Hypoxia and Chromatin, a focus on transcriptional repression mechanisms.

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Abstract

Hypoxia, or reduced oxygen availability, has been studied extensively for its ability to activate specific genes. Hypoxia induced gene expression is mediated by the HIF transcription factors, although not exclusively so. Despite the great knowledge on the mechanisms by which hypoxia activates genes, much less is known about how hypoxia promotes gene repression. In this review, we discuss the potential mechanisms underlying hypoxia-induced transcriptional repression responses. We highlight HIF-dependent and independent mechanisms, but also the potential roles of dioxygenases with functions at the nucleosome and DNA level. Finally, we discuss recent evidence regarding the involvement of transcriptional repressor complexes in hypoxia.

Keywords: Hypoxia; Chromatin; Transcriptional repression; Repressor Complexes; JmjC, Histone methylation, HIF
Introduction

Hypoxia

Decreases in oxygen availability are generally termed as hypoxia. These can occur at the organism level, such as when climbing high mountains, or at the cellular level, when oxygen supply is reduced and/or metabolic activity is high [1-3]. Changes at the cellular level in response to hypoxia are paramount for cellular and organismic survival [4].

To achieve a cellular response to hypoxia, cells have evolved mechanisms that impinge at all levels of gene expression regulation [2, 5], as well as energy conservation processes. These involve blocks in translation and the cell cycle, and switches in metabolic processes such as moving from oxidative phosphorylation to glycolysis [2]. A major coordinator of the cellular response to hypoxia is the transcription factor family, Hypoxia Inducible Factor (HIF).

The HIF family is composed by three different heterodimers, encompassing HIF-1β (gene name Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT), shared by all dimers), and HIF-1α; HIF-2α (gene name Endothelial PAS Domain Protein 1 (EPAS1)) and HIF-3α. Oxygen sensitivity is conveyed to HIF via the action of dioxygenases, most specifically Prolyl Hydroxylase Domain-Containing Proteins (PHDs) and Factor Inhibiting HIF (FIH) (reviewed in [4]). Proline hydroxylation of HIF-α in their oxygen degradation domain create a high affinity binding site for the tumour suppressor protein von Hippel Lindau (VHL), which is part of the E3-ubiquitin ligase complex containing cullin-2, elongin B/C and Ring-Box 1 (RBX1) [6]. VHL-dependent ubiquitination signals HIF-α for proteasomal and autophagy mediated degradation [6, 7]. On the other hand, FIH-dependent hydroxylation of HIF-α, results in an impairment
of the recruitment of the key coactivator protein CBP/p300, which is required for a minimum of 40% of all HIF-dependent genes to be expressed [8-11].

HIF mediated gene expression is largely achieved by direct binding of HIFs to the Hypoxia Response Elements (HREs) present in the regulatory region of target genes [12-19]. However, HIF binding is limited to just a few hundred of the 3.1 million RCGTG motifs present in the human genome [20]. Therefore, chromatin accessibility is one of major determinants of HIF binding, although not the only one, which provides a potential explanation for the differential genome-wide HIF binding profile and gene expression patterns in response to hypoxia observed across cell lines.

As mentioned above, HIF’s role in coordinating the cell’s response to hypoxia is achieved by transcriptional regulation. For the majority of cases analysed, HIF is an activator of transcription, with very few cases of direct transcriptional repression being described (see below). To activate gene expression, HIF has to engage with chromatin, in order to access its DNA binding sites across the genome. Several studies have analysed genomic wide binding site for HIFs in a variety of cellular backgrounds, identifying binding to genes HIF is known to transactivate but also in genes who’s expression is not altered in hypoxia [14]. As such, chromatin should be considered as an important player in the cellular response to hypoxia.

**Chromatin**

In mammalian cells, DNA is stored as chromatin. Chromatin is a complex and highly dynamic structure containing a mix of DNA and proteins. Chromatin is thus a fundamental regulator of cellular processes requiring access to DNA, including DNA-repair, DNA replication, and transcription. As mentioned before, gene transcriptional changes are a key part of the hypoxia response, and as such, delineating the crosstalk...
between chromatin structure and transcription is essential in understanding the cellular response to low oxygen stress.

