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Sumoylation of hypoxia inducible factor-1α and its significance in cancer

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Hypoxia-inducible factor-1 (HIF-1) is a key heterodimeric transcription factor for the cellular adaptive response to hypoxia, a common feature of the microenvironment in solid tumors. The transcriptional activity, protein stabilization, protein-protein interactions and cellular localization of HIF-1α, an oxygen-sensitive subunit of HIF-1, are mainly modulated by various post-translational modifications. Recently, we reported that polycomb chromobox 4 (Cbx4) governs the transcriptional activity of HIF-1α by enhancing its sumoylation at K391 and K477, through which Cbx4 potentiates angiogenesis of hepatocellular carcinoma. This review summarizes the current knowledge of HIF-1α sumoylation and its roles in the pathogenesis of cancer.

HIF-1α, sumoylation, cancer

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Hypoxia, a common characteristic of the microenvironment for cancer cells, promotes changes in metabolism (e.g., changes from oxidative phosphorylation to glycolysis), and the progression and metastasis of solid tumors [1–3]. Transcription factors known as hypoxia-inducible factors (HIFs) bind hypoxia responsive elements (HREs) within the promoter regions of their target genes [4,5]. HIF target genes include those involved in the regulation of hematopoietic and vascular development, cellular glucose uptake and metabolism, cell proliferation and differentiation, pH homeostasis, cell death and autophagy [6–14].

HIF heterodimer comprises one of three major oxygen-sensitive HIF-α subunits (HIF-1α, HIF-2α or HIF-3α) and a constitutively expressed β-subunit (HIF-1β, also known as aryl hydrocarbon receptor nuclear translocator, ARNT). These subunits join together to form the HIF-1, HIF-2 and HIF-3 transcriptional complexes, respectively. The hypoxic response is primarily mediated by the hypoxia-inducible HIF-1 and HIF-2 complexes, which have both overlapping and unique target genes [5]. Their transcriptional activities, protein stabilization, protein-protein interactions, and cellular localization are mainly modulated by post-translational modifications such as hydroxylation, ubiquitination, acetylation, phosphorylation and *S*-nitrosylation [15,16]. Recently, we reported that polycomb chromobox 4 (Cbx4, Figure 1A) governs the transcriptional activity of HIF-1α by enhancing its sumoylation, and hence potentiating angiogenesis of hepatocellular carcinoma (HCC) [17]. Here we review the current understanding of HIF-1α sumoylation, and its effects on protein stabilization and transcriptional activity of HIF-1, as well as its roles in the pathogenesis of cancer.

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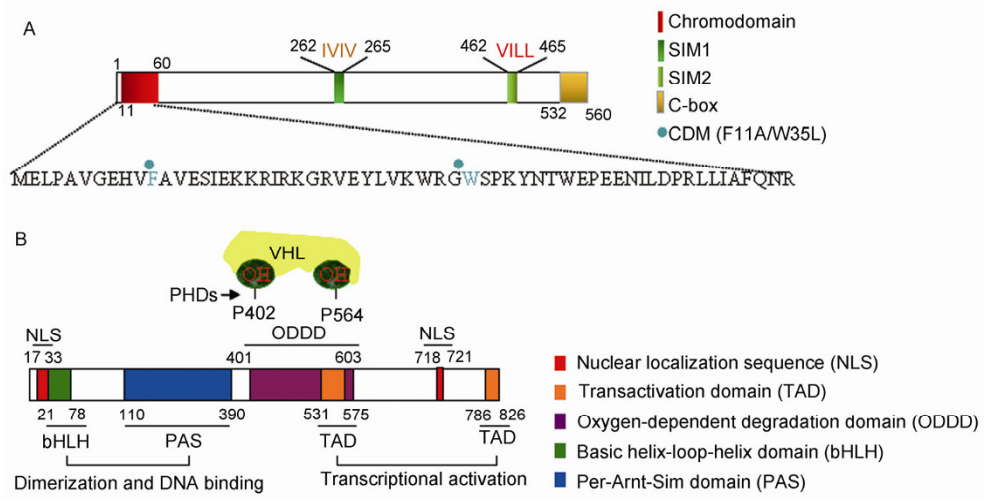

Figure 1 The schematic structure of Cbx4 (A) and HIF-1α proteins (B).

