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The regulation of transcriptional repression in hypoxia

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Abstract
A sufficient supply molecular oxygen is essential for the maintenance of physiologic metabolism and bioenergetic homeostasis for most metazoans. For this reason, mechanisms have evolved for eukaryotic cells to adapt to conditions where oxygen demand exceeds supply (hypoxia). These mechanisms rely on the modification of pre-existing proteins, translational arrest and transcriptional changes. The hypoxia inducible factor (HIF; a master regulator of gene induction in response to hypoxia) is responsible for the majority of induced gene expression in hypoxia. However, much less is known about the mechanism(s) responsible for gene repression, an essential part of the adaptive transcriptional response. Hypoxia-induced gene repression leads to a reduction in energy demanding processes and the redirection of limited energetic resources to essential housekeeping functions. Recent developments have underscored the importance of transcriptional repressors in cellular adaptation to hypoxia. To date, at least ten distinct transcriptional repressors have been reported to demonstrate sensitivity to hypoxia. Central among these is the Repressor Element-1 Silencing Transcription factor (REST), which regulates over 200 genes. In this review, written to honor the memory and outstanding scientific legacy of Lorenz Poellinger, we provide an overview of our existing knowledge with respect to transcriptional repressors and their target genes in hypoxia.

Keywords: Hypoxia, Repressor, Transcription, REST

1- The activation and resolution of adaptive responses to hypoxia.

The evolution of mitochondrial respiration along with the associated increase in cellular metabolic capacity, allowed the development of multicellular organisms[1]. Oxygen, the final electron acceptor in the oxidation of organic compounds, is thus essential to sustain essentially all complex life. Hypoxia is the condition which arises when metabolic oxygen demand exceeds it's supply. Exposure to hypoxia can elicit adaptation, such as that observed during ascent to high altitude, where erythropoiesis is induced to counteract the reduced atmospheric oxygen supply[2].

Hypoxia is commonly associated with a range of pathophysiological states including ischemia, chronic inflammatory disease and cancer[3]. Taking inflammatory diseases as an example, upon tissue invasion by a pathogen, resident and recruited inflammatory cells mount

a protective response that requires the oxygen-demanding synthesis of inflammatory mediators and oxidative burst therefore creating a hypoxic environment[4]. When inflammation chronic, the associated fibrosis and thrombosis also results in diminished blood delivery, thus exacerbating the hypoxic state[5]. Physiological hypoxic microenvironments are also common and can occur in the developing embryo and the adult[3]. Hypoxic regions arise in a spatio-temporally controlled way in the developing embryo and function as signal to orchestrate embryonic development[6,7]. Physiological hypoxia is also important in the adult where hematopoietic stem cells reside in the most hypoxic niches of the bone marrow[8,9]. Therefore, hypoxia is a relatively commonly encountered threat to the maintenance of metabolic homeostasis in health and disease.

Adaptation to hypoxia involves 3 major responses (Figure 1): (1) increased glycolysis to cope with ATP depletion, (2) increased oxygen delivery to restore oxidative phosphorylation, and (3) inhibition of energy-demanding processes, to direct the scarce energetic resources to key house-keeping functions. This can be achieved by acute responses that rely on the modification of pre-existing proteins, translational inhibition and transcriptional changes (Figure 1) that a will be discussed in detail in the next sections.

1.1-Modulation of the activity of pre-existing proteins

The strategies used by a cell to adapt to hypoxia are dependent upon the extent and duration of the challenge. Acute hypoxia can induce rapid and transient effects on the activity of pre-existing proteins[10]. This can be achieved through the regulation of post-translational modifications such as hydroxylation[11-13] and phosphorylation[14], or through a direct effect on enzymes that requires oxygen such haem oxygenase-2 (HO-2). HO-2 activity is reduced in the carotid bodies in response to systemic hypoxia, leading to the modulation of ion channel activity and the subsequent generation of neuronal signals that promote increased depth and rate of breathing to restore systemic oxygen levels to normal[15]. Finally, hypoxia-induced changes in cellular metabolism can indirectly affect enzyme activity. For example, the AMP-activated protein kinase (AMPK), a sensor of AMP:ATP ratios, is activated when ATP levels drop in response to hypoxia, and promotes energetic homeostasis by activating catabolic process and inhibiting anabolic metabolism[16,17]. While the acute responses to hypoxia are mostly mediated by changes in the activity of pre-existing proteins, long lasting adaptation to chronic hypoxia (e.g. high altitude) is achieved through delayed, yet sustained, changes in translation and transcription.

1.2-Inhibition of translation

Down-regulation of energy consuming processes under hypoxic conditions is thought to be part of an energetic adaptation strategy, aimed at redirecting the available energy supply

(ATP) to house-keeping functions, especially the maintenance of ionic and osmotic homeostasis in order to prevent membrane depolarization and subsequent cell swelling and necrosis[18]. Protein translation is one of the most energy consuming processes that occurs in cells. In rat cardiomyocytes it can take up to 27% of the total cellular energy capacity. RNA synthesis and Na+/K+ ATPase activity are the two other major energy sinks under normoxic conditions taking 20% and 22% of total energy capacity, respectively[19]. When energy capacity is reduced, translational rates are among the first cellular processes to be down-regulated.[20-22] Hypoxia induces translational arrest by activation of PERK and inhibition of mTOR, two major modulators of the translational machinery[22,23]. Therefore, by reducing the rate of protein translation in hypoxia, energy is conserved and reserved for essential processes.

1.3-Transcriptional adaptation

1.3.1-Gene induction in hypoxia

The discovery of the hypoxia inducible factor (HIF)[24,25] opened the way to the study of transcriptional regulation as a means for adaptation to hypoxia. HIF is responsible for adaptation to hypoxia through mechanisms including the induction of angiogenesis and erythropoiesis. HIF promotes such adaptive processes through the induction of VEGF and EPO respectively. HIF also promotes an increase in glycolysis to compensate for loss of ATP production during oxidative phosphorylation, through the induction of many of the enzymes in the glycolytic pathway (e.g. LDHA), and also through the induction of PDK1, which inhibits the conversion of pyruvate to acetyl Coenzyme A and entry into the tricarboxylic acid cycle[26].