The structural repeating unit of chromatin is a nucleosome, which consist of 147 base pairs of DNA wrapped 1.65 times around a histone octamer (reviewed in [21, 22]). The histone octamer consists of two copies each of the four core histones (Histone (H) 2A, H2B, H3 and H4), which forms as a result of tetramer dimerization between a H3/H4 and a H2A/H2B tetramer (reviewed in [23]). Nucleosomes are linked by linker DNA and linker histone H1 and condense to higher order chromatin structures, eventually forming chromosomes (reviewed in [24]). There are two major functional states of mature chromatin, heterochromatin and euchromatin (reviewed in [25]). Heterochromatin is a highly compact state which constitutes a barrier to DNA binding and is associated with silenced loci. Conversely, euchromatin has a more open conformation and is associated with actively transcribing and poised loci. Further to these two major conformations, microscopy techniques and biochemical assays have shown that chromatin structure is more complex, with additional chromatin compaction states [26-28]. In *Drosophila* cells, five distinct chromatin states have been identified through DamID assays followed by Chromatin Immunoprecipitation (ChIP) and microarrays [27]. These five states differ in protein binding, histone modifications, biochemical characteristics and transcriptional activity. On the other hand, researchers using a live cell quantitative FLIM–FRET based assay for chromatin compaction, reported three chromatin states based on spatial characteristics [26]. This study also found altered variations in the relative signals of the three types of chromatin state in response to ATP depletion, Trichostatin A (TSA) treatment and different stages of the cell cycle, supporting previous work on chromatin compaction dynamics. More recently the aforementioned technique has been used to measure chromatin
compaction in the model organism *C. elegans*, finding heterogeneous chromatin compaction at the whole organism level with nanoscale spatial and temporal resolution [28]. These studies among others demonstrate the complexity of chromatin organization in metazoan organisms, indicating the existence of intricate control mechanisms.

There are various interrelated mechanisms by which chromatin structure is regulated, including Chromatin Remodeller Complex (CRC) functions (reviewed in [29]), post translational modifications to histones (reviewed in [30]), incorporation of histone variants (reviewed in [31]), DNA methylation (reviewed in [32]), action of non-coding RNAs (ncRNAs) (reviewed in [33]) and chromatin architectural proteins (reviewed in [24]) (Figure 1). These mechanisms dictate the chromatin landscape, which is a key determinant in the transcriptional output of the cell and thus cell fate decisions. Chromatin is responsive to numerous stimuli and developmental cues [34] and is often deregulated in disease (reviewed in [35]).

**Figure 1. Chromatin structure.** Simplified linear diagram of chromatin highlighting the main mechanisms by which chromatin structure is regulated. Chromatin Remodeller Complex (CRC), post translational modification (PTM), non-coding RNAs, (ncRNAs).
An emerging field is the study of chromatin structure in response to hypoxia, where some experimental evidence is now being published.

**Hypoxia induced chromatin changes**

Hypoxia has been shown to induce changes in chromatin structure, most notably histone methylation, acetylation, and DNA methylation. In this review, we will focus on methylation.

Less is known pertaining to chromatin compaction states in response to low oxygen stress. Through the use of Single Molecule Localisation Microscopy (SMLM) and *in situ* DNA digestion coupled with fluorescent microscopy, a rapid change in chromatin architecture and increase in chromatin compaction has been reported in human cardiomyocytes deprived of oxygen and nutrients [36]. Interestingly, the change in chromatin architecture was found be rapidly reversible in response to reoxygenation and replenishment of nutrients, demonstrating the dynamic capacity of chromatin to sense and respond to oxygen and metabolic changes [36]. Another study determined that A431 cancers cells treated with 0.1% oxygen for 48 hours have reduced sensitivity to Mononuclease digestion, suggesting increased heterochromatin composition [37]. Through the use of proteomics, this study also identified an increase in Heterochromatin Protein 1 Binding protein 3 (HP1BP3) in the chromatin bound fraction of cells treated to hypoxia. HP1BP3 has previously been shown to function in maintaining heterochromatin integrity, thus could be a player in inducing hypoxic chromatin compaction [37, 38].

Chromatin looping, which brings distal sequence regions together, represents additional mechanisms by which transcription is regulated by chromatin architecture.
Further to proximal promoter binding at HRE sites, HIF-1α and HIF-2α also bind to intergenic regions of the genome [12, 14, 41, 42] and there is evidence of HIF binding regulating distal gene expression through Promoter Enhancer Interactions (PEIs) [14]. Work from the Ratcliffe and Mole laboratories, utilising ChIP sequencing and Capture C in MCF7 cells treated to 0.5% for 16 hours, has revealed genome wide HIF binding-HIF regulated gene PEIs [41]. This study, and others, also elucidated that HIF promoter binding in hypoxia is predominantly at pre-established and primed, promoter enhancer loops [41, 43].

Despite the increase in evidence for chromatin regulation in hypoxia, there is still a great deal of unknowns. The use of imaging and sequencing technologies to study chromatin spatial organization should be used to gain further insight into the dynamic interplay between hypoxia, chromatin and gene transcription. This would help elucidate how chromatin contributes to gene repression in hypoxia.

