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Hypoxia-induced and stress-specific changes in chromatin structure and function

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Abstract

Cellular adaptation to stress relies on specific, regulated responses to evoke changes in gene expression. Stresses such as hypoxia, heat shock, oxidative stress and DNA-damage activate signaling cascades that ultimately lead to either induction or repression of stress-responsive genes. In this review, we concentrate on the mechanisms by which stress-induced signaling promotes alterations in chromatin structure, whether the read-out is activation or repression of transcription. Specific alterations in chromatin are highly regulated and dictated by the type of imposed stress. Our primary focus is on the types of chromatin alterations that occur under hypoxic conditions, which exist within a majority of tumors, and to compare these to changes in chromatin structure that occur in response to a wide variety of cellular stresses.

Introduction

The transcriptional machinery responsible for the activation of stress-responsive genes must overcome the natural barriers of chromatin [1,2]. Conversely, mechanisms acting in repression of gene expression, in response to stress, likely involve creating such barriers by altering chromatin structure. Chromatin modifiers fall into two main categories: ATP-dependent chromatin remodelers that mobilize or eject nucleosomes and histone modifying complexes that add covalent modifications to histones [3]. These post-translational modifications include, but are not limited to, acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, and polyADP-ribosylation. The overall effect that each of these histone moieties has on gene expression depends on numerous factors: the type of modification, the target residue, the number of moieties added to each residue, the neighboring modifications present in *cis* or *trans* and the location of the modified histone within the gene. Histone modifications such as acetylation and phosphorylation, regulate gene expression in part by altering the charge of the targeted amino acid, thus altering chromatin structure. These and other modifications create protein binding sites or platforms recognized by specific structural domains such as bromo- or chromo-domains [4–8]. Proteins interacting with remodeled or modified chromatin include transcription factors, additional chromatin modifiers and stress-induced proteins involved in processes such as DNA repair [9–11].

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Hypoxia is a well-studied cellular stress that involves genome-wide changes in gene expression. Tissue hypoxia occurs when oxygen tension drops below what is required for normal cellular function [12]. This may be the result of inadequate blood flow to tissues or reduced oxygen transport capacity. Hypoxia leads to cell cycle arrest, apoptosis, and necrosis. Advanced solid tumors often have areas of hypoxia and even anoxia. Oxygen partial pressure (pO_2) values of ≤ 2.5mm Hg (0.3% O_2) are found in advanced solid tumors from a wide-range of cancers, compared to normal air oxygen concentration of 21% O_2 (159mmHg). Hypoxic tumors are associated with resistance to typical cancer treatments. Radiation therapy, chemotherapeutic reagents and immunotherapy are much less effective in tumors with pO_2 values of < 25–30mm Hg [13].

Tumor hypoxia occurs as the tumor proliferates and grows, increasing the distance between tumor tissue and nearby blood vessels; thus, simple oxygen diffusion is no longer sufficient [12,14,15]. In order for the tumor to survive the hypoxic environment, genes involved in angiogenesis, glycolysis, erythropoiesis, cell survival and proliferation are induced. These changes in gene expression are observed during oxygen concentrations of ≤ 1% oxygen (7mm Hg) [16]. The expression of hypoxia-inducible genes, along with those that promote cell detachment and tumor invasiveness, results in a more clinically aggressive phenotype, as reviewed in [17–23].

The transcription regulator, hypoxia-inducible factor 1 (HIF-1), is critical in regulation of a majority of genes in response to hypoxia. HIF-1 is a heterodimer of an oxygen-regulated alpha subunit and an oxygen-independent beta subunit. Under normoxic conditions, HIF-1α is hydroxylated at proline residues 402 and 564. The von Hippel Lindau (VHL) protein recognizes these modifications and targets HIF-1 for ubiquitylation and subsequent degradation. HIF-1α is negatively regulated under normoxic conditions in a variety of other ways, including lysine acetylation and asparaginyl hydroxylation [24–26]. However, under hypoxic conditions, HIF-1α and β heterodimerize and bind to hypoxic response elements (HREs) within the promoters of HIF-1 target genes to promote transcription. HIF-1α has a bHLH domain required for DNA binding and both N-terminal (N-TAD) and C-terminal (C-TAD) transactivation domains [17,27–29]. HIF-1-mediated response to hypoxia involves the cooperation of chromatin modifying complexes, discussed below and noted in Table 1.

Regulation of gene expression through covalent modification of histones

Histone acetyltransferases (HATs) and regulation of gene expression in response to hypoxia

Histone acetyltransferases (HATs) are defined by a catalytic domain that facilitates transfer of acetyl groups to lysine residues within the N-terminal tails of histones, as well as other protein substrates. HATs fall into two main classes: type A nuclear HATs and type B cytoplasmic HATs. Of the nuclear HATs, three families are identified: the GNAT, MYST, and p300/CREB binding protein (CBP) families. All HATs have an acetyl-CoA binding site and function in larger complexes with distinct subunits that may specify their protein substrates and the biological functions in which they are involved [3]. Histone hyperacetylation is most commonly associated with active transcription [30]. Acetylation of histones may augment transcription by neutralizing the positive charge on lysines, thus reducing histone association with negatively charged DNA. Addition of the bulky acetyl groups to histones also decreases compaction between histones within nucleosomal arrays. Furthermore, acetylated lysines may provide a platform for the recruitment of other transcription factors that aid in gene expression [3].