The HIF pathway evolved as an adaptive mechanism, but it is maladaptive in cancer, where it plays an oncogenic role through the up-regulation of numerous genes that promote tumor growth. For example, increased glycolysis is thought to facilitate diversion of glycolytic intermediates into nucleoside and amino acid biosynthetic pathways facilitating cellular proliferation[27], and increased expression of the collagen remodeling LOX genes promotes metastasis[28]. While HIF is the master regulator of gene induction in hypoxia (transcriptomic studies have revealed that HIF regulates 29% to 52% of induced genes[29-31]), other transcription factors also contribute to the full-spectrum of transcriptional changes. Indeed, at least 20 other transcriptional activators are known to be regulated by hypoxia (Table S1)[32].

A fundamental aspect of the HIF response is its resolution, which is necessary to prevent detrimental effects on cells and tissues, including tissue acidification, increased haematocrit[33-35] and chronic HIF stabilization which has been associated with a number of cancers[33]. An example of a non-resolving HIF response is causative in the von Hippel-Lindau (VHL) disease, a hereditary human cancer syndrome predisposing patients to highly angiogenic

tumours[36]. In these patients loss of VHL causes HIF stabilization under normoxic conditions and expression of tumor promoting HIF target genes[36].

1.3.2-Gene repression in hypoxia

It is clear form transcriptomic studies that in response to hypoxia, concomitant with transcriptional up-regulation, there is transcriptional repression of comparable numbers of genes (Table S2). The mechanisms underpinning transcriptional repression is poorly understood relative to gene induction where HIF transcription factors are of central importance. Nonetheless transcriptional gene repression in hypoxia are a central part of the overall adaptive transcriptional response, as illustrated by the common repression between different transcriptional studies of several gene families involved in energy-demanding processes such as transcription, translation, mRNA processing and splicing, cell cycle and proliferation (Table S3). Hypoxia-repressed (but not hypoxia-induced) *in vitro* derived transcriptional signatures have prognostic value in breast cancer[37]. This fact alone highlights the need for a better understanding of the mechanisms of gene repression in hypoxia. The clinical importance of hypoxia-repressed genes has not been as thoroughly investigated as the importance of induced genes. This historical bias is probably influenced by the large amount t of attention focused on the HIF pathway which regulates the transcriptional induction of hundreds of target genes[37].

Transcriptional down-regulation of these energy-demanding processes under hypoxic conditions might be an essential part of a defense mechanism to direct the scarce energy available to essential house-keeping functions[18]. As described above, protein translation is regulated by hypoxia due to the action of the mTOR and PERK (Figure 1). However, long-term changes in translation and determined at the transcriptional level, likely so that the cells don't waste valuable resource in making new RNA that cannot be translated into protein due to the energy deficit. Consistent with this notion, genes related to translation are among the ones most commonly found to be repressed in transcriptomic studies (Table S3). For example, the translation initiation factor *EIF5A* is one of the five most down-regulated genes in hypoxia (>6 fold)[31].

Most transcriptomic studies measure steady state mRNA level, but changes in mRNA stability influence the expression levels. Transcriptomic studies on the effect of hypoxia on *de novo* (nascent) mRNA pools revealed 196 differentially expressed genes, 43 of which where repressed in RAW264.7 macrophages[38]. In another study performed in kidneys from ischemic mice, 1219 genes were differentially expressed (642 up- and 577 down-regulated), combinatorial analysis of steady state and nascent mRNA revealed that the majority of the genes (947) where *de facto* transcriptional changes rather than mRNA

stabilization/destabilization[39]. Thus, gene repression in hypoxia is at least in part mediated by *de facto* transcriptional changes, resulting from the activation of transcriptional repressors.

A better understanding of the mechanism leading to repression of the genes involved in multiple energy-demanding processes (Table S3), would allow a better understanding of the adaptation to hypoxia, and potentially new avenues for therapeutic intervention. In this review we aim to provide an overview of the known existing mechanisms for transcriptional gene repression in hypoxia mediated by the activation of transcriptional repressors, an area that has been much less under the scrutiny of the scientific community, than gene induction in hypoxia and its master regulator HIF. Initially we will focus on REST, a transcriptional repressor that has been implicated by several studies to be a key transcription factor in hypoxia, and possibly a master regulator of gene repression[40].

2-Mechanisms of transcriptional repression in hypoxia

Transcriptional repressors are a large and diverse group of proteins[41]. They can repress transcription through one, or a combination of several mechanisms: inhibition of the basal transcriptional machinery, inhibition of transcriptional activators or remodeling of chromatin structure[41]. Repressors play essential roles in physiology, from embryonic development[42,43] to adaptation to stress[44], and pathophysiology from neurological disorders[44] to cancer[45]. Hypoxia is a major stress to the cells, that requires gene repression for adaptation. In the following sections we will summarize existing evidence for roles plays by transcriptional repressors in hypoxia.

2.1-The Repressor Element 1-Silencing Transcription Factor (REST)

REST, also known as neuron restrictive silencer factor (NRSF), is a C2H2- or Kru□ppel-type zinc finger, one of the largest classes of transcription factors in humans[46]. REST is recruited to target genes containing the 21 base pair Repressor Element 1 (RE1), and inhibits transcription by interaction with the basal transcriptional machinery or by regulation of chromatin structure[42]. REST was originally discovered as a master regulator of neuronal development[47,48], and is the first example of a transcriptional repressor that regulates a large repertoire of cell-type specific genes[49]. During embryonic development, neuronal precursor cells gradually loose REST expression through proteasomal degradation, as a result, REST target genes are expressed in mature neurons, conferring them their unique phenotype, while repressing it everywhere else[42].