1 Ubiquitin-dependent and independent degradation of HIF-1α protein

The human HIF-1α subunit contains 826 amino acids with an approximate molecular weight of 120 kD [18]. The schematic structure of HIF-1α protein is shown in Figure 1B. It carries two nuclear localization sequences that are responsible for translocation of the accumulated HIF-1α protein to the nucleus under hypoxic conditions [19]. The transcriptional activity of HIF-1α is determined by two transactivation domains (TADs) respectively in its N-terminal and C-terminal [20–22]. Deletion of amino acids 576–785 of HIF-1α increases its transcriptional activity [20], suggesting that this region possibly contains an inhibitory domain. HIF-1α also contains a basic helix-loop-helix (bHLH) domain that mediates its dimerization with HIF-1β subunits, and a Per-Arnt-Sim domain (PAS) to be responsible for DNA binding [21]. Intriguingly, most post-translational modifications occur within the oxygen-dependent degradation domain (ODDD) which plays a crucial role in the hypoxia-dependent stabilization of HIF-1α [23].

Under normoxic conditions, prolyl hydroxylases 1–3 (PHD1–3) hydroxylate the proline residues at positions 402 and 564 within the ODDD of HIF-1 [24,25]. HIF-1 hydroxylation is essential for the binding of the von Hippel-Lindau (VHL) tumor suppressor protein, one component of a multiple protein ubiquitin-ligase complex VHL/elongin B/elongin C [26–28]. This E3 ubiquitin ligase complex links a ubiquitin chain to HIF-1α and directs it to the proteasome complex for proteolytic degradation [29]. As PHDs use oxygen as a cofactor for their hydroxylation activity, these enzymes become inactive under hypoxic conditions, resulting in non-hydroxylation of prolines 402 and 564, and inhibition of VHL binding. Consequently, the HIF-1α protein is stabilized and translocated into the nucleus [19], where it binds to HREs to regulate expression of its target genes (Figure 2). In addition to VHL, murine double minute 2 [30] and Jab1 [31] have also been reported to affect the ubiquitination and stability of HIF-1α. It should be pointed out that the protein stability and transcriptional activity of HIF-1 can be regulated by several other factors besides hypoxia, such as the accumulation of intermediate metabolites, the loss of tumor-suppressor function and the gain of oncogene function [32].

HIF-1α protein can be degraded by a mechanism that is independent of VHL, hypoxia and the ubiquitination machinery [33]. SHARP1, a crucial regulator of the invasive and metastatic phenotype in triple-negative breast cancer, was reported to inhibit the aggressiveness of the subtype of cancer through binding to HIF-1α and HIF-2α. SHARP1 acts as the HIF-presenting factor to the proteasome resulting in the proteasomal degradation of HIF-1α and HIF-2α (Figure 2). Remarkably, histone deacetylase (HDAC) inhibitors have also been shown to induce the proteasomal degradation of HIF-1α in a mechanism that is independent of VHL or the ubiquitin system. The mechanism involves the enhanced interaction of HIF-1α with heat shock protein (HSP) 70 and is secondary to a disruption of the HSP70/HSP90 axis function that appears to be mediated by the activity of HDAC-6 [34].

2 Sumoylation and HIF-1α

The small ubiquitin-related modifiers (SUMOs) share only about 18% sequence identity with ubiquitin, but SUMOs and ubiquitin are structurally quite similar. Four SUMO paralogues (designated SUMO-1, -2, -3, and -4) have been identified in mammals [35]. Like ubiquitin, SUMOs can be covalently attached to lysine residues within their target proteins via an enzymatic cascade involving a heterodimeric