CBP, p300, and steroid receptor cofactor-1 (SRC-1) are specific HATs involved in hypoxia-mediated gene regulation [31,32]. Although CBP and p300 have a high degree of homology, some functional differences between them are found that may be due to HAT-specific

associating proteins [33]. Mice were engineered with mutations in the CH1 domain of either p300 or CBP. The p300 homozygous mutant mice were born and thrived at near normal frequencies, while the CBP homozygous mutant mice died shortly after birth, demonstrating that CBP and p300 CH1 domains are genetically non-redundant [34].

CBP/p300 binds to the C-TAD of HIF-1α through its cysteine-histidine rich (CH1) domain and enhances HIF-1 transactivation of several genes, including erythropoietin (Epo), vascular endothelial growth factor (VEGF), and lactate dehydrogenase-A (LDH-A), in a hypoxia-dependent manner [32,34–36]. Inactivation of p300, via transfection of the E1A oncoprotein, abolishes hypoxia-mediated induction of these three genes [35,36]. Likewise, overexpression of a polypetide consisting of the C-TAD portion of HIF-1α that binds CBP/p300, acts in a dominant-negative manner, inhibiting hypoxia-induced expression of an erythropoietin promoter by 80–90%, as well as diminishing tumor growth [37]. Initial studies by the Brindle group corroborated that the C-TAD function of HIF-1α requires the CH1 domain of CBP/p300 by measuring the activity of a tethered Gal4-DNA binding domain-HIF-1α fusion protein on a 5XGal-luciferase reporter gene. However, they also found that the CH1 domain of p300/CBP is required only for 35%–50% of all endogenous, HIF-1-responsive genes. The necessity of p300/CBP's CH1 domain for HIF-1 function is gene-dependent: vital for some and dispensable for others. Interestingly, histone deacetylase (HDAC) activity is essential for 70% of HIF-1 responsive genes, as discussed later in this review [34].

SRC-1, a member of the p160 protein family, is another HAT that potentiates HIF-1 activity. SRC-1 interacts with a variety of nuclear receptors in a hormone-dependent manner, and is thought to play a role in bridging the activated receptor with basal transcription machinery, as well as acetylating histones [31]. CBP mediates colocalization of HIF-1 and SRC-1 [38], presumably by the complex formed between CBP/p300 and SRC-1, which amplifies CBP/p300-mediated activation of HIF-1 responsive promoters [31,39]. While all three of these HATs enhance HIF-1 function, there are few reports of investigators directly analyzing histone acetylation at the promoters of HIF-1 target gene. One publication documents an increase in histone acetylation at the promoter of VEGF during hypoxia [40]; although, it is not known the degree to which hypoxia-induced acetylation is attributed to HAT activity of p300/CBP versus SRC-1.

To assess directly if the HAT activity of p300/CBP is essential for its function as a HIF-1 coactivator, a mutation was made in the HAT domain of p300. Cotransfection of p300ΔHAT with the C-TAD of HIF-1α resulted in no enhanced HIF-1 activity; whereas, wild-type p300 enhances the C-TAD function of HIF-1α. In contrast, p300ΔHAT did enhance HIF-1 N-TAD activity a modest two-fold. This slight increase in HIF-1 N-TAD activity may be attributed to p300/CBP's ability to serve as a bridge between activators and the general transcription machinery. In the case of SRC-1-amplified HIF-1 activity, full-length p300, but not p300ΔHAT, further augmented SRC-1 enhancement of C-TAD function two-fold, indicating that the HAT activity of p300 contributes to the C-TAD activity of HIF-1 even in the presence of SRC-1 [31]. Whether the HAT-catalytic domain of SRC-1 is essential for its enhancement of HIF-1-mediated transactivation in hypoxia remains to be determined.

Interactions between HATs and their target proteins are themselves subject to upstream signaling and regulation. For example, the MAPK (mitogen-activated protein kinase) signaling cascade is known to be essential in promoting interaction between HIF-1α and p300/CBP and increasing HIF-1α–mediated transactivation. MAPK enzymes can phosphorylate p300 *in vitro*, which may directly or indirectly facilitate HIF-1-p300 interaction and increase HIF-1 transactivation, although the exact mechanism has not been determined [41]. Additionally, nitric oxide (NO) signaling induces covalent modification of HIF-1 protein. NO stimulates S-

nitrosation of cysteine 800, a critical residue within the C-TAD of HIF-1α, which enhances interaction of HIF-1 with p300/CBP and increases HIF-1 activity [42,43].

Negative regulators of p300/CBP-HIF-1α interactions are also vital components of HIF-1-regulating pathways. Among these p53, p35srj/CITED2 (CBP/p300 interacting transactivator with ED-rich tail) and CITED4 compete with HIF-1α for binding to p300/CBP and inhibit HIF-1 activity [44–49]. *In vitro* protein binding assays reveal that p53 competes with HIF-1 at the CH1 domain of p300, and p53 over expression promotes HIF-1α degradation [44]. CITED2 also competes with HIF-1α for p300 binding *in vitro* and *in vivo*. Interestingly, the CITED2 gene contains HREs within its promoter and is activated by HIF-1 itself during hypoxia, suggesting that CITED2 participates in a negative feedback loop with HIF-1 [48, 49]

Factor Inhibiting HIF-1 (FIH-1) disrupts HIF-1-p300/CBP interaction by hydroxylation of an asparagines residue (Asn 803) in the C-terminus of HIF-1α. FIH-1 is an Fe(II)-dependent enzyme that uses molecular O_2 as a cofactor. Thus, FIH-1 serves as an oxygen sensor, along with prolyl hydroxylases that hydroxylate HIF-1α and mark it for proteosomal-mediated degradation [25,26,50–52].