Several lines of evidence indicate that the simple picture of REST being a neuronal switch, is far more complex, with REST being expressed in some neurons within the brain, where it serves a neuro-modulatory role, in response to stimuli including seizure[50,51], ischemia[52,53], oxidative stress and ageing[44]. In addition, in non-neuronal cells REST is involved in

immunity[54], cardiac function[55] and cellular proliferation[56], being also heavily involved in cancer development[45].

Calderone and collaborators, first reported on the contribution of REST up-regulation (mRNA and nuclear protein) to ischemia-induced neuronal cell death[52], the mechanisms involved repression of glutamate receptor subunit GluR2[52,57]. Subsequent studies revealed that REST induction by ischemia, had more pronounced effects on gene expression, leading to the epigenetic remodeling and suppression of several neuronal genes, which ultimately lead to cell death[53]. Studies using cultured neuroblastoma cell lines, subjected to an in vitro model of ischemia, oxygen and glucose deprivation, also exhibited increased REST mRNA and protein[58]. These studies were the first to identify a potential link between low oxygen and REST regulation (Table 2). Recently it has been shown that REST is a hypoxia-responsive transcriptional repressor, acting as a master regulator of gene repression in hypoxia, regulating 20% of all hypoxia-repressed genes in HEK293 cells exposed to 1% oxygen[40]. Upon exposure to hypoxia, REST accumulates in the nucleus (almost 4-fold increase over normoxia at 16 and 24 hours), and this can be rapidly reversed by re-oxygenation, REST protein stability was not affected by hypoxia, and REST mRNA was only modestly and transiently increased (at 2 hours of hypoxia), suggesting that the main mechanism for REST nuclear accumulation involves a nuclear translocation regulated by a post-translational regulation mechanism[40]. A recent study by Rios and collaborators, has also reported REST accumulation in the nucleus of Marrow-isolated multilineage inducible (MIAMI) cells exposed to 3% oxygen for 7 days, with only a very modest <1.5-fold increase in REST mRNA.[59]. While the mechanisms responsible for nuclear translocation of REST in hypoxia is currently unknown, REST has been shown to require several proteins such as dynactin p150(Glued), huntingtin, HAP1, and RILP/Prickle1 to form a complex involved in its the translocation into the nucleus[60]. REST is post-transcriptionally regulated by phosphorylation[61,62] (Casein-kinase II[63]) and ubiquitylation (β-TRCP[61,62], HAUSP[64] and DYRK1A[65]), a promising topic to study the regulation of REST in hypoxia, as these post-translational modifications are used by hypoxia to modulate the activity of several hypoxia responsive proteins (e.g. HIF and NFκB pathways)[66-68]. Two studies reported a decrease in REST expression, in total cell lysates after 72 hours of 2% oxygen, in prostate cancer LNCaP cells[69], and 5% oxygen in prostate cancer PC-3 cells and neural crest cells[70]. The mechanism for REST down-regulation involved hypoxia-induced miR-106b~25[70]. This suggests that similarly to the HIF response which is resolved in prolonged hypoxia, to prevent over-activation of its target genes[71,72], the REST response to hypoxia also needs to be resolved. In summary, several lines of evidence suggest that REST is regulated by oxygen deprivation, in both hypoxic cell lines and ischemic rodents (Table 2).

2.2.1-Physiological relevance of REST regulation by hypoxia

Originally discovered as regulator of neuronal development, REST has since be found to be a very versatile and context specific transcription factor[45,73]. For example, in non-neuronal cancers of the breast, lung and colon REST is a tumor suppressor[45,73], while in neuronal cancer REST is an oncogene[73-75]. In the adult brain REST can also play contradictory roles, being neurotoxic in ischemia[52,53,57], epilepsy[50,51] and Huntington's disease,[76,77] and neuroprotective in aging and Alzheimer's disease[44].

Regarding the role of REST in hypoxia and in oxygen-deprived conditions such as ischemia, the roles and genes regulated by REST also point to diverse and cell-type specific roles (Table 3). Global ischemia is able to induce REST protein and mRNA in hippocampal neurons, REST-epigenetic reprograming and repression of neuronal genes (e.g. GluR2, GluA2) leading to cell death[52,53,57]. In HEK293 cell we have showed it contributes to the resolution of the HIF-response, and regulates HIF-dependent metabolic adaptation to hypoxia (glycolytic genes)[72]. HIF-signaling is also negatively regulated by REST in prostate cancer cells[69]. Thus regulation of HIF-signaling is a key feature of REST in hypoxia.

In a study by Lin and collaborators, REST downregulation after 3 days in hypoxia (2% oxygen) was required for the neuroendocrine phenotype of prostate cancer cells exposed to hypoxia, namely neuron-like extensions and AMPK activation (phohspho-Thr172)[69]. Suggesting that tumor hypoxia in solid prostate cancer down-regulates REST as a mechanism to induce the neuroendocrine phenotype, which is observed in the most advanced, treatment-refractory stages of this cancer[69]. Notably, their comparison of the transcriptomic changes induced by REST RNAi in normoxic cells, and cells exposed for 3 days of hypoxia, revealed a 20% overlap in the induced genes, suggesting that REST down-regulation in prolonged/chronic hypoxia is a mechanism involved in late transcriptional up-regulation in hypoxia[69].