**Histone methylation-focus on repression**

Histone methylation is a dynamic and reversible post translational modification at lysine (K) and arginine (R) N terminal tails of histones. These modifications can provide binding sites for chromatin binding proteins and the histone methylation landscape is predictive of gene transcriptional state, transcription factor binding and chromatin compaction [44, 45]. H3K4, H3K9, H3K27, H3K36, are amongst the most common and well-studied histone methylation sites. H3K9 di-methylation (me2)/tri-methylation (me3) and H3K27me3 are linked to transcriptional repression and are key players in cell fate decisions and tissue specific transcriptional control (reviewed in [46]). H3K9me2/3 are markers of heterochromatin and are found at coding and non-coding regions (reviewed in [47]). Both modifications are associated with gene
silencing via crosstalk with DNA Methyl Transferases (DNMTs) and recruitment of
other chromatin modifying protein such Heterochromatin protein 1 (HP1) which can
which can regulate heterochromatin formation (reviewed in [48]). H3K27me3 is
located primarily at gene promoters of open chromatin and is involved in gene
repression through recruitment of Polycomb Repressive Complexes (PRCs) (reviewed
in [49]). H3K27me3 also marks poised enhancers and co-occupies promoters with the
active histone modification H3K4me3, termed bivalent promoters (reviewed in [50].)

Histone Methyl Transferases (HMTs) add methyl groups to histones, transferring a methyl group from S Adenosyl Methionine (SAM) (reviewed in [46]). Two families of enzymes remove histone methylations. LSDs target H3K4me1/2 and H3K9me1/2 through a Flavin Adenine Dinucleotide (FAD) dependent amine oxygenase reaction [51]. Jumonji C (JmjC) histone demethylases target a much broader range of histone targets (reviewed in [52, 53]). The later are molecular dioxygenases, requiring oxygen, iron and 2-oxoglutarate for demethylation. This oxygen dependency of JmjC histone demethylases provides an important link to chromatin structure and oxygen sensing (reviewed in [52, 53]). The writers and erasers of H3K9 and H3K27 methylation are shown in Table 1.
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**Table 1. Writers and erasers of H3K9 and H3K27 methylation.** Histone Methyltransferases (HMTs), Jumonji C (JmjC) histone demethylases and Lysine Specific Demethylases (LSDs) targeting H3K9 and H3K27 are shown (targets are in brackets). Euchromatic Histone Lysine Methyltransferase 2 (EHMT2, G9α), G9α Like Protein 1 (GPL), Suppressor Of Variegation 3-9 Homolog (SUV39H), SET Domain Bifurcated 1 (SETDB1), PR/SET Domain (PRDM), Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2), Lysine Demethylase (KDM), PHD Finger Protein (PHF), Myc-Induced Nuclear Antigen (MINA).
Hypoxia induced increases in both active and repressive histone methylations have been shown in several human cancer lines, mouse embryonic fibroblasts and human tumour samples [54-59]. With regards to repressive modifications, H3K9me2 and H3K27me3 increased in Hepa 1-6 cells exposed to 0.2% oxygen for 48 hours [54]. H3K9me3 increases were also observed in RKO cells exposed to 2% and 0.1% oxygen, these changes were rescued upon reoxygenation [59]. Additionally, H3K9me2/me3 increases have were shown in mouse macrophages exposed to 1% oxygen for 24 hours [56]. Total H3K9me2/me3 levels were also elevated in A549 cells exposed to 0.5% oxygen, as well as site specific increases at the hypoxia repressed gene promoters, MutL Homolog 1 (MLH1) and Dihydrofolate Reductase 2 (DHFR2) [58]. Here researchers detected increased H3K9me3 after 90mins of 0.5% oxygen exposure, this is the only such study which has investigated histone methylation levels in response to acute hypoxia. Whilst the aforementioned studies investigated total levels of histone modifications and some site-specific changes, Prickaerts et al elucidated specific increases in H3K27me3 and H3K4me3 in response to hypoxia on a genome wide scale through the use of ChIP sequencing integrated with microarray analysis in MCF7 cells exposed to oxygen deprivation and reoxygenation [57]. Many of these changes were reversible upon reoxygenation and showed correlation with transcriptional changes in hypoxia. Interestingly, the researchers uncovered evidence for hypoxia acquired promoter bivalency modulating poised/active gene transcriptional control. Xenografts of human breast and lung cancer were also found to have increased H3K27me3 and H3K9me3 respectively [57, 59].

