (Bertout, Patel and Simon, 2008; Semenza, 2013). In this regard, one of the primary cellular apparatus that organisms developed to cope with reduced oxygen availability is represented by the hypoxia-inducible factor transcription factors (HIF) family. It consists of key regulators of the cellular response to low oxygen levels, whose aim is to promptly coordinate a series of metabolic changes (i.e. from aerobic to anaerobic metabolism) that allow the body to prevent metabolic shutdown and consequent death (Elks et al., 2015). Indeed, if this mechanism either fails or is maladaptive can lead to a wide range of pathological conditions including stroke, inflammation, tissue ischemia, and the growth of solid tumours. This is because, hypoxia shares an interrelated connection with inflammation, as inflammatory states during hypoxic conditions usually occur as a consequence of a broad range of human diseases (Bartels, Grenz and Eltzschig, 2013).

In this regard, synthetic GCs, which are analogous to naturally occurring steroid hormones, have been used for years as anti-inflammatory medications for treating pathological conditions that are linked to hypoxia (i.e., asthma, rheumatoid arthritis, chronic obstructive pulmonary disease, ischemic injury, etc.).
This is because GCs represent a well-known group of lipophilic steroid hormones synthetized and released by the interrenal tissue in teleosts, in response to stress. In particular, the latter is regulated by the hypothalamus-pituitary-interrenal (HPI) axis, which is the equivalent of the mammalian hypothalamus-pituitary-adrenal (HPA) axis. Moreover, cortisol is the main form of GC both in humans and teleosts and control a variety of physiological processes comprising inflammation, glucose homeostasis, stress response and intermediary metabolism.

In this respect, while several in vitro studies highlighted the presence of a crosstalk between hypoxia-inducible factors and GCs, (Kodama et al., 2003; Leonard et al., 2005; Wagner et al., 2008; Zhang et al., 2015, 2016) many questions remain unanswered and the argument is still debated. To this end, this study builds on previous knowledge regarding the interplay between these two major signalling pathways and provides further insights related to the role of HIF signalling on GC responsiveness and vice versa. Of note, it is particularly important to study and shed light on this discussed topic for the following reasons: a) by teasing out how this crosstalk occurs in vivo researchers might find new routes to downregulate HIF signalling for clinical purposes; b) by understanding how GCs act on HIF and vice versa, it will be also possible to reduce both the presence of adverse effects and the GC resistance, which nowadays limit their clinical use. Therefore, extending the research on how precisely this interplay occurs in vivo, may have a wide physiological significance in health and disease and may help researchers to develop more effective anti-inflammatory drugs in the future.

To this end, the zebrafish (Danio rerio) has been selected because it is an effective in vivo model organism to study how and to what degree hypoxic signalling affects the endogenous GC response and vice versa. Indeed, zebrafish share all the
components of the human HIF and GC signalling pathways and it has been demonstrated to be a very informative and genetically tractable organism for studying both hypoxia and the stress response both in physiological and pathophysiological conditions (van Rooijen et al., 2011; Santhakumar et al., 2012; Ziv et al., 2012; Elks et al., 2015; Faught and Vijayan, 2018b). In this regard, this thesis presents novel in vivo data, based on the use of zebrafish larvae, focused on the crosstalk between these two major signalling pathways.

As a base for starting the genetic analysis of this crosstalk, the generation both of arnt1 and gr null mutant lines was warranted in order to downregulate both HIF and GR signalling respectively. To achieve this, a discriminative experiment was designed to insert each mutation in the well-established vhl+/−(phd3:eGFP) mutant background (Rooijen et al., 2009; Santhakumar et al., 2012), where HIF signalling is strongly upregulated. The following step was to determine the importance both of arnt1 and arnt2 in the overall HIF response. To this end, phenotypic analysis carried out on 5 dpf double mutant larvae derived from the arnt1+/−;vhl+/− mutant line allowed to show the presence of a mild upregulation of the HIF signalling coupled to a more rescued phenotype in them, compared to the one observed in vhl−/− larvae. However, arnt1+/−;vhl−/− larvae beyond 5 dpf displayed only partial recovery from the vhl phenotype, as they developed well till 15 dpf, but then failed to grow and thrive compared to their wild-type siblings.