Specific prolyl hydroxylases, which are associated with negative regulation of HIF-1α, paradoxically may interact with identified members of HAT-protein complexes. For example, a candidate tumor suppressor protein, Inhibitor of Growth 4 (ING4), inhibits angiogenesis, tumor growth, and loss of contact inhibition [53]. ING4 was co-purified in a complex containing the HAT, HBO1, but also found in association with a prolyl hydroxylase at an HRE. In contrast to CBP/p300 and SRC-1, the ING4-complex ING4 directly interacts with HIF prolyl hydroxylase 2 (HPH2) but does not alter HPH2-catalytic activity. ING4, HPH-2 and HIF-1 were found by chromatin immunoprecipitation (ChIP) to associate with an HRE-containing promoter under hypoxia and suppress HIF-1-mediated regulation of a stably transfected, synthetic HRE-driven reporter under hypoxia [53]. In this context, it is not known if ING4 is bound to chromatin as a member of an HBO1-containing HAT complex, nor if HAT activity is required for ING4-mediated HIF-1 suppression.

Deacetylation and HDACs during hypoxia

Severely hypoxic and anoxic cells enter anaerobic glycolysis to generate ATP, whereby pyruvate is converted into lactate instead of acetyl-CoA. Lower cellular levels of acetyl-CoA during hypoxia [54] may, hypothetically, lead to a global decrease in histone acetylation levels [55]. Additionally, specifically regulated, enzymatic removal of acetyl-groups from histones and other proteins is provided by HDACs. HDACs are organized into three categories: Class I HDACs 1,2,3, and 8 are similar in structure and are ubiquitously expressed. HDACs 4–7,9 and 10 are designated as class II, due to a conserved domain within a nuclear export signal. Class III HDACs are related to the yeast Sir2 HDAC, in that they act in an NAD-dependent manner. The newest HDAC, HDAC 11, contains conserved regions of both class I and class II, but is distinct enough from either of the two to form a new class IV category [56–59].

In order to ascertain if hypoxia induces histone hypoacetylation, histones were purified from human fetal lung type II cells exposed to 2% oxygen. Analysis by immunoblotting revealed a global reduction of acetylated H3-K9 in response to hypoxia, as well as decreased CBP protein levels and increased expression of HDACs 1,2,4 and 11 [60]. If histone deacetylation contributes to regulation of transcription under hypoxia, one would expect to find hypoacetylated histones at the promoters of hypoxia-repressed genes. Hypoacetylated histone H3 is indeed found at the promoters of hypoxia-repressed genes, novel immunogenic protein 3 (NIP3) and survivin [61,62]. Furthermore, hypoxia-induced repression of mismatch repair gene, *Mlh1*, is reversed in the presence of the broad-based, HDAC-inhibitor TSA,

demonstrating that deacetylation is likely essential for this hypoxia-regulated response though direct analysis of the acetylation status of *Mlh1*-chromatin was not performed [63].

During severe hypoxia (0.2% oxygen), p53 protein accumulates; however, in this environment, p53 does not activate its previously characterized cell-cycle arrest- and apoptosis-promoting target genes. Hypoxia-activated p53 associates with transcriptional corepressor, mSin3A, rather than transcription coactivator p300, and may effect histone deacetylation. mSin3A is generally found as a member of HDAC-protein complexes and functions as a transcriptional corepressor by mediating interactions between HDACs and transcription regulatory proteins [64–66]. Recent global analysis of p53-interactions with chromatin in response to stress, including hypoxia, showed little change in promoter-association of p53 although gene expression patterns change markedly [67]. These and other studies underscore the importance of corepressor or coactivator association with chromatin-bound, transcription factors to effect alteration of chromatin structure and gene expression.

HDAC activity has been correlated with hypoxia-induced repression of specific genes but, paradoxically, HDACs are also associated with activation of HIF-1 responsive genes [34]. Several studies report inhibition of HDAC-activity as a means of anti-angiogenesis therapy [68–72]. One potential mechanism to explain this phenomenon is that HDACs reportedly repress tumor suppressors, VHL and p53. Over-expression of HDAC1, which has been shown to be hypoxia-inducible, decreases p53 and VHL gene expression and, in parallel, stimulates angiogenesis. Further, TSA, an inhibitor of HDAC activity, increases p53 and VHL expression and down-regulates HIF-1α, VEGF and, consequently, angiogenesis [71]. However, others have now reported that TSA-mediated inhibition of HIF-1α is independent of VHL and p53 [73]. Many groups agree that a deacetylation event is necessary for HIF-1-mediated response; however, the target of deacetylation is a point of disagreement. To resolve these questions, histone modification analyses of hypoxia-regulated genes, as well as the modification status of potential protein targets of regulated acetylation, *e.g*, p53, HIF-1α, p300 and Hsp90, must be determined. Deacetylation of one or more of these targets may be critical in control of angiogenesis [24,73,74].

Acetylation/deacetylation in response to other stresses

A variety of cellular stresses, in addition to hypoxia, regulate gene expression by shifting acetylation and deacetylation equilibria (Table 1). These include oxidative stress that is created by increases in reactive oxygen species (ROS). ROS signaling in hypoxia is a topic of great debate, some reports citing an increase in ROS, while others claim that a decrease in ROS occurs during hypoxia [75,76]. Oxidative stress enhances inflammation by increasing expression and activities of redox-sensitive transcription factors, such as NFκB and AP-1, as well as inhibiting HDAC activity. Further, oxidative stress promotes interactions between CBP/p300 and the p65 subunit of NFκB, which correlates with increases in acetylated H4 at the promoters of NFκB-target genes and activated transcription. Glucocorticoids may act as anti-inflammatories by inhibiting these functions and promoting recruitment of HDACs to the promoters of inflammatory genes, though the exact mechanism of this process remains undetermined [77,78].