Mechanistically, hypoxic induction of histone methylation levels has been attributed to inhibition of JmjC histone demethylases [57, 58]. JmjC histone
demethylases are oxygen dependent enzymes and also have a catalytic fold similar to that of FIH [60, 61]. Whilst oxygen dependency of the majority of JmjC enzymes has not been established, *In vitro* histone peptide methylation assays and histone methylation assays from cell lysates have demonstrated the potential of some of these enzymes to function as bona fide oxygen sensors [57, 58, 62, 63]. Among these, the H3K27 demethylase KDM6B and the H3K9 demethylase KDM4E were inhibited over physiologically relevant oxygen concentrations, the latter displaying similar oxygen dependency kinetics to PHD2 [63]. Whilst the H3K9 demethylase KDM4A has a reported km for oxygen of 173 +/- 23µM, placing it between FIH and PHDs with regards to oxygen dependency [62]. Furthermore, increased H3K9me3 in U2OS cells overexpressing KDM4A was reduced in a stepwise fashion upon exposure to 5, 1 and 0.1% oxygen [62]. These studies demonstrate a role of JmjC enzymes in functioning as cellular oxygen sensors.

Many JmjC histone demethylases are transcriptionally upregulated in hypoxia, some of which are direct HIF target genes (reviewed in [53, 64]). It is speculated that upregulation of JmjC histone demethylases in response to low oxygen may be a feedback mechanism to help maintain to retain histone methylation status of the cell. However, it should be noted that there is evidence for JmjC histone demethylases remaining active in response to low oxygen stress and mediating the hypoxic response via histone demethylation [65, 66]. KDM4C and KDM3A were found to bind HIF-1α and function as an enhancer of HIF-1α transactivation activity in hypoxia via H3K9 demethylation at hypoxia responsive promoters. Thus, determining the dynamics of JmjC histone demethylase oxygen sensitivities, activities in different oxygen environments and cell backgrounds, and consequences this has on hypoxic gene transcription is needed. Non histone targets and histone methylation independent
functions of JmjC histone demethylases should also be considered when investigating their possible roles in hypoxia induced transcriptional repression (reviewed in [53]).

Pertaining to HMTs and histone methylation in hypoxia, G9α (gene name Euchromatic Histone Lysine Methyltransferase 2 (EHMT2)) protein levels are induced post transcriptionally in hypoxia [58]. G9α was recently identified as a PHD1 target for hydroxylation and is degraded by in a VHL dependent manner [67]. Moreover, via promoter H3K9me2, G9α mediates transcriptional repression at a subset of hypoxia repressed genes and there is growing evidence for chemotherapeutic benefit in targeting G9α through dysregulation of hypoxic gene expression [67-69]. SET Domain Bifurcated 1 (SETDB1) and Suppressor Of Variegation 3-9 Homolog (SUV39H) 2 protein levels are also stabilised is low oxygen stress and hypoxia induced, SETDB1 mediated H3K9me3 upregulation on the ATM And p53-Associated KZNF Protein (APAK) gene reduces its expression [70]. This triggers an increase in p53-dependent hypoxia-induced apoptosis and manipulation of this pathway deregulates cell viability in hypoxia [70]. Elevated H3K9me3 in hypoxia is also required for Ataxia telangiectasia mutated (ATM) activation in the absence of DNA damage, facilitating DNA replication in a low oxygen environment [59]. This study suggests a mechanism for H3K9me3 mediated activation of ATM in hypoxia involving transcriptional repression of ATM-specific phosphatases, including Protein Phosphatase 2 (PP2A). Developmental importance of H3K9 methylation and hypoxia is shown by SUV39H1 and SUV39H2, which are hypoxia inducible, and loss of their expression is associated with the epigenetic changes required during foetal lung development [71].

Given the recent evidence of hypoxia induced histone methylation changes, further analysis on how histone methylation contributes to the transcriptional repression observed in hypoxia is therefore necessary.
Chromatin remodellers in hypoxia-focus on repression