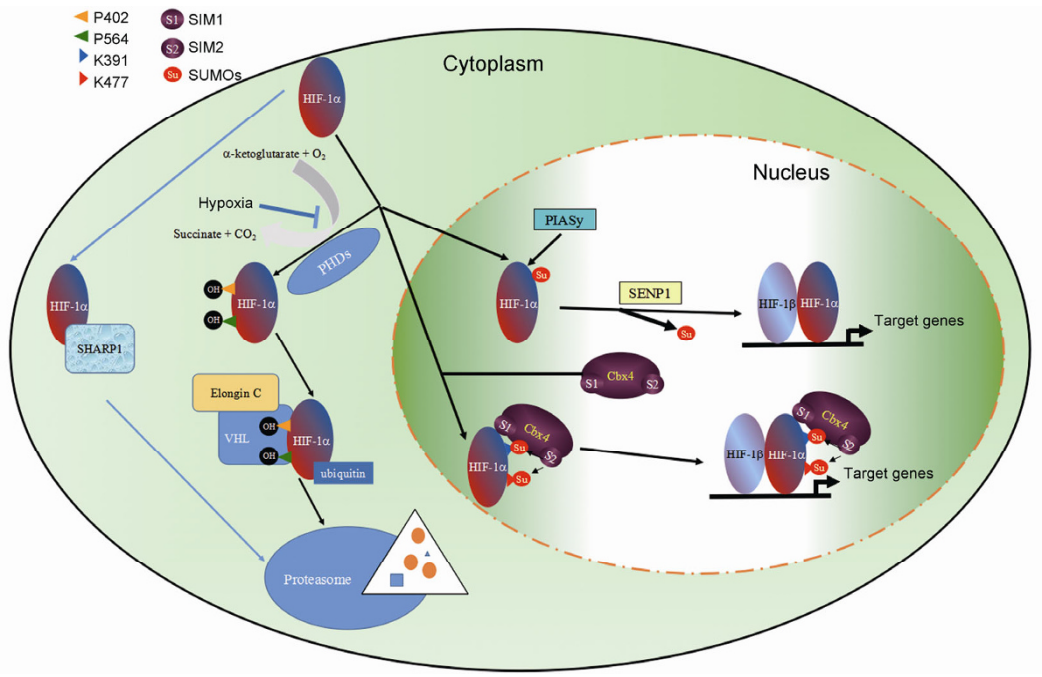

Figure 2 Regulation of the stability and transcriptional activity of HIF-1α protein by sumoylation.

E1-activating enzyme (SAE1/2), an E2-conjugating enzyme (Ubc9), and a growing number of distinct E3 ligases (Figure 3), which can increase the efficiency and specificity of the sumoylation process [36]. The activated SUMOs are transferred from the heterodimeric E1 to Ubc9 through a thioester linkage between diglycine residues at the extreme C terminus of mature SUMO proteins and the active site cysteine of Ubc9. Subsequently, the SUMO moiety is ligated onto an acceptor lysine residue of a substrate in a process that can be enhanced by a SUMO E3 ligase. However, Ubc9 is sufficient to promote substrate sumoylation, in the absence of ligase, at least *in vitro* [36,37].

Sumoylation has emerged as an important regulatory posttranslational event in mammals, as evidenced by the embryonic lethality of mouse mutants lacking Ubc9 [38]. Sumoylation modulates a diverse range of biological processes such as gene transcription, DNA replication and repair, chromosome segregation, and the transport of proteins through the nuclear pore [36,39,40]. In addition to the covalent attachment of SUMOs to lysine residues in target proteins, some specific motifs mediate non-covalent interactions with SUMOs [41–44]. In general, these motifs comprised a hydrophobic core, which is often flanked by acidic residues. The best-characterized SUMO interaction motifs (SIMs) have the consensus sequence, V/I-X-V/I-V/I or V/I-V/I-X-V/I/L, where X is any amino acid [43,44]. Structural studies have demonstrated that these hydrophobic SIMs bind to the second β-strand and the first α helix of SUMOs. With increased attention focused on the noncovalent interaction of SUMOs with SIM-containing proteins, it has become clear that there is some flexibility in