Another important aspect to consider is that unlike both vhl and arnt2 mutants, which are embryonic lethal by 8–10 dpf (Hill et al., 2009; Rooijen et al., 2009), arnt1−/− larvae showed to be viable and fertile. Furthermore, my data confirmed that even if Arnt1 is not essential for survival, it is necessary in the liver and in organs outside the central nervous system for HIF–α function. By contrast, it was possible to verify that
Arnt2 isoform is especially required in the developing central nervous system, in accordance with previous study (Hill et al., 2009). As a consequence of the above considerations, the similarities observed in terms of HIF reporter brightness both in \( arnt1^{-/-};vhl^{-/-} \) and \( arnt2^{-/-};vhl^{-/-} \) larvae (obtained via CRISPant technology) suggested that there is not a strong functional separation between the two isoforms. It is also possible to conclude that both Arnt1 and Arnt2 have partially overlapping functions \textit{in vivo} and that both contribute to assure the HIF response. However, a more in-depth analysis of the expression pattern of these two isoforms coupled to molecular analysis will help to elucidate which is their involvement in the HIF signalling at early developmental stages in zebrafish larvae.

With respect to the effect of HIF signalling and GC responsiveness, it was particularly interesting to note that a strong activation of the HIF signalling (in \( vhl^{-/-} \) larvae) can blunt GR transcriptional regulation as judged by RTqPCR analysis, whereas \( arnt1 \) loss of function is able to derepress it. Moreover, as my experiments have been performed at normal atmospheric oxygen conditions, it was possible to deduce that normoxic HIF activity nevertheless suffices to mitigate GR transcriptional regulation. This is important because there were no clear indications so far about the control mechanism performed by HIF on GC responsiveness. Indeed, the majority of the data about it derived from \textit{in vitro} studies, where no functional HPI axis with its GC-GR mediated negative feedback loop are present (Kodama et al., 2003; Leonard et al., 2005; Wagner et al., 2008; Gaber et al., 2011; Zhang et al., 2015). For this reason, showing and confirming the presence of this mechanism would be able to clarify how the crosstalk between HIF and GC pathway occurs \textit{in vivo}. Moreover, this would underscore a novel model of interaction between these two major signalling pathways.
In this regard, an initial confirmation of this hypothesis was obtained by quantifying the steroidogenesis-related genes expression (pomca and cyp17a2) both in vhl and in arnt1 mutant lines via whole-mount in situ hybridization. Indeed, both vhl\(^{-/-}\) and in arnt1\(^{-/-}\) larvae displayed a downregulated pomca expression at the level of the pituitary gland. However, arnt1\(^{-/-}\) larvae displayed upregulated cyp17a2 expression, which was instead downregulated in vhl\(^{-/-}\) larvae. Furthermore, by considering the quantitative analysis on GR target genes in these mutants, it was possible to infer that in an arnt1 knock-out scenario (where HIF is suppressed), pomca downregulation occurs as a result of the GC/GR-mediated negative feedback loop aimed to restrain cortisol biosynthesis. This was also confirmed by the presence of upregulated basal cortisol levels coupled to an increased GC responsiveness in this mutant line (figure 5.6). On the other hand, in vhl\(^{-/-}\) (where HIF is upregulated) the fact that both cortisol levels and GC response were downregulated in the presence of pomca and cyp17a2 inhibition, strengthened the hypothesis that this might occur due to a direct HIF-mediated activity on pomca expression.

Indeed, GCs control a plethora of physiological processes, act on almost all the tissues and organs in the body and have a strong anti-inflammatory and immunosuppressive actions. For these reasons, their production must be finely controlled by the HPA/I axis (R. Oakley, 2013). Moreover, even if it is rare and counterintuitive that a GC-resistant condition coincides with low cortisol levels (as observed in BME treated vhl mutants), I speculate that HIF signalling can act as a second controller of the GC-mediated stress response (in addition to cortisol levels themselves). Indeed, as previous work in my laboratory highlighted that GCs also act as HIF activators (Santhakumar et al., 2012; Vettori et al., 2017) I infer that HIF may in turn control cortisol levels by acting on pomca expression. This would allow HIF
signalling not only to manage its own levels, but also to assure both stress resolution and homeostasis. Of note, the reason for hypothesising that HIF signalling would counteract the anti-inflammatory GC activity, resides in the fact that the simultaneous expression of both upregulated HIF and GC pathway would be detrimental to homeostasis. Indeed, HIF is a master regulator of cellular pro-inflammatory responses to hypoxia, whereas GCs have a potent anti-inflammatory and immune suppressive activity.