REST is overexpressed in neuronal cancers, and has oncogeneic properties, it inhibits apoptosis and promotes tumorigenisis[74,75]. Interestingly, hypoxic neuroblastoma tumors and cells exposed to hypoxia (1% oxygen) down-regulate neuronal markers[78,79], and REST protein is induced in oxygen/glucose deprived SK-N-SH neuroblastoma cells[58]. Thus, it is plausible that REST induction by the hypoxic microenvironment known to be present in neuronal tumours[80], contributes to its oncogenic role. Interestingly our RNA-seq analysis revealed REST dependent repression of cell adhesion genes, which might be important for metastasis as hypoxic suppression of adhesion molecules allows cancer cells to escape their stressed environment[81,82]. Several of the REST target genes we identified in our RNA-seq analysis where part of cancer pathways (RB1, MET, MYCBP, WNT5A, HDAC1, PRKCB, LAM4A,

LAMC1 and LAMB1). HIF itself is implicated in cancer, and has been show to be repressed by REST in HEK293[72] and prostate cancer cells[69]. The physiological relevance of REST regulation of all these cancer-related genes in hypoxia, beyond the in vitro cell culture paradigm, will most certainty be context- and cell- type specific as REST is well known to play both tumor suppressor and oncogenic roles, a feature it shares with NOTCH signalling[83], E-cadherin[84] and MYC[85]. Overall, existing evidence suggest that the regulation of REST protein expression levels by hypoxia in cancer cells, both its induction in neuronal cancer and its down-regulation in prostate cancer, plays an pathogenic role.

During neuronal development, neuronal stem cells require REST down-regulation for the expression of neuron-specific genes[42]. Hypoxia is well known to be a signal required for proper embryonic development[3], and is reported to promote neuronal development through an unknown mechanism[86]. Using neural crest cells as a model to study neuronal development, Liang and collaborators showed that hypoxia promotes REST down-regulation through up-regulation of miR-106b~25[70]. Hypoxia and REST RNAi treated cells induced to a similar level the expression of several neuronal markers: the transcription factor ASH-1, the downstream protein paired-like homeobox 2a (Phox2a), and tyrosine hydroxylase (TH)[70]. Overall, this study suggest that hypoxic down-regulation of REST in neuronal precursor cells, is an important mechanism for neuronal development.

We recently published a deep analysis of the effect of REST on hypoxia regulated gene repression, we analyzed the transcriptomic changes by RNAseq, and found that REST RNAi rescued the hypoxic repression of 201 genes, 20% of the total hypoxia repressed genes[40]. Thus we showed for the first time that REST is a master regulator of gene repression in hypoxia. Gene ontology analysis of the REST-repressed genes in hypoxia suggests that REST plays an important role in the cellular adaptation to hypoxia by suppressing genes involved in proliferation, cell cycle progression and transcription[40]. Our own ChIP experiments confirmed the hypoxic regulation of SYJN1 encoding synaptojanin 1, a protein involved in clathrin-mediated endocytosis and ATP-intensive process[40]. Thus repressing energy demanding processes in hypoxia is a plausible function for REST.

As discussed above, REST regulates HIF and HIF-signalling[59,72], including HIF-target genes involved in glucose metabolism[72], biosynthesis of lipids and nucleic acids, and protein catabolism[40]. Thus, REST seems to be heavily involved in the regulation of the metabolism of hypoxic cells.

2.2.2-Mechanism of REST-mediated gene repression in hypoxia

In ischemic neurons, REST is recruited to the promoter of MOR-1[57] and GluA2[53], as assessed by Chromatin immunopreceptitation assays. Furthermore, GluA2 promoter exhibited recruitment of the REST co-repressor complexes CoREST and mSin3, and marks of

epigenetic silencing, that where dependent on REST[53]. CoREST and mSin3 are multiprotein co-repressor complexes that contain both chromatin remodelers (BRG1, SWI/SNF) and histone modifiers (histone deacetylases 1 and 2, histone methyl-transferase G9a)[42] This suggests that REST recruitment, with co-repressor complex assembly and epigenetic silencing is a mechanism for hypoxic gene repression.

In hypoxic HEK293 cells, we have observed REST recruitment to the HIF-1α[72] and SYNJ1[40] promoters, with co-repressor complex components CoREST and mSin3 also being recruited to the HIF-1α promoter[72]. Interestingly, the GANAB gene had REST bound to the promoter in hypoxia, but there was no increase of REST binding with hypoxia compared to control cells (21% oxygen)[40]. Despite the absence of REST recruitment, GANAB was regulated by REST in hypoxia, as assessed by rescue of GANAB repression in hypoxia by REST RNAi[40].
Thus, we propose that, while REST recruitment is required for transcriptional repression of some genes (e.g. SYNJ1, HIF-1α), for other genes (e.g. GANAB) REST might already be bound to its target gene in normoxia in a low affinity state, "poised to act," with its repressive activity triggered only in hypoxia, possibly by co-factor recruitment[40].
This hypothesis finds support in studies by Otto and colleagues[87], where many of REST-occupied genes were still expressed while others were repressed, potentially due to their functional relevance to the cell.
Using publically available REST ChIP-seq datasets we found that 64% of our identified normoxic REST target genes and 18% of our hypoxic REST target genes were previously described in these datasets[40]. This suggests that in hypoxia, REST regulates more genes indirectly than by direct binding. Consistent with indirect regulation we found that REST repressed the expression of transcription factors and transcriptional machinery in hypoxia[40].
In conclusion, 3 potential mechanisms account for REST mediated gene repression in hypoxia, direct REST recruitment to the gene promoter, activation of REST activity in genes where REST is "poised-to-act", and indirect mechanisms.

3- Other hypoxia responsive transcriptional repressors

3.1 DEC1 and DEC2

DEC1 (Differentially expressed in chondrocytes protein 1) was found to be inducible by hypoxia in a screen for factors dependent on the VHL- HIF pathway, and its expression is dependent on HIF-1α, as cells lacking functional HIF1α do not up-regulate DEC1 in response to hypoxia[88]. The role of DEC1 activation in hypoxia is best described in melanocytes, where HIF-1α induced DEC1 is recruited to the M-MTIF promoter (melanocyte-specific isoform of

the microphthalmia-associated transcription factor) and represses its transcription, resulting in an inhibition of the oncogenic properties of M-MITF (the)[89].