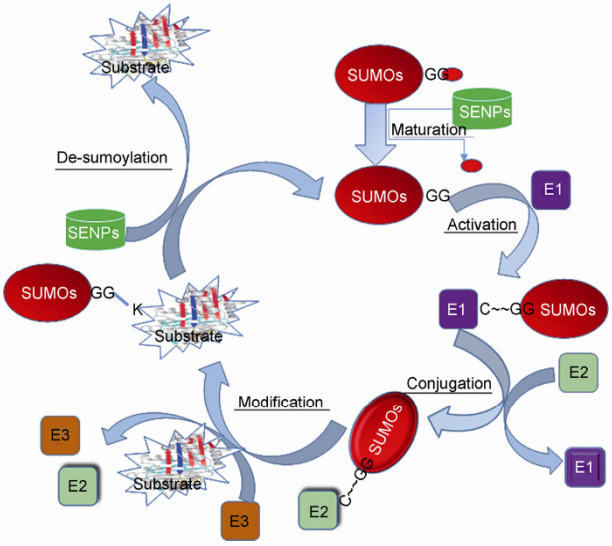

Figure 3 The sequential activation of an enzymatic cascade for protein sumoylation from SUMO maturation by SENPs, SUMO activation by the E1 enzyme, conjugation to the E2 enzyme UBC9, SUMO linkage to the protein substrate by E3 ligase and SUMO deconjugation.

the precise sequence of the hydrophobic core of the SIMs. For example, the corepressor CoREST1 binds to SUMO-2 via a SIM with a five amino acid core in which positions 1, 3 and 5 are hydrophobic residues [45]. Such results suggest that further variation in consensus SIMs will be found, emphasizing the importance of testing the functionality of potential SIMs.

Previous studies of HIF-1α sumoylation have produced conflicting results [46–49]. Originally, Bae et al. [46] found

that ectopic SUMO-1 expression in HEK 293T cells increases the stability of cotransfected HIF-1α, thus enhancing the transcriptional activity of HIF-1 protein in which HIF-1α is sumoylated at lysines 391 and 477. Carbia-Nagashima et al. [48] reported that the RWD-containing sumoylation enhancer (RSUME) is induced by hypoxia and enhances overall SUMO-1, -2, and -3 conjugation. Moreover, RSUME enhances HIF-1α sumoylation, thus promoting the stabilization and transcriptional activities of HIF-1 during hypoxia.

Berta et al. [47] revealed that HIF-1α undergoes sumoylation by SUMO-1, -2 and -3 *in vitro*, in proximity to, and within the ODDD domain, in a process promoted by the SUMO E3 ligase RanBP2/Nup538. However, contrary to the studies mentioned above, they found that *in vivo* sumoylation does not change HIF-1α turnover rate and decreases its transcriptional activity. The SUMO E3 ligase PIASy was also reported to enhance hypoxia-induced HIF-1α sumoylation and to negatively regulate its stability and transactivation [50]. Furthermore, sentrin/SUMO-specific protease 1 (SENP1), a desumoylation enzyme, stabilizes HIF-1α and enhances its transcriptional activity by removing SUMO. This was proposed to be because sumoylation of HIF-1α promotes its binding to VHL through a proline hydroxylation-independent mechanism, leading to HIF-1α ubiquitination and degradation (Figure 2) [49].

3 Cbx4 and HIF-1α sumoylation

Polycomb group (PcG) proteins are major transcriptional repressors that epigenetically modify chromatin. PcGs include structurally diverse and functionally related proteins, many of which interact with each other to form multimeric, chromatin-associated protein complexes called polycomb repressive complexes (PRC), e.g., PRC1 and PRC2 in mammals. These complexes are important for the regulation of many biological processes such as the cell-division cycle, DNA repair, cell differentiation, senescence and death. Cbx proteins, including Cbx2, Cbx4, Cbx6, Cbx7, and Cbx8, are the core components of the PRC1 complex. The classification of members of the Cbx family is based on the presence of a single N-terminal chromodomain (Figure 1A), which, in at least some members of the family, can bind to histone proteins via methylated lysine residues [51]. More interestingly, human Cbx4 (also known as polycomb 2, Pc2) also has SUMO E3 ligase activity [51], for which a limited repertoire of substrates have been identified, including C-terminus binding protein 1 [52], DNA methyltransferase 3a [53], Smad-interacting Protein 1 [54], centrin-2 [55], and homeodomain-interacting protein kinase 2 [56].

