Modulation of *WHSC2* expression in human endothelial cells

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Abstract ***WHSC2*, a novel gene recently isolated within the critical region of Wolf–Hirschhorn syndrome, is expressed in endothelial cells. *WHSC2* is downregulated by HIV-1 Tat, whereas it is not modulated by angiogenic and pro-differentiative molecules. *WHSC2* encodes a basic polypeptide of 528 amino acids. The in vitro translated protein shows a molecular weight of 57 kDa. WHSC2 has two nuclear translocation sequences which actively mediate its transport to the nucleus, as shown in whsc2-GFP-transfected NIH-3T3. We also found a helix-loop-helix (HLH) motif in region 130–185. Since members of the HLH family control differentiation and cell cycle progression, we hypothesize that WHSC2 may function as a transcriptional repressor.**

Key words: *WHSC2*; Endothelial cell; HIV-1 Tat; Angiogenesis

1. Introduction

The Tat protein of the human immunodeficiency virus type 1 (HIV-1) is essential for efficient HIV-1 gene expression [1]. Moreover, Tat is secreted by HIV-1-infected cells [2], and biologically significant levels of Tat have been detected in the sera of HIV-1-infected subjects [3]. Extracellular Tat binds to and is rapidly taken up by neighboring cells [4] and can influence the expression of cellular genes in both infected and uninfected cells [5–12].

Our interest lies in the regulation of gene expression in endothelial cells (EC). The normal function of the endothelium is impaired in HIV-1 infection. Disturbances of the local cytokines as well as the release of Tat by infected mononuclear cells play a role in endothelial dysfunction. Interestingly, in vascular EC, Tat has been shown to specifically bind and activate the Flk-1/kinase insert domain receptor (Flk/kdr) [13], a vascular endothelial growth factor (VEGF)-A tyrosine kinase receptor, which is a major regulator of vasculogenesis and angiogenesis [14]. This finding may explain Tat angiogenic activity [15]. Indeed, Tat has been postulated to have a role in the pathogenesis of Kaposi's sarcoma (KS) because it is angiogenic and it enhances the proliferative effects of basic fibroblast growth factor (bFGF) [16]. Moreover, *tat* transgenic mice develop KS-like lesions [17]. In addition, Tat activates ECs by stimulating the expression of E-selectin and IL-6 [18]. Because of the multiple effects of Tat on EC, we isolated differentially expressed genes in Tat-treated EC to yield insights into the molecular mechanisms contributing to endothelial dysfunction in HIV-infected individuals, in KS and, more generally, in angiogenesis. Here we report that Tat downregulates the expression of *whsc2* [19] in human EC. *WHSC2* has recently been isolated within the critical region of Wolf–Hirschhorn syndrome (WHS), a multiple malformation syndrome characterized by mental and developmental defects resulting from the deletion of the short arm of one chromosome 4 (4p16.3) [19]. We show that *WHSC2* is a nuclear protein expressed in various tissues and in different cell lines. In addition, *WHSC2* is not modulated by angiogenic factors other than Tat.

2. Materials and methods

2.1. In vitro transcription and translation

WHSC2 was cloned in pBluescript and used for in vitro transcription. *WHSC2*-pBluescript was incubated for 30 min at 37°C with proteinase K (50 μg/ml) in 0.01 mM Tris–HCl pH 7.8 containing 0.5% SDS and 5 mM EDTA. 600 ng of proteinase K-treated DNA were incubated with rabbit reticulocyte lysate and 0.5 μl [^{35}S]Met for 90 min at 30°C. The sample was then run on 10% SDS–PAGE, fixed and autoradiographed. In vitro translation of the plasmid alone did not yield any band (not shown).

2.2. Green fluorescence protein (GFP) fusion plasmid construction

pEBFP-N1/WHSC2 was constructed by inserting *Hin*dIII and *Bam*HI cut-Whsc2 which had been obtained by PCR using Whsc2 cDNA as template and the following oligomers: sense 5′-CCC AAG CTA TGG CGT CCA TGC GGG AG-3′ and antisense 5′-CGC GGA TCC CCG GAC ACA TTG GTC ATG GG-3′ (11). PCR reaction was as follows: 1 min at 95°C, 2′ at 65°C and 3′ at 72°C for 30 cycles. The correct sequence and orientation of the construct were confirmed by sequencing.