Hypoxia engagement with chromatin remodellers has been analysed mostly in the context of transcriptional activation. Four main families of chromatin remodeller can be found in mammals. These are SWItch/Sucrose Non-Fermentable (SWI/SNF), Chromodomain Helicase DNA-binding CHD (CHD), Inositol-requiring 80 and Imitation SWI families (Figure 2) [52, 53]. Many more sub-complexes exist, giving rise to increased complexity in function and regulation (reviewed in [72]).
Figure 2. Mammalian Chromatin remodeller complexes. Chromatin Remodeller Complexes (CRCs) from the 4 subfamilies of CRCs based on ATPase domains are shown. A) SWItch/Sucrose Non-Fermentable (SWI/SNF), B) Imitation SWI (ISWI), C) Inositol-Requiring 80/ Sick With RSC (INO80/SWR), D) Chromodomain Helicase DNA-binding CHD (CHD). Rectangles represent ATPase domain. Brahma (BRM) BRM Related Gene 1 (BRG1), BRG1/BRM Associated Factor (BAF), Polybromo
Associated BAF Complex (PBAF), Snf2 Related CREBBP Activator Protein (SRCAP), Tat Interacting Protein 60 (TIP60), Nucleosome Remodeling Deacetylase (NuRD), Chromatin Accessibility Complex (CHRAC), Remodeling And Spacing Factor (RSF), Nucleolar Remodeling Complex, Cat Eye Syndrome Chromosome Region Candidate 2 (CERC2), CECR2 Containing Remodeling Factor (CERF), Nucleosome Remodeling Factor (NURF), B-Cell CLL/Lymphoma (BCL), Bromodomain Containing (BRD), Polybromo 1 (PBRM1), RuvB Like AAA ATPase (RUVBL) (RVB), Actin related protein (ARP), Ubiquitin C-Terminal Hydrolase L5 (UCH37 Microspherule Protein 1 (MCRS1), Nuclear Factor Kappa B Subunit (NF κβ), DNA Methyltransferase 1 Associated Protein 1 (DMAP1), Glioma-Amplified Sequence 41 (GAS41), Vacuolar Protein Sorting 72 (VPS72), Zinc Finger HIT-Type Containing 1 (ZNHIT1), H2A Histone Family Member Z (H2AZ), Transformation/Transcription Domain Associated Protein (TRRAP), MORF-Related Gene 15 Protein (MRG15), MRG Domain Binding Protein (MRGBP), Esa1 Associated Factor 6 (EAF6), Inhibitor Of Growth Family Member 3 (ING3), Histone Deacetylase, (HDAC), Metastasis Associated (MTA), Retinoblastoma-Binding Protein (RBAP), ATP-Dependent Chromatin Assembly Factor 1 (ACF1), Sucrose Nonfermenting Protein 2 Homolog (SNF2H), Sucrose Nonfermenting 2-Like Protein 1 (SNF2L), Williams Syndrome Transcription Factor (WSTF), Bromodomain PHD Finger Transcription Factor (BPTF).

With relevance to the hypoxia signalling pathway, the SWI/SNF family seems to be particular important, with high level of human mutations found in renal clear cell cancer, where VHL is also found highly mutated [73]. SWI/SNF complex (Figure 2A) can be subdivided into two sub-complexes called BRG1/BRM Associated Factor (BAF) and Polybromo Associated BAF Complex (PBAF). These are defined not only by their catalytic subunits (BRM Related Gene 1 (BRG1) or BRM) but also by differences in assessor factors such as BAF250/BAF250B (BAF), and Polybromo 1 (PBRM1) (PBAF) [74].

It is known that hypoxia engages and requires SWI/SNF for activation of HIF and its targets [53, 75]. However, there is no indication so far that SWI/SNF can be...
involved in hypoxia mediated repression and has been associated only with transcriptional activation in hypoxia.

Perhaps the chromatin remodelling family most often associated with transcriptional repression is ISWI (Figure 2B). In mammalians, it is subdivided into several additional complexes depending on assessor partners for the two catalytic subunits Sucrose Nonfermenting Protein 2 Homolog (SNF2H) and Sucrose Nonfermenting 2-Like Protein 1 (SNF2L) (Figure 2B). ISWI is known to control nucleosome spacing and this has been shown to have a cooperative action with another family of remodellers called CHD [76]. ISWI also associates with additional factors and is important for the function of CTCF in establishing chromatin barriers [77].

ISWI has been shown to contribute to the cellular response to hypoxia by controlling levels of FIH, and hence some of the HIF-1 dependent targets [78]. In addition, it also controls the levels of FIH-independent targets suggesting a broader role in transcriptional regulation in hypoxia [78]. Interestingly, ISWI was identified as part of a complex containing the transcriptional corepressor C-terminal Binding Protein (CtBP) [79]. CtBP has been associated with the transcriptional response to hypoxia through a variety of studies [80-84]. It is thus possible that ISWI action in hypoxia, is connected to CtBP, however, this has not been formally addressed.