Recently, we reported that Cbx4 directly interacts with, and enhances overall SUMO-1, -2, and -3 conjugation to HIF-1α protein in HCC cells. HIF-1α can be desumoylated by ectopic SENP1 expression. Under hypoxic conditions, the silencing of Cbx4 by its specific shRNAs inhibits HIF-1α sumoylation in HCC cells [17]. Recent analyses have shown that for Cbx4 two functional SIMs (SIM1 and SIM2, Figure 1A) are prerequisites for its SUMO E3 ligase activity [57,58]. For HIF-1α sumoylation, SIM1 of Cbx4 contributes to its interaction with HIF-1α protein, and SIM2 is essential for its SUMO E3 ligase activity. Furthermore, we found that Cbx4 promotes HIF-1α sumoylation at lysines 391 and 477 within the ODDD of HIF-1α. Intriguingly, HIF-1α with mutations of these two sumoylation sites still interacts with Cbx4 with similar binding affinity to that of wild-type HIF-1α, and it can also be recruited to the promoter region of HIF-1 targeted gene vascular endothelial growth factor (VEGF) together with Cbx4. However, the DNA binding ability and transcriptional activity of mutated HIF-1α cannot be enhanced by Cbx4. Therefore, we conclude that Cbx4 upregulates the transcriptional activity of HIF-1α through enhancing the sumoylation of HIF-1α at K391 and K477, as depicted in Figure 2 [17].

4 HIF-1α sumoylation and cancer

As described above, HIF-1α has been established as a strong promoter of tumor progression by effects on processes including neovascularization, glucose metabolism, energy metabolism, migration, invasion, autophagy and cell death [59]. As sumoylation might regulate the stability and/or transcriptional activity of HIF-1α, the regulators of HIF-1α sumoylation are presumed to play crucial roles in tumorigenesis. Indeed, PIASy was reported to inhibit the angiogenic activity of endothelial cells, and its expression is inversely correlated with tumor angiogenesis in colon cancer [50]. SENP1 is highly expressed in both precancerous prostate intraepithelial neoplasia lesions and prostate cancer tissues, and its expression correlates with prostate cancer aggressiveness and recurrence [60,61]. With *in vitro* and *in vivo* assays, SENP1 is recognized as a promoter of colony formation, migration, invasion, tumor growth and metastasis in nude mice of prostate cancer cell lines [61]. In addition, SENP1 inhibition promotes the apoptosis of Burkitt lymphoma cells [62].

It has been reported that SUMO-1 is highly expressed in HCC cell lines and clinical HCC samples, while the expression level of SUMO-1 in non-neoplastic liver tissues was significantly lower [63]. Analysis of a large cohort of HCC tissues allowed us to report that Cbx4 is highly expressed in most of these tissues, and that elevated Cbx4 protein is positively correlated with poor overall survival of HCC patients [54]. Wang et al. [64] also showed that Cbx4 expression was upregulated in multiple HCC cell lines and clinical samples, and that HCC patients with a high level of cyto-

plasmic Cbx4 have a shorter overall and recurrence-free survival. Intriguingly, our further investigations demonstrated that Cbx4 expression is also positively correlated with VEGF expression and microvessel density (MVD) [17]. In line with these clinical observations, overexpression of Cbx4 but not other members of the Cbx family promotes VEGF expression and *in vitro* angiogenesis in several HCC cell lines under hypoxia. Conversely, silencing of Cbx4 by shRNAs dramatically blocks hypoxia-induced VEGF production and *in vitro* angiogenesis. The Cbx4-CDM mutant (F11A/W35L double-mutation in the chromodomain of Cbx4) has lost its polycomb function but still possesses SUMO E3 ligase activity. Ectopic expression of the Cbx4-CDM mutant increases hypoxia-induced VEGF production and the hypoxia-induced tube-forming ability of vascular endothelial cells to a similar degree to wild-type Cbx4. In contrast, no such increases are seen for all three ΔCbx4-SIM mutants, which maintain the polycomb function but lose SUMO E3 ligase activity [17]. Conversely, the knockdown of HIF-1α or HIF-1β with their specific shRNAs almost completely abolishes hypoxia-induced VEGF expression, supporting the essential role of HIF-1 sumoylation in Cbx4-dependent VEGF expression.