Stress in the endoplasmic reticulum (ER-stress) is caused by a number of factors including translational inhibition, exposure to protein denaturing agents, and conditions that cause an accumulation of unfolded or incorrectly folded proteins. Glucose-regulated protein 78 (GRP78/BiP) is an ER chaperone that is activated by the unfolded protein response and promotes cellular survival. Upon ER stress, p300 is recruited to the promoter of GRP78/BiP, which correlates with an increase in acetylated H4 and expression of GRP78/BiP [79]. ER-stress may also occur under hypoxic conditions, due to inhibition of protein translation or decreased metabolic functions. To adapt to the inefficient energy-producing state created by hypoxia, the mTOR-

signaling pathway is repressed. This in turn induces ER-stress, as an attempt to conserve energy within stressed cells and survive [79,80].

Phosphorylation and Phosphoacetylation during stress

Phosphorylation of histone H3, at serines 10 and 28, correlates with active transcription of stress-induced genes, as well as mitosis. For example, toxicants such as anisomycin, arsenite, and DNA-damaging cisplatin all induce phosphorylation of H3-S10 at stress-response genes [10]. Stress-induced phosphorylation of H3 is mediated through the MAPK pathway. Four main MAPK pathways are known: extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), c-Jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38, and big mitogen-activated protein kinase (BMK)/ERK 5 pathway. Stress-induced phosphorylation of H3, however, is only mediated through the ERK or p38 pathways. The ERK pathway is activated by growth factors and TPA; while, the p38 pathway is induced by stresses such as anisomycin, arsenite and UV-irradiation [81]. Both the ribosomal S6-kinase (RSK2) and mitogen and stress activated kinase (MSK1/2) have been implicated in directly phosphorylating H3 and the chromatin-associated high mobility group protein (HMG-14) through MAPK pathways. More recent data support MSK1/2 as the most likely stress-induced H3 kinase [81,82].

The amount of histone H3 that is phosphorylated, in response to stress, is a small percentage of total H3, but the phosphorylated fraction is more likely acetylated to create phosphoacetylation [83], including acetylated K-9 and/or K-14 and phosphorylated S-10, of an N-terminal tail of H3 [84]. The chromatin of immediate-early genes is a known target of phosophoacetylation in response to stress induced by toxicants and UV [81,82,84]. This histone phosphoacetylation may be responsible for transient relaxation of chromatin, revealed by DNase I sensitivity analyses, and trans-activation of immediate-early genes in cells exposed to stress [85,86]. Likewise, oxidative stress induces phosphoacetylation of NFκB-target gene chromatin to potentiate gene activation [77,78].

To our knowledge, there is no published report of hypoxia-induced phosphorylation of H1, phosphorylation of H3, or phosphoacetylation of H3 at hypoxia-regulated genes. Hypoxia and oxidative stress both induce NFκB activity; therefore, it is plausible that the chromatin of NFκB target genes is phosphoacetylated in response to hypoxia, as well as oxidative stress. In addition to ERK1/2's role in activating a histone H3 kinase, MSK1/2, ERK1/2 also upregulates HIF-1 activity [41,81]. One potential mechanism for ERK 1/2-mediated activation of HIF-1 activity is through phosphorylation of H3 at HIF-1 target genes. Phosphorylated H3 may be a preferred substrate of p300/CBP [81], or it may facilitate recruitment of a secondary protein that enhances p300/CBP binding at HIF-1 target genes or both. Alternatively, MAPK signaling enhances HIF-1 activity by promoting HIF-1-p300/CBP interaction; therefore, this may be the sole mechanism of ERK1/2-mediated activation of HIF-1 target genes [41].

Histone methylation during hypoxia

Histone methylation contributes to gene activation, gene repression, gene silencing, X-inactivation and changes in chromosomal structure. The varied effects of histone methylation are controlled through residue-specific histone methylation and the number of methyl groups added to each amino acid. Methylation occurs at arginine and lysine residues of histones H3 and H4. Arginine can be either mono- or di-methylated; whereas, lysine can be mono-, di- or tri-methylated [87–89]. Unlike histone acetylation, methylation does not regulate gene expression by changing the charge of a lysine or arginine. Histone methylation regulates gene expression by creating binding sites for specific proteins and potentially antagonizes or augments the effects that other histone modifications have on gene expression [87].

Activation-associated modifications of histones include methylated arginines, created by PRMT1 at H4-R3 and PRMT4/CARM1 at H3-R2, R3 and R17. Histones methylated on lysine residues are found within both euchromatin and heterochromatin. Methylated lysines 4, 36, and 79 of histone H3 are found in actively transcribing genes; whereas, mono- and di-methylated H3-K9 and trimethylated H3-K27 associate with transcriptionally repressed genes within euchromatin. In contrast to dimethylated H3-K9, trimethylated H3-K9, along with trimethylated H4-K20 and monomethylated H3-K27, is found in heterochromatin [87,88].