INO80 family (Figure 3C) comprises of a large family of remodellers with the catalytic subunits INO80 and Snf2 Related CREBBP Activator Protein (SRCAP), characterised by Rec-like helicase domains [85]. This family is known to be involved in nucleosome sliding and histone exchange [86]. Associations with the hypoxia response have been found, but whether the chromatin remodelling aspect of the complex is required is still not clear. Components of the Tip60 complex, more
specifically Pontin (gene name RuvB Like AAA ATPase (RUVBL)1) and Reptin (RUVBL2) have been associated with the modulation of the cellular response to hypoxia. In hypoxic conditions, Reptin is methylated at lysine 67 by the methyltransferase G9α and this modification allows its binding to HIF-1α and its recruitment to the promoters of hypoxia-responsive genes where it negatively affects their transcription [87]. Consequently, Reptin deletion results in enhanced induction of a subset of HIF targets, suggesting that the axis G9α/Reptin may work as a negative feed-back loop that acts to limit HIF activity. However, since these studies focused on the role of Reptin on genes upregulated by HIF, its potential role on gene repression under hypoxia remains unexplored. Similarly, methylation of Pontin by G9α induced by hypoxia, also potentiates HIF-mediated activation [88], however, no data was provided regarding the role of Pontin in hypoxia mediated repression in this study.

At present there are no studies concerning the role of CHD family in the response to hypoxia.

**DNA methylation in hypoxia**

One of the most studied aspects of chromatin changes and transcriptional repression mechanisms is DNA methylation. DNA methylation, classically occurs at CpG islands present at promoters, leading to inhibition of promoter activity via failing to recruit specific transcription factors or by active recruitment of transcriptional repressor complexes [89]. DNA methylation is set by DNMTs, of which three have been described in humans, DNMT1, DNMT3A, and DNMT3B [90, 91]. As with most processes in the cell, DNA methylation is a reversible state, through the involvement of specific enzymes called Ten-Eleven-Translocation (TET) [5, 90]. These enzymes remove DNA methylation by hydroxylation of 5-methylcytosine, followed by further
potential oxidation reactions. These additional modified bases are removed by thymine DNA glycosylase and base excision repair pathways [5].

DNA methylation in hypoxia has been recently studied due to the realisation that TET (TET1, TET2 and TET3) enzymes are dioxygenase enzymes, requiring oxygen, 2-oxoglutarate and iron for their catalytic activity [5]. A study using in vitro models, but also tumour hypoxia in patient samples, demonstrated that hypoxia does alter global levels of DNA methylation, irrespectively of proliferation or metabolism [92]. This study showed that hypoxia increased DNA methylation across promoters, in a manner that was dependent on TET enzymatic activity [92]. Since, TET enzymes require oxygen for their function, by default hypoxia should increase DNA methylation across the genome.

DNA methylation changes at particular loci may also be altered by hypoxia in an indirect manner via altered recruitment of DNMTs. For example, DNMT3 enzyme are targeted to loci marked with unmethylated H3K4, or H3K36me3 [90] and H3K9 methylation is strongly linked to DNA methylation via DNMT recruitment [48]. Since, JmjC containing histone demethylases are also oxygen sensitive induces H3K4me2/me3, H3K36me3 and H3K9me3 [53], it is possible to speculate that hypoxia would lead to decreased recruitment of DNMT3 at promoters marked H3K4me3, but would increase the methylation across gene bodies, marked with H3K36me3 and loci marked with H3K9me2/me3 (Figure 3). However, such studies have not been performed and as such further investigation is required for this to be formally demonstrated.
Figure 3. Dynamics of DNA methylation. DNA methylation is performed by DNMT enzymes and removed by Ten-Eleven-Translocation (TET) enzymes. DNMTs target areas of unmethylated H3K4 and trimethylated H3K36 or di/trimethylated H3K9.