Tumor hypoxia down regulates key genes of the DNA mismatch repair system, leading to genomic instability and tumor progression. A study in multiple cancer cell lines, has shown that hypoxic repression of the key mismatch repair gene (MLH1) is transcriptionally regulated by DEC1 and DEC2, which are induced by HIF-1α in hypoxia [89-91].

3.2 Bach1

The transcription repressor Bach1 (BTB and CNC homology 1, basic leucine zipper transcription factor 1), is transcriptionally induced by hypoxia in several human cell lines, and to promotes transcriptional repression of HO-1 (heme oxygenase-1). The physiological relevance of this mechanism is not known. HO-1 is induced by and promotes degradation of heme, in a protective response against oxidative stresses[108].

3.3 ID1 and ID2

The protein inhibitor of DNA binding 2 (ID2), while not being a transcriptional repressor, is a negative regulator of the activity of bHLH transcription factors[78]. ID2 is involved in normal neural crest development and inhibits pro-neuronal bHLH proteins. Hypoxia leads to a reduction of neuronal gene expression in neuroblastoma, and ID2 mRNA is quickly induced (3-fold after 2 hours) in SK-N-BE(2) neuroblastoma cell lines, and transcriptionally dependent on HIF-1α[78]. Hypoxic induction of ID2 may contribute to the neuroblastoma de-differentiation into a neuronal crest-like phenotype (loss of neuronal markers) associated with increased tumor growth and aggressiveness[78]. ID1 is also induced by hypoxia in multiple cell types, but with unreported function[78,92].

3.4 ZEB1/ZEB2

E-cadherin loss of function is a hallmark of metastatic cancer. Renal clear cell carcinoma (RCC) is characterized by loss of function of the von Hippel-Lindau tumor suppressor (VHL), which negatively regulates (HIF-1α)[93]. Loss of E-cadherin expression in VHL-null RCC4 cells can be corrected by rescue of VHL expression, or inhibition of HIF-1α using a dominant negative mutant or RNAi[93]. Because the mRNA expression of ZEB1 and ZEB2, well-described transcriptional repressors of E-cadherin, where reduced upon HIF inhibition, the authors suggest that loss of E-Cadherin in Renal clear cell carcinoma is due to HIF-dependent induction of ZEB1/ZEB2.[93]

3.5 Snail

Snail, like ZEB1 and ZEB2, is a well-described transcriptional repressor of E-Cadherin. Studies in ovarian cancer cell lines have shown that Snail mRNA in induced by hypoxia, suggesting it might contribute to hypoxia induced epithelial mesenchimal transition and metastasis in ovarian cancer by repressing E-cadherin.[94]

3.6 CtBP

The transcriptional corepressor C-terminal-binding protein (CtBP) is able to sense free nuclear NADH and transduce this into changes in gene expression[42]. In cancer cell lines, hypoxia increased free NADH levels, promoting CtBP recruitment to the E-cadherin promoter. In addition, hypoxic repression of E-cadherin and increased cell migration, where rescued with CtBP RNAi[95]. Thus, hypoxic recruitment of CtBP to the E-Cadherin promoter is a mechanism for E-Cadherin repression and increased cancer cell migration in hypoxia.

3.7-HIF-dependent gene repression

Microarray data combined with siRNA against HIF-1/-2 indicate that there are HIF-dependent and HIF-independent hypoxia repressed genes[31]. For example, *EIF5A* is 6-fold repressed in hypoxia, but only 1.5-fold repressed in the presence of HIF-2 siRNA[31]. While there are some cases of hypoxic gene repression by HIF binding to gene promoters (e.g. CFTR[96], ADK51[97] and APC[98]), these seem to be the exception rather than the rule, as studies combining genome wide ChIP-seq and microarray data indicate that while HIF is enriched in the promoters of genes induced in a HIF-dependent way, it is not enriched in the promoters of genes that are repressed in hypoxia, thus indicating the presence of HIF-independent and/or indirect HIF-dependent mechanisms governing gene repression in hypoxia[99]. Supporting this notion, transcriptional regulation in hypoxia exhibits temporal waves of regulation, with a second late wave of transcriptional regulation by transcription factors that were transcriptionally induced by hypoxia in the first early wave of regulation[66].

3.8-Hydroxylase dependent gene repression

Some studies have revealed that hypoxia repressed genes can also be repressed by DMOG, a 2-oxoglutarate analogue, commonly used as a "hypoxia mimetic" drug that activates HIF through the inhibition of the Prolyl hydroxylase domain containing (PHD) enzymes. An example is the DNA repair gene *BRCA2,* in hypoxic breast cancer cells[100]. Transcriptomic studies comparing the effect of hypoxia and DMOG, revealed a sticking overlap, with 77% of the hypoxia induced genes also induced by DMOG[31]. This is consistent with the perceived central role of PHD/HIF in hypoxia induced gene expression[26]. When the effects on hypoxia repressed genes where analyzed, the overlap was 36%[31]. This suggests that the PHD/HIF pathway does not play such a predominant role in gene repression as it does in gene

induction. In addition, PHD and other 2-oxoglutarate-dependent enzymes could contribute to gene repression in hypoxia, possibly thought the hydroxylation of transcriptional repressors. This exciting hypothesis remains to be tested.

Another class of hydroxylases that can play a role in gene repression in hypoxia are the jmjC demthylases, reviewed in[101]. These hydroxylases require molecular oxygen to de-methylate histones, leading to epigenetic silencing. Interestingly, more than twenty jmjC demethylases have been shown to be induced by hypoxia / HIF, thus raising the possibility that they contribute to gene repression in hypoxia through epigenetic silencing mechanisms[101].

Thus, while the underlying mechanisms are only recently being explored, hydroxylases might play a role in gene repression in hypoxia.