Of note, my hypothesis would also be in accordance with a previous research showing that hypoxia exposure resulted in the downregulation of steroidogenic genes (StAR, cyp19b, cyp19a, cyp11c1, hsd17b2 and hmgcr) in 72 hpf larvae, whilst zHIF-α loss of function stimulated the upregulation specifically of StAR, cyp11b2 and cyp17a1 (Tan et al., 2017). Importantly, the fact that cortisol levels were reduced in vhl/− and upregulated in arnt1/− is consistent with my assumption. In addition, the fact that in teleost GCs biosynthesis is finely regulated by the hypothalamus-pituitary gland activity in a circadian way and multiple studies showed a direct connection between HIF signalling and circadian rhythms (Egg et al., 2013, 2014; Pelster and Egg, 2015; Peek et al., 2017), this further confirmed the tight interplay observed between GC and HIF signalling pathways. As a consequence of the above considerations, the HIF-mediated pomca negative regulation seems to be a logic homeostatic response.

Consequently, another important aspect to analyse in the future is related to the HIF signalling regulation of the GC responsiveness in the presence of arnt1 and/or gr loss of function (arnt1/−; vhl/− and gr/−; vhl/− larvae), where HIF pathway is partially attenuated. This would be particularly interest to achieve via cortisol quantification coupled to molecular analysis, because it will allow to deepen the knowledge on the HIF-GC crosstalk also in these intermediate scenarios.
With respect to the role of GCs in the HIF signalling, it is important to notice that 
gr mutation is able to generate an efficient, but not complete rescue of the vhl 
phenotype. Moreover, the fact that gr/;vhl/ survived much longer than vhl mutants 
(>21 dpf), and that similarly to arnt1/;vhl/, failed to grow and thrive when compared 
to siblings, confirmed it. It is also interesting to note that previous work from my lab 
(Vettori et al., 2017) established that activation of the GC signalling negatively 
regulates VHL in human liver cells. However, my current data derived from in vivo 
 genetic analysis on these mutants reveal that in zebrafish larvae there must be a 
different interaction’s point between these two pathways. This is because a further 
activation of the HIF reporter was observed following BME treatment in the absence 
both of Vhl and Vll. Cumulatively, my data allowed to show for the first time in an in 
vivo animal model that GR is fundamental to ensure high HIF signalling levels. This is 
interesting because by knowing the importance of GCs in ensuring HIF pathway, it 
would allow to use GR inhibitors to attenuate an overstated HIF response, and to elicit 
a more controlled reinstatement of the homeostasis. Nevertheless, since at present the 
precise molecular mechanism behind it is still unknown, more research towards this 
direction (i.e. ChIP seq, pull-down assay) is warranted.

Importantly, data related to the effect of betamethasone treatment in arnt1/- 
larvae revealed that although betamethasone can upregulate fkbp5 expression (Vettori 
et al., 2017), unexpectedly it failed to stimulate the HIF signalling. This was also 
observed in the arnt1/-;vhl/ larvae whose phd3:eGFP expression unvaried after BME 
treatment. Here, I speculate that this would be best explained by assuming that a BME- 
Gr complex would preferentially act on a HIFα/ARNT1 but not a HIFα/ARNT2 complex. 
However, more analysis is needed to test whether this occurs also in mammalian cells.
Finally, the importance of GCs in the HIF pathway was further analysed by studying the Mr contribution to the HIF signalling itself. Indeed, nowadays nothing is known about it. Moreover, a recent work by Faught and Vijayan, 2018 demonstrated that both Gr and Mr are involved in the regulation both of zebrafish stress axis activation and function. Surprisingly, the fact that mr CRISPR injection in a vhl\textsuperscript{-/-} background triggered a significant reduction of HIF reporter brightness, compared to uninjected vhl\textsuperscript{-/-} larvae highlighted for the first time \textit{in vivo} the importance of mineralocorticoid receptor in assuring high HIF levels. In addition, the further reduction of the HIF reporter expression following the additional removal of mr in a gr\textsuperscript{-/-};vhl\textsuperscript{-/-} background allowed to strengthen these data.