HIF-dependent mechanisms of repression

As mentioned above, in contrast to gene upregulation, the mechanisms by which hypoxia leads to gene repression are not well understood. In some sporadic cases, direct HIF binding could mediate the transcriptional repression, as suggested for the regulation of Carbamoyl-Phosphate Synthetase 2, Aspartate Transcarbamylase, And Dihydroorotase (CAD) [93], Alpha Fetoprotein (AFP) [94], Adenosine Kinase (AK) [95], Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) [96] and APC, Adenomatous Polyposus Coli (APC) [97] genes. It has been proposed that HIF binding could displace transcriptional activators, such as MYC Proto-Oncogene, BHLH Transcription Factor (MYC), or recruit co-repressors that could account for down-regulation of gene expression [98]. However, these cases appear to be sporadic as genome-wide mapping of HIF-binding sites was unable to detect a significant association between gene repression and proximal HIF binding [12, 13, 15-19]. Interestingly, knock-down of HIF isoforms prevents the majority of the
effects of hypoxia on both gene induction and repression [12, 99]. Thus, for the large
majority of genes, hypoxia-triggered gene repression is likely to be indirectly mediated
by HIF probably through trans-acting elements. In keeping with this hypothesis, HIF-
1α directly regulates the expression of several sequence-specific repressors such as
the MAX Interactor 1, Dimerization Protein (MXI1) gene, which encodes a repressor
of MYC, thus leading to the repression of MYC targets such as PPAR-Gamma
Coactivator 1-Beta (PGC-1β) [100]. Similarly, the transcriptional repressors Basic
Helix-Loop-Helix Family Member E40 (BHLHE40) [101] and BTB Domain And CNC
Homolog 1 (BACH1) [102] are a direct HIF targets. BHLHE40 induction by hypoxia
results in the repression of Peroxisome Proliferator Activated Receptor Gamma 2
(PPARG2) [103], Sterol Regulatory Element Binding Transcription Factor 1 (SREBP1)
[104], Signal Transducer And Activator Of Transcription 1 (STAT1) [105] and
Melanogenesis Associated Transcription Factor (MITF) [106] genes, while induction
of BACH1 results in Heme Oxygenase 1 (HMOX1) repression [102]. Although these
individual gene studies suggest a role for these repressors in the response to hypoxia,
their relative contribution to the global transcriptional repression has only been
analysed for MXI1 [107, 108]. These studies found that MXI1 knockdown had very
little effect on the hypoxic transcriptome, suggesting functional redundancy with other
repressors of the Mad family. Alternatively, it could be that MXI1 is just one of many
repressors downstream of HIF, each one acting on a small fraction of the genes
repressed by hypoxia, additional studies are therefore required to fully answer this
question.

Interestingly, BHLHE40 was shown to inhibit myogenic differentiation in
response to hypoxia through the induction of Myogenin (MYOG) independently of HIF
[109], suggesting that BHLHE40 has a major role in hypoxia-triggered gene repression
acting in pathways both HIF-dependent and independent. In addition to BHLHE40, the Repressor Element 1-Silencing Transcription Factor (REST) accumulates in the nucleus in response to hypoxia and acts as a key repressor of the hypoxic transcriptome in a HIF independent manner [110].

Finally, several studies have established a role of hypoxia and HIF in the regulation of specific microRNAs, particularly miR-210, which act to repress gene expression by inducing mRNA decay and/or inhibiting their translation [16, 111, 112]. Thus, at least part of the gene repression observed under hypoxia, could occur at the post-transcriptional level.

Transcriptional repression complexes in hypoxia

Sin3A-HDAC

Although it is known that HIF recruits Histone Deacetylases (HDACs) and, intriguingly, HDAC inhibitors prevent HIF-mediated transcription (reviewed in [113]), little attention has been paid to the role of these co-repressor complexes in the transcriptional response to hypoxia. The class I HDACs HDAC1, HDAC2 and HDAC3 are ubiquitously expressed nuclear enzymes that are components of multiprotein repressor complexes. These include SIN3 Transcription Regulator Family Member A (SIN3A), nucleosome-remodeling HDAC (NuRD) and CoREST; the two highly related HDAC3-containing complexes nuclear receptor co-repressor (NCoR or NCOR1) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT or NCOR2) [114]. However, to date the only the HDAC-containing co-repressor complex whose function in hypoxia has been analyzed at global scale is SIN3A.

The SIN3 protein is highly conserved from yeast to mammals. It is a central component of the SIN3 corepressor complex that participates in a wide variety of
processes including development, energy metabolism, cell growth and differentiation and as well as several pathological conditions including oncogenic transformation [115]. In mammals there are two SIN3 isoforms, SIN3A and SIN3B, encoded by separate genes, that are widely expressed and bind common as well as distinct transcriptional repressors and complexes (reviewed in [115]).