4-Cross-talk between REST and HIF: master regulators of gene repression in hypoxia

As mentioned above, REST negatively regulates HIF-expression and HIF-signaling in HEK293[72] and MIAMI[59] cells. Other reports in the literature suggest that REST and HIF cross-talk to fine tune target gene expression. Namely, the NCX1 gene (Na^+/Ca^{2+} exchanger 1), coding for a plasma membrane protein regulating intracellular calcium and sodium homeostasis in the brain, is epigenetically repressed in a rat model of brain ischemia by the REST/Sp3 complex and induced in brain ischemic preconditioning by the HIF-1/Sp1 complex[102]. Thus, in addition to regulating specific cohorts of hypoxia induced and hypoxia repressed genes, HIF and REST, share some transcriptional targets, working together to fine-tune the transcriptional response to hypoxic challenges.

5-Conclusions

Cellular adaptation to hypoxia involves multiple responses ranging from changes in the activity of pre-existing proteins, inhibition of protein synthesis, and also modulation of the transcriptional landscape (Figure 1). Our understanding of the mediators of transcriptional changes in hypoxia is rapidly expanding. While HIF has earned and deserves to retain it's place as the master regulator of gene induction in hypoxia, evidence also exists for at least 20 other transcriptional activators in hypoxia (Table S1). Transcriptional repression in hypoxia, on the other hand, is less well understood. We now know that at least 10 transcriptional repressors are regulated by hypoxia (Table 1). Most of these factors have been identified to regulate cancer related genes M-MTIF, MLH1 and E-Cadherin, and to contribute to hypoxic modulation of cancer cell metastasis and DNA mismatch repair response (Figure 2). For most transcriptional repressors (DEC1, DEC2, ID1, ID2, ZEB1, ZEB2, Snail, Bach1, REST) hypoxia regulates their expression (protein or mRNA level) using HIF-dependent and

independent mechanisms (Figure 2). In the case of DEC1, CtBP and REST, hypoxia also seems to promote their recruitment to the promoter of its target genes (Figure 2). REST is undoubtedly the most studied and best understood transcriptional repressor in contexts of oxygen deprivation, including hypoxic exposure of cell lines *in vitro* and ischemia in rodents. REST induction by oxygen deprivation plays a pathogenic role in ischemic neurons and oxygen/glucose deprived neuroblastoma cells (Table 2), where REST-repression of neuronal genes contributes to cell death (CART, MOR-1, GluR2, GluA2). In addition to these genes, REST is recruited to the promoter of 12 other genes in ischemic neurons, the relevance of which is unknown (Table 3). In HEK293 cells and MIAMI cells, REST regulates HIF signaling, promoting resolution of the HIF-1α response, which can be damaging when over-activated[33-36]. Another mechanisms for REST-mediated gene regulation in hypoxia, is repression of REST protein and subsequent de-repression (induction) of target genes. This mechanisms seems to play a role in the neuroendocrine and neuronal differentiation of prostate cancer cells and neuronal crest cells, respectively (Table 3). However, evidence is lacking for this mechanism, as no gene was yet identified as induced by hypoxia in a REST-dependent manner using genetic modulation of REST expression. The most common mechanism for REST regulation of gene expression in hypoxia, seems to rely on the induction of REST expression (mRNA and/or protein), nuclear accumulation and recruitment to the target gene (as accessed by Chromatin immunoprecepitation assays). Several genes have been identified in which REST inhibition (RNAi or dominant negative REST expression) rescues their repression in hypoxia (Table 3). These include HIF-1α, with effects on glycolytic metabolism and the neuronal genes CART, MOR-1, GluA2, GluR2, with effects on neuronal cell death. Other genes validated to be REST-repressed in hypoxia include GANAB, RAB3C, and SYNJ1, the last one being involved in endocytic processes and energy demanding process, suggesting that REST might contribute to the transcriptional repression of energy-demanding processes in hypoxia. This is further substantiated by our recent transcriptomics (RNAseq) analysis, where we found that REST RNAi rescued the hypoxic repression of 201 genes, including genes involved in energy demanding processes such as transcription, metabolism, proliferation and cell cycle progression.

The hypoxia-repressed transcriptomic signature of cancer cells has prognostic value in cancer[37]. Expanding our understanding of the mechanisms of gene repression in hypoxia will open new avenues for developing diagnostic, prognostic and potentially therapeutic approaches. REST inhibitors are already being developed for the treatment of nuerological disorders, where REST plays a pathogenic role[103]. These are expected to be useful in situation where inhibiting REST activity in hypoxia might be beneficial, for example, REST

contributes to neuronal cell death in ischemic neurons, thus its inhibition is predicted to be beneficial in ischemic diseases (e.g. stroke).

We are only starting to unravel the physiological relevance and the mechanisms of gene repression in hypoxia. The transcriptional repressor REST, seems to play a prominent role in gene repression in hypoxia, it is the most studied and best described repressor, and can regulate up to 20 % of hypoxia repressed genes[40]. Thus, REST is the first master regulator of gene repression in hypoxia.