To our knowledge, there are no publications reporting analyses of activating histone methylation marks at arginines or lysines of hypoxia-induced genes. As discussed previously, ER stress can be induced by hypoxia, Ca^{++} depletion, blocked N-linked glycoslyation of proteins and exposure to protein denaturing agents. Activated transcription of the ER chaperone gene, GRP78/BiP, is correlated with recruitment of PRMT1, an arginine histone methyl transferase, and increased dimethylated H4-R3 [79]. If this mechanism is universal in activation of GRP78/BiP gene expression, then an increase in arginine methylation is likely induced by ER stress during hypoxia. CARM-1-mediated histone methylation at arginine residues enhances NFκB-mediated transactivation in response to inflammatory triggers, in addition to facilitating nuclear hormone receptor mediated activation [90]. We may find that hypoxia-induced, NFκB-target genes likewise have increased histone arginine methylation, as well as hypoxia-induced, histone acetylation. Moreover, due to findings that histone methylation, associated with active transcription, facilitates histone acetylation [91,92], it is probable that histone methylation occurs on all hypoxia-induced genes marked with increased histone acetylation.

Interestingly, a global increase in di- and tri-methylated H3-K9 was observed in human fetal type I lung cells and A549 human lung carcinoma cells subjected to hypoxia, concomitant with a global decrease in acetylated H3-K9 and H3-K14 [55,60]. This same combination of histone modifications, normally correlated with inactive gene expression, was found at the hypoxia-repressed surfactin protein A (Sp-A) gene, indicating that histone hypoacetylation and histone H3-K9-methylation are involved in hypoxia-induced transcription repression [60]. It will be interesting to see if methylated H3-K9 and HP1-binding are associated with all hypoxia-repressed genes, alongside activating histone methylation marks on lysines and arginines of hypoxia-induced genes. The rapid changes in gene expression that follow cellular exposure to cycles of hypoxia and reoxygenation may rely on a specific subset of histone modifications that likewise are easily reversible or built in a combinatorial manner. Analyses of chromatin structure of an increased number of hypoxia-responsive genes must be performed to discern potential regulatory motifs and mechanisms of gene activation or repression under conditions of oxygen fluctuation.

Stress response dictated by chromatin remodeling complexes

Chromatin remodeling and hypoxia

The human SWI/SNF complex, which is the founding member of a family of ATP-dependent chromatin remodeling enzymes, contributes to HIF-1-mediated activation of target genes in hypoxia. Both catalytic subunits of human SWI/SNF, Brahma (Brm) and Brahma/SWI-2 related gene 1 (Brg1), enhance HIF-1-mediated activation of an erythropoietin (Epo)-driven reporter and a synthetic 6XHRE-driven reporter during hypoxia [93]. These two ATPases are highly homologous; however, the two remodeling complexes are targeted to distinct promoters by specific transcription factors and are involved in different cellular pathways [94,95]. Knock-down of both enzymes reduced Epo expression greater than either alone, indicating a lack of absolute redundancy between the two enzymes. Both enzymes are recruited, along with HIF-1, to the promoter of Epo in a hypoxia-dependent manner. Additionally, both Brm and Brg enzymes are recruited to the VEGF promoter, but are not essential for hypoxia-induced

expression of VEGF [93]. The bromodomains of Brg-1 and Brm bind to acetylated lysines, and the promoters of Epo and VEGF are enriched in acetylated histones after hypoxic exposure [40,93]. HATs and chromatin remodeling complexes likely work together to create an environment on the Epo promoter, required for binding of transcription factors; whereas, chromatin remodeling may not be essential for transcription factor interactions at the VEGF promoter. Analyses of nucleosomal distribution along the Epo promoter and/or the VEGF promoter under normoxia and hypoxia should be undertaken to determine the importance of Brm/Brg-1-mediated chromatin remodeling in activation of these genes.

Chromatin remodeling and gene regulation during other stresses

Although very little is known about chromatin remodeling in response to hypoxic stress, it has been proposed as a mechanism of gene regulation in response to other stresses (Table 1). For example, genome-wide localization of Rsc9, a component of the RSC chromatin-remodeling complex, to stress-regulated genes occurs during treatment with H_2O_2 or rapamycin, either of which can inhibit TOR-signaling and induce ER-stress. Rsc9-relocalization potentially promotes remodeling of chromatin structure at these target genes. Expression analysis of genes, known to be regulated by TOR signaling, showed that either repression or activation of TOR-regulated mRNAs and rRNA occurred under conditions that led to Rsc9 interaction with chromatin [96]. Analysis of chromatin structure and potential remodeling at specific Rsc9-occupied, TOR-regulated genes has not been performed to confirm that Rsc9 association alters nucleosomal positioning and binding of transcription activators or repressors.

In contrast, the role that chromatin remodeling plays in response to the stress of heat shock and activated transcription of heat shock protein 70 (hsp70) genes is well characterized. RNA polymerase II is paused at the promoter of inactive *hsp*-genes and is released for elongation by heat shock factor 1 (HSF1) binding to specific regulatory elements. This heat shock-induced release of RNA polymerse II is more efficient when SWI/SNF is present than in its absence. Mutation of the activation domain of HSF1 impairs recruitment of SWI/SNF and decreases association between Brg1 and HSF1. SWI/SNF recruitment by HSF1, and subsequent remodeling of positioned nucleosomes and/or compacted chromatin at the promoter of hsp70, may be essential for HSF1-mediated stimulation of Pol II elongation [97].

Covalently modified histones as protein recruitment marks during stress

DNA-damage and damage-independent responses

Histone modifications can influence gene expression by providing a binding site for transcriptional activators and repressors. Under stresses that induce DNA damage, H2AX (a variant of histone H2A) is phosphorylated and recruited to sites of DNA-strand breakage, and is essential for formation of DNA damage-induced foci. Phosphorylated H2AX is important for retention and accumulation of repair and checkpoint proteins, as well as recruitment of chromatin remodelers, INO80 and SWR1, and the HAT complex, NuA4 [9,11,98]. INO80 and SWR1 facilitate interactions between DNA and end-processing enzymes required for DNA repair. Furthermore, phosphorylated H2AX is connected to recruitment and spread of cohesin at DNA double-strand breaks. Cohesin links sister chromatids together during S-phase and may facilitate DNA repair by keeping the sister chromatids close to each other for homologous recombination [9,11].