A recent bioinformatics approach found that the factor SIN3A was overrepresented in the proximity of genes whose transcription is repressed by hypoxia [108]. This study also identified enrichment for HDAC1, HDAC2 and Sap30, all components of the SIN3A co-repressor complex, and the sequence-specific repressors MXI1, Max, E2F4 and E2F6, known to interact with the SIN3A complex. In agreement with these computational predictions, knock-down of SIN3A significantly attenuated the repression of over 75% of the genes that were down-regulated in control cells, suggesting a major role for this co-repressor complex in this process [108]. However, several lines of evidence indicate that the function of SIN3A in the control of transcription is more complicated than previously anticipated. On the one hand, the genome-wide binding pattern of SIN3A showed a strong enrichment for this factor in active promoter regions, with SIN3A signal centered at the transcription start site (TSS) of actively transcribed genes and absent from genes with low or undetectable expression. On the other hand, SIN3A depletion not only affected gene repression, but also diminished the induction of about 47% of the genes upregulated by hypoxia. These results suggest that, beyond its function as co-repressor, SIN3A has wider role on transcriptional regulation. In agreement, SIN3A was initially described in yeast as a protein with dual functions as activator and repressor [116, 117] and recent studies are putting forward its role as an activator of specific genes [86], including targets of the aryl hydrocarbon receptor (ARNT), a transcription factor
related to HIF, known to mediate the transcriptional response to xenobiotics [118]. Moreover, the dual role of the Sin3A in transcriptional repression and activation in response to hypoxia is not unique to this repressor complex; it has been reported that knock-down the repressor HEXIM1 affects similar number of genes repressed and induced by hypoxia [119]. Furthermore, close analysis of results obtained by Casciello et al [67] indicate G9α also possess dual functions. In fact, emerging models propose that recruitment of both co-repressor and co-activator complexes is needed for gene induction in general [120]. As such further scrutiny is required for the understanding of how these complexes promote or repress transcription at particular target genes.

**REST**

Perhaps the transcription silencer mostly associated with hypoxia in recent years has been REST. Initially associated with neuronal development [121], its functions have now been extended to multiple cell types including cancer cells [122], cardiac [123] and beta cells [124].

REST mediates transcriptional repression via several mechanisms. Via its zinc fingers it is able to bind to repressor elements; via its N-terminus recruits SIN3A complex and via its C-terminus recruits CoREST, HDACs, LSD1, G9α and Methyl-CpG Binding Protein 2MeCP2 (reviewed in [124]). A role for CoREST and its associated histone demethylase LDS1, has been described for the repression of the gene *MLH1*, a key component of the DNA mismatch repair system, in response to hypoxia [125]. Interestingly, the CoREST complex is recruited by REST sequence-specific factor to repressed genes and, in addition to LSD1, CoREST complexes also interact with the G9α histone methylase. Thus, given the role of CoREST in *MLH1* down-regulation and the effects of REST and G9α in the repression of genes under
hypoxia described above, it would be interesting to investigate the role of this co-repressor in the global response to hypoxia.

REST was shown to be repressed by an hypoxia induced micro-RNA, miR-106b~25 cluster, in advance prostate cancer [126]. However, more recent studies have shown that REST is involved in transcriptional repression in hypoxic conditions [110, 127-129]. Cavadas et al [110] demonstrated that hypoxia can induce nuclear translocation of REST and importantly, REST is required for the repression of around 20% of hypoxia repressed genes in HEK293 cells. This data indicate the importance of this transcriptional repressor but also highlight that other mechanisms are important for transcriptional repression in hypoxia. Furthermore, it is tempting to speculate that REST’s importance in contributing to transcriptional repression following hypoxia, might be dependent on the cell type. Given REST importance in neural and cardiac tissue (two tissue very sensitive to changes in oxygen supply), it is possible that REST contribution to the hypoxia response in these tissues could be even more prevalent. However, further studies are necessary to establish if this is the case.

**Perspectives/Conclusions**

As mentioned above, although much is known regarding hypoxia induced gene expression, less is so for hypoxia induced gene repression (Figure 4). With the discovery of a variety of dioxygenases impinging on several aspects of gene regulation such as histone and DNA methylation, there is now a suggestion for these to be involved in transcriptional repression in response to reduced oxygen. However, further studies are needed to address this. Similarly, some repressor complexes have been associated with the regulation of the hypoxia response. However, these are still just the tip of the iceberg. Whilst H3K27me3 is levels are induced by hypoxia [54, 57], and
H327me3 is associated with Polycomb repressive complexes (PRCs) [49], there is no evidence for activation of gene silencing mechanisms involving PRCs in response to hypoxia. This is also extended to chromatin remodelers associated with repression of transcription, where much more work is needed to explain how hypoxia is able to repress as many genes as it induces.

Figure 4. Mechanisms of hypoxic gene repression in the context of chromatin structure. Hypoxia Inducible Factor (HIF), Repressor Element 1-Silencing Transcription Factor (REST), SIN3 Transcription Regulator Family Member A (SIN3A), Histone Deacetylase, (HDAC), G9α Like Protein 1 (GPL), Suppressor Of Variegation 3-9 Homolog (SUV39H), SET Domain Bifurcated 1 (SETDB1), Chromatin Remodeller Complex (CRC), Jumoni C (JmjC), DNA Methyl Transferase (DNMT), Ten-Eleven-Translocation (TET), Lysine Specific Demethylase 1 (LSD1).
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Author Contributions

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Conflicts of Interest

The authors declare no conflict of interest.

References