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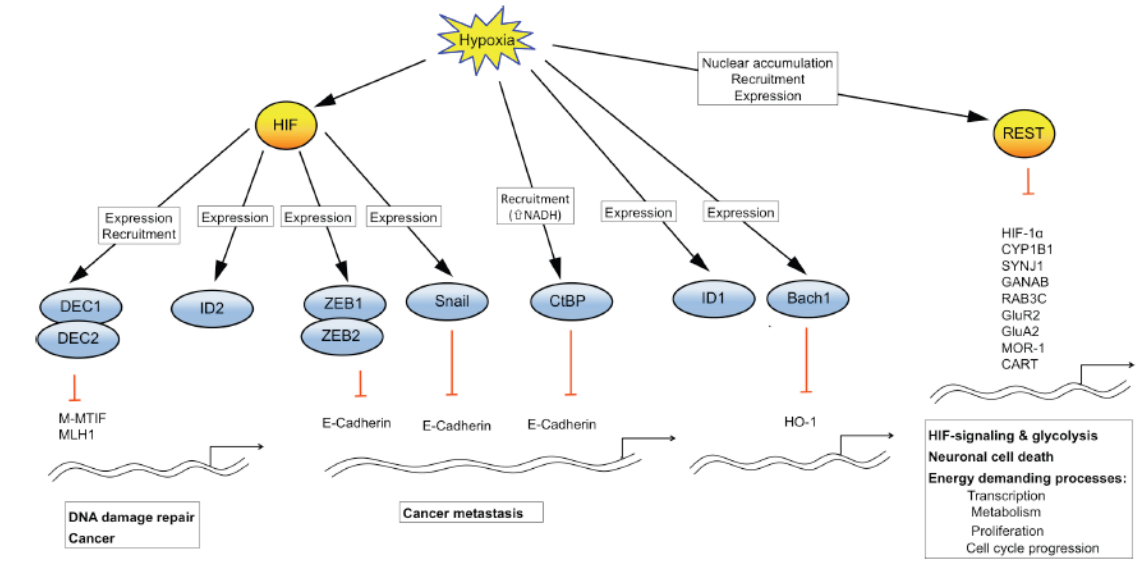
Figure 1. Molecular mechanisms of adaptation to hypoxia. Adaptation to hypoxia involves 3 major responses: increased glycolysis to cope with ATP depletion, increased oxygen

delivery to restore oxidative phosphorylation, and inhibition of energy-demanding processes, to direct the scarce energetic resources to key house-keeping functions. This can be achieved by acute responses that rely on the modification of pre-existing proteins (e.g. AMPK and HO-2), translational inhibition (mediated by mTOR and PERK) and transcriptional changes (mediated by HIF and largely unknown mechanisms for transcriptional repression). Depicted are examples of these mechanisms (from left to right): **(1)** Hypoxia decreases ATP production (energetic stress), this activates AMPK that restores energetic homeostasis by inducing catabolic pathways and inhibiting anabolic pathways. Adapted from[104,105]. **(2)** Haem oxygenase-2 (HO-2)-generated carbon monoxide (CO) is reduced under hypoxia, CO inhibits cystathionine-γ-lyase (CSE)-regulated hydrogen sulfide generation, thus in hypoxia more H2S is generated, inhibiting potassium channels (K+ channel), this leads to increased Ca2+ influx through Ca2+ channels, which initiates neuronal signaling to the central nervous system, ultimately increasing the rate and depth of breathing. Abbreviations: NT, neurotransmitter(s); and NT-R, neurotransmitter receptor. Adapted from[15]. **(3)** Hypoxia activates TSC1/2 via AMPK or HIF-1α/REDD1, TSC1/2 inhibits mTOR leading to an inhibition of 4EBP1, a negative regulator of the eIF4E, thus suppressing protein translation. mTOR also activates P70S6K, which activates ribosomal protein S6. Adapted from[23,106,107]. **(4)** Alternatively hypoxia can activate the effector of the UPR, the kinase PERK, which phosphorylates and inactivates eIF-2α, thus leading to suppression of protein translation. Adapted from[23,106,107]. **(5)** HIF is the master regulator of gene induction in hypoxia. HIF is stabilized by inhibition of the oxygen-dependent PHD enzymes. **(6)** Transcriptional repression is an essential component of adaptation to hypoxia through the inhibition of energy demanding process. However, the mechanisms for transcriptional gene repression in hypoxia are poorly understood.

Figure 2. The landscape of transcriptional repressors in hypoxia. Depicted are the known hypoxia-regulated transcriptional repressors, their target genes and the biological processes they regulate in hypoxia (see text for details). For most transcriptional repressors (DEC1, DEC2, ID1, ID2, ZEB1, ZEB2, Snail, Bach1, REST) hypoxia regulates their expression (protein or mRNA level) using HIF-dependent and independent mechanisms. In the case of DEC1, CtBP and REST, hypoxia also seems to promote their recruitment to the promoter of its target genes. REST is the best-described transcriptional repressor in hypoxia. The most common mechanism for REST regulation of gene expression in hypoxia, seems to rely on the induction of REST expression (mRNa and/or protein), nuclear accumulation and recruitment to the target gene. At least 9 genes are regulated by REST in hypoxia, these where validated using chromatin immunoprecepitation assays to show REST recruitment to their promoters, and REST RNAi to rescue their hypoxic repression.