Phosphorylated H2AX is observed under hypoxic conditions, as well as hypoxia followed by reoxygenation. The hypoxia-induced presence of H2AX relies on ATR signaling and not ATM, the kinase generally involved in DNA damage-response. ATR signaling promotes cell arrest, and phosphorylation of H2AX was observed in severely hypoxic cells that were experiencing replication arrest. The exact function of phosphorylated H2AX in hypoxia or in cell cycle arrest

has not been deciphered. Hypoxia is not thought to induce DNA damage; therefore, phosphorylated H2AX likely does not have the same function during hypoxia as other DNA-damaging stresses [99]. It will be interesting to see if phosphorylation of H2AX also occurs in hypoxic cells that escape surveillance mechanisms and continue to proliferate, as opposed to hypoxic cells that have undergone replication arrest.

Checkpoint proteins delay cell cycle progression in response to DNA damage, allowing time for DNA repair. In *S. cerevisae*, Rad6-Bre1-directed ubiquitylation of H2B-K123 is essential for Rad9-mediated activation of the checkpoint kinase, Rad53. H2B-K123 ubiquitylation is also essential for Dot1-mediated methylation of H3-K79. Interestingly, mutations in Dot 1 or H3-K79 also result in Rad53 defects. It is reasonable to hypothesize that H2B-K123 facilitates checkpoint kinase, Rad 53, activation by promoting H3-K79 methylation. In mammals, methylated H3-K79 provides a binding site for the Rad9 homologue, p53 binding protein (53BP1), and methylation of H3-K79 is essential for 53BP1 foci formation [9,11]. However, foci formation is not known to occur in response to hypoxia but may be promoted by cycles of hypoxia and reoxygenation, which cause DNA-damage [100], in a growing tumor.

Global, structural changes to chromatin induced by stress

Hypoxia-mediated cellular responses

Global changes in chromatin structure are hallmarks of cellular processes such as apoptosis, necrosis, mitosis and cell cycle arrest; whereas, localized changes in chromatin structure facilitate gene-specific regulation and DNA repair. The fate of hypoxic cells is dependent upon multiple factors. Hypoxia promotes apoptosis and growth inhibition and, at the same time, also imposes a strong selective pressure for survival of cells with impaired cell cycle arrest and apoptotic processes. This selective process promotes genomic instability and the transcriptional activation of genes that enhance survival and growth during tumorigenesis [12,13].

Apoptosis is characterized by chromatin condensation, DNA fragmentaion, release of nuclear proteins, cytoplasmic shrinking and membrane blebbing [101]. Necrosis, rather than apoptosis, occurs when cellular ATP levels are below a threshold required to initiate the apoptotic machinery. Necrotic cells swell until they burst, inducing an inflammatory response [102]. Exposure to severe hypoxia induces apoptosis of normal fibroblasts and tumor-derived, cultured cells [103–105]. Normal, wild type neuronal cells undergo both apoptosis and necrosis in rats subjected to bilateral occlusion of the common carotid arteries. Chromatin extracted from these cells exhibited non-specific DNA fragmentation, indicative of necrosis, as well as internucleosomal cleavage of chromatin, an increase in electron density and pycnotic condensation, which are associated with apoptosis. Although both apoptotic and necrotic characteristics were observed, the two events may take place in different neuronal cells and/or at different times [106]. Rat1a fibroblasts are apoptotic during anoxia, but undergo necrosis if subjected to hypoxia and glucose deprivation. These oxygen- and glucose-deprived cells fail to maintain a level of ATP critical for apoptosis to occur [102].

Structural changes in chromatin during apoptosis include covalent modifications of histones. Phosphorylation of H2AX, triggered by hypoxia, is observed in apoptotic cells, concomitant with appearance of high molecular weight-DNA fragments but before internucleosomal cleavage occurs [107][100]. It is not known whether H2AX exists in apoptotic cells due to a failed attempt at DNA repair or if it has a separate function. Apoptosis-specific phosphorylation of H2B on serine 14 occurs via Mst1, a mammalian sterile 20 kinase, and is proposed as a direct cause of chromatin condensation. H2B-modification and chromatin condensation precede genomic fragmentation during apoptosis [108].

Phosphorylation of H3-S10 and hyperphosphorylation of H1 are required for mitotic chromatin condensation [109]. Because apoptosis is also associated with chromatin condensation, several investigations were undertaken to determine if these marks also occur during apoptosis. In thymocytes, phosphatase inhibitors induce apoptosis and increase phosphorylation of proteins predicted to be histones H3, H2, and H1[110]. Phosphorylation of H3 at serine 10 occurs in cells undergoing apoptosis, triggered by the fungal toxin, gliotoxin. However, cells treated with apoptosis-inducing agents, other than gliotoxin, do not undergo rapid phosphorylation of H3-S10 [111]. Likewise, dephosphorylation of H1 occurs during apoptosis, prior to internucleosomal cleavage, in contrast to phosphorylation of H1 observed during mitosis [112] Therefore, mitosis-associated chromatin condensation marks do not appear to be general markers of apoptosis.