Cumulatively, it was possible to show that both the glucocorticoid receptor and mineralocorticoid receptor play a key role in enforcing the HIF signalling in the zebrafish. Moreover, this hypothesis is consistent with Faught and Vijayan, 2018 work, showing that both Gr and Mr signalling are involved in the regulation of the GC negative feedback. For these reasons, since HIF signalling plays a pivotal role in tumour growth and is proven difficult to downregulate \textit{in vivo}, this outcome can have a wider significance in health and disease. In this regard, my research indicates that the modulation of Gr and Mr might be a potential avenue.

Another important clinical application could be related to the mitigation of GC resistance. Indeed, it is well known that the loss of GC negative feedback, followed by hypercortisolaemia is linked to increased physiological stress and seems to contribute to anxiety, depression and post-traumatic stress disorders (Griffiths \textit{et al.}, 2012). However, recent studies have debated about cortisol’s role as pathophysiological benchmark of major depressive disorder, by stating that the link between GCs and depression is correlative rather than causal (Qin \textit{et al.}, 2019; Nandam \textit{et al.}, 2020). In
this regard, the use of Mr inhibitors in combination with iron chelators (i.e EDTA, deferiprone)/PHD inhibitors (i.e dimethyloxalylglycine, DMOG) could help stabilize HIF in order to both counteract the exacerbated GC production and to simultaneously avoid any possible cortisol-MR mediated activity. This could also be useful to reduce long-term use GC related side effects. On the other hand, since synthetic GCs are commonly used for acute and chronic inflammatory disorders treatments, a combined use with HIF blocking chemicals and/or oxygen treatment could help reducing HIF overexpression and to potentiate GC-related effects. In accordance with this assumption, the fact that dexamethasone (GC agonist) has been recently shown to decrease mortality in hospitalised patients with COVID-19 that require mechanical ventilation, is promising and deserves further researching.

Of note, future work that could bring to any potential drug development would include additional preclinical testing both in vitro and in vivo to better elucidate how HIF-GC interaction occurs specifically at molecular level. In particular, I believe that a multidisciplinary study characterized by molecular biology, biophysics (in vitro) and inflammation model (in vivo) approach could be exploited to this purpose. Firstly, the use of ChIP sequencing, proximity ligation assay and Fluorescence Resonance Energy Transfer (FRET) assay, could help uncovering which genes, domains, HRE/GRE are involved and if GC-GR and HIF physically interact. Secondly, to understand whether HIF-GR interplay occurs at DNA level or, as predicted by Kodama et al., 2003, via LBD, a GR-LBD plasmidic construct could be injected into gr<sup>-/-</sup> (phd3:eGFP) incross-derived zebrafish embryos following treated with a HIF activator drug (i.e FG4592 compound). Indeed, since gr<sup>-/-</sup> showed low HIF levels, the chemical treatment should not be able to upregulate HIF pathway in the uninjected group. Vice versa, if this occur in the injected group, it will mean that the HIF-GC interaction requires the GR-LBD to take place.
Thirdly, a mouse model for inflammation would help corroborating how HIF affects GC signalling and may have a strong clinical relevance. Indeed, what is clinically often an issue is that people with severe inflammatory phenotype are usually resistant to GCs. In this regard, since I observed that upregulated HIF levels suppress GR activity, being able to efficiently block HIF in the inflamed tissue could increase GC treatment efficacy, especially in resistant patients.

In conclusion, my study stresses the importance of the GC pathway in driving HIF signalling. In addition, a negative regulatory role performed by HIF in regulating both GR responsiveness and steroidogenesis was uncovered as shown via RTqPCR, WISH and steroid hormone quantification. A novel mineralocorticoid receptor contribution to the HIF-GC crosstalk was also highlighted. Here, although Mr contribution to HIF response in other organisms remains unclear, my data suggests that research into its function is warranted. Finally, original zebrafish mutant lines (gr+/-;vhl+/-, arnt1+/+;vhl+/- and arnt1+/-;gr+/-;vhl+/-) which helped to better comprehend how the interplay between HIF and GCs occurs in vivo were created and deeply described. For these reasons, I believe that this work could pave the way for further in vivo analysis aimed to accurately identify the broad crosstalk behind these two major signalling pathways.
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