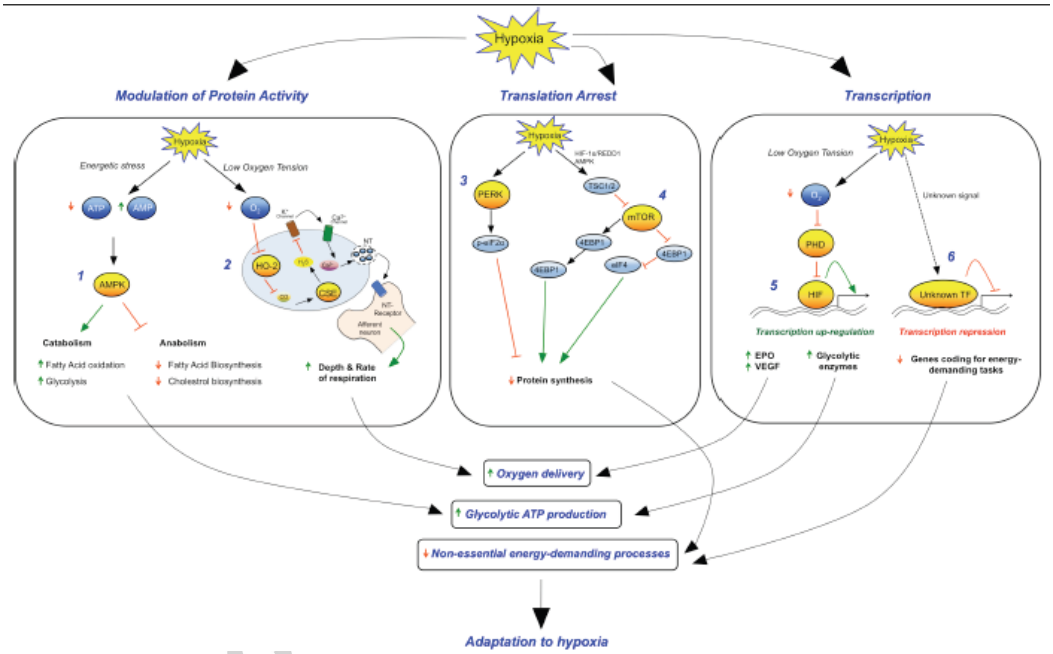


Table 1. Hypoxia responsive transcription repressors

Name	Description
DEC1/2	Induced by HIF-1 in hypoxia, repress MITF and MLH1 [89-91]
BACH1	Induced by hypoxia, represses HO-1 (haem oxygenase-1) [108]
ID2	Induced by hypoxia, HIF-1 dependent, may contribute to hypoxic neuroblastoma de-differentiation into a neuronal crest-like phenotype. [78]
ID1	Induced by hypoxia in multiple cell types. [78,92]
ZEB1 / ZEB2	Repressed in RCC4 cells in response to dn-HIF-1α or VHL overexpression, but may be repressed by hypoxia. Known repressors of E-cadherin. [93]
SNAIL	Induced by hypoxia in ovarian cancer, may contribute to hypoxia induced EMT/metastasis by repressing E-cadherin. [94]
REST	Induced by hypoxia and ischaemia, it represses multile genes in hypoxia, including HIF-1α. See Table 2 and 3 for details.
CtBP	This co-repressor is be recruited to the E-cadherin promoter, in response to increased NADH levels in hypoxia.[95]

Table 2. REST regulation in oxygen-dprived conditions

Experimntal condition	Effect on REST	Function	Ref
Ischemic neurons [(a)]	↑mRNA ↑nuclear protein	Represses gluR2 leading to neuron death	52,57
Ischemic neurons [(a)]	↑ mRNA ↑nuclear protein	Epigentic remodelling and repression of neuronal genes	53
Oxygen/glucose deprived SK-N-SH cells[(b)]	↑mRNA ↑protein	Represses the neuropeptide CART leadint to cell death	58
1% O_2 (24 hrs), HEK293 cells	↑ nuclear protein	Represses >200 genes	40
3% O_2 (7 days), MIAMI cells	↑ mRNA ↑ nuclear protein	Hypoxia-induced stem cell self-renewall	59
2% O_2 (3 days), LNCaP cells[(c)]	↓ total protein mRNA not affected	Neuroendocrine phenotype of prostate cancer cells (AMPK activation, neurite growth)	69
5% O_2 (3 days), LNCaP, PC-3 cells[(c)]	↓ total protein ↓ tmRNA	Neuroendocrine phenotype of prostate cancer cells	70
5% O_2 (3 days), primary neural crest cells	↓ total protein ↓ tmRNA	Neuronal diferentiation of neural crest cells	70

Notes and abbreviations: [(a)] Studies performed on hypocampal CA1 neurons from mouse model of ischaemia; [(b)] SK-N-SH cells are a neuroblastoma-derived cell line; [(c)] LNCaP and PC-3 are prostate cancer derived cell lines.

Table 3. REST target genes in oxygen-deprived conditions

Experiment	Target	Evidence	Ref
Ischemic neurons [(a)]	GluR2	REST RNAi rescues ischemic repression *Western Blott and Immunoflurescence*	52
Ischemic neurons [(a)]	MOR-1	Ischaemia promotes REST recruitment to the promoter [(b)] *Chromatin-Immunoprecepitation*	57
Ischemic neurons [(a)]	GluA2	Ischaemia promotes REST recruitment to the promoter and epigenetic silencing *Chromatin-Immunoprecepitation* REST RNAi or dnREST[(c)] rescues ischemic repression *WesternBlott and qRT-PCR*	53
Ischemic neurons [(a)]	Peg12 Slc22a12 Nfkb2 GluA2 Chrnb2 Grin1 Csrnp3 Nppa Slc22a13S cg2 Fdxr Nefh Syp	Ischemia (24 and 48 hours) promotes REST recruitment to the promoter[(d)] *ChIP-on-chip*	53
OGD, SK-N-SH[(e)]	CART	REST RNAi rescues hypoxic repression *qRT-PCR*	58
1% O_2, HEK293	Hif-1α	REST RNAi increases expression and activity in hypoxia *WesternBlott and qRT-PCR* Hypoxia promotes REST recruitment to the promoter *Chromatin-Immunoprecepitation*	72

1% O_2, HEK293	201 genes	REST RNAi rescues hypoxic repression *RNA-seq*	[40]
	GANAB SYNJ1	REST is enriched in the promoter Hypoxia promotes REST recruitment to the promoter *Chromatin-Immunoprecepitation*	
	GANAB CYP1B1 RAB3C	REST RNAi rescues hypoxic repression *qRT-PCR*	
2% O_2 ,LNCaP	AMPK	REST RNAi increases AMPK activation *WesternBlott*	[69]

Notes and abbreviations: [(a)] Studies performed on hypocampal CA1 neurons from mouse model of ischaemia; [(b)] MOR-1 is a well known REST target gene, that is repressed in ischaemia[57]; [(c)] dominat negative REST; [(d)] 58 targets at 24 hrs, 50 targets at 48 hrs; [(e)]OGD, oxygen/glucose deprivation.

Highlights:

- Gene repression accounts for approximately 50% of the transcriptional response to hypoxia.
- A number of transcriptional repressors have been identified as hypoxia-sensitive.
- REST is a key transcriptional repressor in hypoxia