During apoptosis, there is a loss of nuclear integrity preceding collapse of the nucleus. There is some debate as to whether chromatin truly undergoes condensation or whether heterochromatin simply aggregates and appears condensed [109]. Loss of acetylated histones from nuclei has been observed in cells undergoing apoptosis, but not during mitosis. Detection of acetylated histones in the cytoplasm led to the hypothesis that hyperacetylated histones located in nuclease-sensitive euchromatin are released into the cytoplasm for further degradation, which the authors speculate is a forerunner of heterochromatin aggregation. However, recent data show that histones released into the cytosol during early apoptosis are hypoacetylated, as well as dephosphorylated and demethylated, compared to histones remaining in the nucleus. Furthermore, released histone H4 is also hypoacetylated and enriched with trimethylated K-20. The origin of released histones, found in the cytosol, has been traced to perinuclear heterochromatin [113]. This group was not able to detect acetylated histones released in the cytosol, at any time prior to hypoacetylated histones. Although the two models of histone mobilization during apoptosis remain unresolved, these studies emphasize an important role for epigenetic histone modifications during apoptosis.

In addition to apoptosis, hypoxia may lead to cell cycle arrest. Under conditions of low oxygen partial pressure (0.2–1 mm of Hg), cells exhibit a lengthened G_1 phase or G_1 arrest. Anoxia causes cells to arrest, by mechanisms that remain ill-defined, regardless of where they are in the cell cycle [12]. A possible role for cyclin-dependent kinase inhibitors, such as KIP1/p27, in mediating hypoxia-induced cell cycle arrest is controversial and may depend on the severity of hypoxia and/or cell type [114,115]. p53-dependent transactivation of cell cycle inhibitors is not likely, as hypoxia-induced p53 is transcriptionally impaired [99] and hypoxia-induced cell cycle arrest is observed in cells lacking wild type p53 [116].

Hypoxia induces developmental and cell cycle arrest in *C.elegans, Drosophila* and zebrafish embryos. In *C. elegans* embryos exposed to anoxia, cells arrest during prophase and metaphase stages of mitosis and during interphase; *Drosophila* embryos arrest during interphase and metaphase; and, zebrafish embryos arrest during interphase. Interphase blastomeres from anoxic embryos of all three species contain condensed chromatin that is not uniformly distributed throughout the nucleus. A more extensive study in *C. elegans*, reveals that just 30 minutes of anoxic exposure (acute anoxia) promotes chromatin condensation in interphase blastomeres. After an intermediate time of 6–12 hours and a longer exposure of 24–72 hours, chromosomes from interphase blastomeres align near the nuclear membrane; whereas, chromosomes from prophase blastomeres align near the nuclear membrane during all phases of anoxia: acute, intermediate and long-term. H3 phosphorylation is detected during acute anoxia and does not become dephosphorylated until exposed to 6 hours of anoxia, suggesting that H3 dephosphorylation is not required for relocalization of the chromosomes to the nuclear membrane. The physiological significance of realigning chromosomes near the nuclear membrane is not known; although, the authors propose that attaching chromatin to nuclear matrix or substructures may restrict chromosome movement and inhibit mitosis [117].

Changes in chromatin structure associated with other stresses

The exchange of histones for histone variants is an additional means of altering chromatin structure. Histone variants are associated with changes in gene expression, DNA repair, meiosis and apoptosis [118]. Besides the aforementioned H2AX variant, the linker histone variant, H1.2, is also a marker of DNA damage-induced apoptosis. DNA double-strand breaks, but not other apoptosis-inducing stimuli, trigger the translocation of H1.2 from the nucleus into the cytoplasm. There, histone H1.2 activates cytochrome c release from the mitochondria, which in turn activates downstream caspases critical for apoptosis. It is not known how histone H1.2 activates cytochrome c release, but it is dependent upon Bak, the proapoptotic bcl-2 family member. The authors propose that cationic amino acid residues in histone H1.2 may interact with anionic phospholipids in the mitochondrial membrane, indirectly activating Bak, and disrupting the membranes. H1.2 is the only isoform of histone H1 capable of inducing cytochrome c release [10]. Because histone H1.2 translocation is specific to stimuli that induce DNA double-strand breaks, and hypoxia does not elicit DNA damage, we would not predict that H1.2 participates in hypoxia-induced apoptosis.

Stress induced by exposure to certain metals results in toxicity. Ni, Cu, Co, and Cr can act as catalytic centers for redox reactions, generating reactive oxygen species, which have been associated with 40 different clinical conditions. Their toxic effects can be attributed to metal-induced, oxidative DNA-damage and the creation of DNA-protein cross-links between the metals and DNA or between DNA and proteins within chromatin. Moreover, morphologic aberrations of chromosomes can occur because of these chromatin cross-links. Nickel exposure results in chromosomal alterations in heterochromatin of the X chromosome in Chinese hamster ovary cells [119].

Oxidative stress by hydrogen peroxide induces higher order chromatin degradation. Severe oxidative stress is associated with acute neurodegenerative disorders and dismantles the genome, causing cell death. Chronic neurodegernative disorders are linked with sublethal, but perpetual oxidative stress. In these cells, oxidative stress results in partial genome fragmentation, which may lead to somatic mutations [120].

In *Drosophila* larval salivary glands, a loosening of the chromatin structure, or puffing, is associated with stress-induced gene activation. These puffs are thought to reflect “open” chromatin structure, which facilitates transcription by creating an accessible template for transcription factor binding. In response to heat shock, ADP-ribose modified proteins accumulate and are thought to strip off nearby chromatin proteins, in order to create chromosome puffs [121]. Because packaging of chromatin is directly related to transcriptional activity and any process with DNA as substrate, exposure to stresses that alter overall structure of chromatin likely have profound, global effects for cells.