In line with these *in vitro* findings, subcutaneously and orthotopically transplanted tumors originated from HCC cell lines ectopically expressing wild-type Cbx4 or its CDM mutant, but not the ΔCbx4-SIM1/2 mutant, grew much faster compared with vehicle controls. Accordingly, ectopic expression of Cbx4 or Cbx4-CDM, but not ΔCbx4-SIM1/2, dramatically increases VEGF expression and MVD in the transplanted tumors [17]. Moreover, orthotopically transplanted SMMC-7721 [17] and MHCC97L cells [65] ectopically expressing Cbx4 and Cbx4-CDM present significantly more lung metastasis nodules than vehicle and ΔCbx4-SIM1/2-infected tumors. Conversely, the knockdown of endogenous Cbx4 causes SMMC-7721 and MHCC97L cells to produce significantly lower tumor burden than cells transfected with vehicle alone in BALB/c nude mice [17].

5 Perspectives

The physiological and pathophysiological roles of HIF-1 have been widely recognized and its post-translational modifications have attracted wide interest. However, HIF-1α sumoylation appears to be very complicated. Dimova and Kietzmann [15] proposed that the effects of sumoylation on HIF-1α vary from cell type to cell type. More intriguingly, we have shown that Cbx4 and PIASy-mediated HIF-1α sumoylations have different effects on the stability and transcriptional activity of HIF-1 in HCC cell lines. Actually, PIASy has no effect on both K391 and K477-mutated HIF-1α, suggesting that Cbx4 and PIASy promote HIF-1α sumoylations at different lysines. Therefore, we propose that different sumoylation patterns produced by specific SUMO E3 ligases might lead to different influences on the stability and transcriptional activity of HIF-1α. However, the sumoylation sites of HIF-1α by PIASy and other SUMO E3 ligases remain to be identified. Alternatively, different sumoylation patterns produced by different SUMO E3 ligases could possibly affect HIF-1α-interacting proteins, which could contribute to the regulation of the stability and transcriptional activity of HIF-1α indirectly. Therefore, it remains to be further explored how Cbx4-induced HIF-1α sumoylation at K391 and K477 increases the DNA binding ability and thus the transcriptional activity of HIF-1.

It has previously been shown that some coactivators and corepressors contribute to the regulation of the transcriptional activity of HIF-1 [66–68]. We presume that sumoylation could affect the interaction of HIF-1 with these known or unknown co-modulators, thus regulating its transcriptional activity. In fact, our preliminary proteomic experiments do indeed reveal different interaction partners for non-sumoylated HIF-1α and HIF-1α proteins with Cbx4-enhanced sumoylation. Therefore, it would be useful to explore these interaction partners and their roles in the regulation of stability and function of HIF-1 to uncover the mechanism by which sumoylation of HIF-1α regulates its transcriptional activity. Regardless of the outcome of such investigations, the development of reagents targeting the SUMO E3 ligase activity of Cbx4, and the interacting sites between Cbx4 and HIF-1α, could be beneficial for inhibiting the tumor angiogenesis essential for cancer cells to fulfill their need for oxygen and nutrients.

Finally, increasing lines of evidence have shown that hypoxia and HIF-1 contribute to differentiation of acute myeloid leukemia [69–75], consistent with the fact that people living at high altitude appear to have a lower risk of leukemia [76–79]. Our investigations showed that HIF-1-mediated leukemic cell differentiation is independent of the transcriptional activity of HIF-1 [80]. In short, hypoxia-stabilized HIF-1α interacts with and increases the transcriptional activity of C/EBPα and Runt-related protein 1 (Runx1, also named acute myeloid leukemia-1, AML1) [81–83]. Therefore, the question of whether sumoylation of HIF-1α contributes to leukemogenesis and leukemic cell differentiation deserves further investigation.

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