Concluding remarks

The unknowns and the knowns in chromatin-mediated stress-response

While there is much knowledge of chromatin modifications that occur in response to stress that induce DNA-damage, those associated with stress that does not induce DNA damage, *e.g*, hypoxia, are not well defined. Globally, hypoxia may reduce cellular levels of acetyl CoA due to anaerobic metabolism; although, it is unknown if acetyl CoA generated during metabolism directly alters levels of histone acetylation. Furthermore, it is important to determine if global changes in histone modifications, such as decreases in histone acetylation, correlate with a global transcription profile. Compaction of chromatin is observed in hypoxic cells undergoing cell cycle arrest and apoptosis, but it is not known if a hypoxia-mediated decrease in histone acetylation creates compacted chromatin, which directly leads to a global decrease in transcription. Clearly, it is plausible that gene-specific modifications such as

phosphoacetylation and arginine methylation, which promote transcriptional activation under other stress conditions, may also contribute to hypoxia-induced gene expression and likely occur at gene targets shared by hypoxia and other stresses.

The impact that chromatin structure has on gene expression and cellular processes, including apoptosis and cell cycle arrest, becomes obvious as identification of histone modifications and chromatin modifying enzymes and their functions continues to expand. Chromatin remodeling, and the enzyme complexes that mediate it, are essential in many stress-related changes in gene expression, yet little is known about how these enzymes contribute to hypoxia-mediated alterations in gene expression. From studies in fission yeast, we have learned that there is specificity between the type of stress and gene-specific chromatin remodeling [122]. Further analysis of the specific signaling pathways that activate distinct chromatin remodeling enzymes will enable researchers to predict which enzymes may act during hypoxia to alter chromatin structure. Clearly, alteration of chromatin structure is integral to cellular response to stress, whether the cell lives by repair or dies by necrosis or apoptosis. Changes in chromatin structure, as any stress-induced process, must be tightly regulated to avoid the pitfalls of tumorigenesis and disaster for the organism.

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Table 1

Stress-induced alterations in chromatin

Stress	Chromatin reponse	Mediator	Ref
Hypoxia	potentiated HIF-1 transactivation	CBP/p300, SRC-1	31–39
	increased H3K9 acetylation of VEGF promoter	unknown	40
	global deacetylation of H3-K9/14 and at the Sp-A promoter	loss of CBP and an increase HDACs 1,2,4,11	55,60
	deacetylation of H3-K9/14 at survivin and NIP3 promoters	unknown	61–62
	global increase of di- and tri-methylated H3-K9 and at Sp-A promoter	unknown	55,60
	repression of the mlh1 gene	HDAC-dependent	63
	activation of hypoxia-induced genes	HDAC-dependent	68–72
	erythropoietin gene activation	Brg1 and Brm	93
	suppresed HIF-1 activity	ING4 (HAT-complex)	53
	phosphorylation of H2AX	ATR	99
	condensation of chromatin	phosphorylation of H2B-S14	108
	increase in internucleosomal cleavage	apoptosis-associated	103–105, 107
	chromatin condensation and perinuclear accumulation along with dephosphorylation of H3 in C. elegans embryos	unknown	117
DNA-damage inducing			
Double-stranded breaks	phosphorylation of H2AX	ATM	9,11,98
	potential opening up of the chromatin	INO81 and SWR1	9,11,98
	H2B-K123 ubiquitiylation	Rad60-Bre1	9,11
	H3-K79 methylation	Dot1	9,11
	H4-K29 methylation	Set9	9,11
	H1.2 translcation into the cytoplasm	apoptosis-associated	10
UV and cisplatin-induced	phosphoacetylation at immediate early genes	MSK1/2 and HATs	10,81–82
Oxidative stress	increased H4 acetylation and activation of NFkB genes	CBP/p300	77–78
	inhibits HDAC activity	reactive oxygen species	77–78
	increased phosphoacetylation	MSK1/2 and HATs	77–78
acute oxidative stress	dismantling of the genome	H2O2	120
chronic oxidative stress	partial genome fragmentation which may cause somatic mutations	H202	120
inflammation-induced	histone arginine methylation	CARM1	90
ER stress	increased H4 acetylation at the GRP78-BiP promoter	p300	79
	increased dimethylated H4-R3 at the GRP78-BiP promoter	PRMT1	79
	transcriptional activation and repression of TOR-regulated mRNAs and rRNA	genome-wide localization of Rsc9	96
Toxicants			
anisomycin and arsenite	increased phosphoacetylation at immediate early genes	MSK1/2 and HATs	10,81–82
metals	oxidative DNA damage	metals	119
	crosslinks between DNA and metals or DNA and chromatin-associated proteins	reactive oxygen species	119
nickel	chromosomal aterations in heterochromatin on the inactive X chromosome	nickel	119
	inhibits GCN5 HAT activity	nickel	119
chromium	perturbs HAT and HDAC binding on polycyclic aromatic hydrocarbon-inducible genes	chromium	119
Heat shock	relief of paused Pol II, facilitates transcription	SWI/SNF	97
	transcriptionally active puffs of chromatin	ADP-ribose modified proteins	121
Hyperosmotic stress	altered chromatin around a CRE-like sequence in the ade6-M26 promoter, increasing transcription	unknown	122
Cation stress and Glucose starvation	altered chromatin structure at the CRE in the cta3+ and fbp+ genes	unknown	122