2.3. Cell culture and transfection

HUVEC-C were from ATCC and cultured in HF12 containing 10% FCS, ECGS (150 μg/ml) and heparin (5 U/ml) on gelatin coated dishes. NIH-3T3 were grown in D-MEM containing 10% FCS. Cells were seeded on glass coverslips and transiently transfected with pEBFP-N1/Whsc2 or pEBFP-N1 alone via the calcium phosphate coprecipitation technique [20]. 24 h after transfection cells were washed, fixed in phosphate-buffered saline (PBS) containing 3% paraformaldehyde and 2% sucrose and labeled with rhodamine-conjugated phalloidin. The signal was evaluated with a fluorescence microscope.

2.4. Western blot analysis

Antiserum was prepared using his-tag polypeptides in the N-terminal region (residues 62–125) and C-terminal region (residues 364–521) of WHSC2. Rabbits were immunized by standard procedures. IgGs against WHSC2 were purified on a protein A Sepharose column.

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Abbreviations: WHS, Wolf–Hirschhorn syndrome; HLH, helix-loop-helix; NLS, nuclear localization sequence; NELF, negative elongation factor

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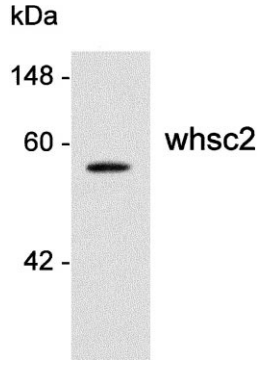

Fig. 1. In vitro translation of *WHSC*2. *WHSC*2 cloned in pBluescript was in vitro transcribed and translated as described. The sample was then run on a 10% SDS–PAGE and autoradiographed.

Nuclei and cytosols were fractionated as described [21]. For Western blotting, cytosolic and nuclear fractions (50 μg/lane) were resolved by SDS–PAGE, transferred to nitrocellulose sheets at 150 mA for 16 h, and probed with anti-WHSC2 IgGs (5 μg/ml). Secondary antibodies were labeled with horseradish peroxidase (Pierce, Rockford, IL, USA). The SuperSignal chemiluminescence kit (Pierce) was used to detect immunoreactive proteins.

2.5. Purification of RNA and Northern blot

Cells were treated with Tat (10 ng/ml) (Tecnogen, Italy), TPA (10 nM), HGF (10 ng/ml), FGF-2 (10 ng/ml), VEGF (10 ng/ml), IL-1 α (10 ng/ml) for different times, rinsed with PBS and lysed in 4 M guanidinium isothiocyanate. RNA was purified as described [20]. RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde, capillary blotted onto nylon membranes and UV-crosslinked. WHSC2 and GAPDH were labeled with a random primer labeling kit (Ambion). Filters were hybridized in 0.5 M sodium phosphate (pH 7.2) containing 7% SDS, 1 mM EDTA and 20% formamide at 65°C for 20 h and extensively washed at high stringency before autoradiography.

3. Results

3.1. Characterization of WHSC2

One of the clones isolated by RNA fingerprinting on EC exposed to HIV-1 Tat [20] was denominated C4. The C4 insert (600 bp) hybridized to a mRNA species of 2.4 kb (see below). To obtain a full length clone, the initial 600 bp C4 fragment was used as a probe to screen a human fetal brain cDNA library to obtain the full length clone. The complete nucleotide sequence of the C4 cDNA is 1584 bp long and contains at the 5′ end a consensus translational initiation sequence [22]. By database similarity, we found that C4 is identical to *WHSC*2, independently isolated by Wright et al. during the course of this work [19]. Wright et al. named this clone WHSC2 since it is located in WHS critical region on chromosome 4, and we will attain to this denomination from now on.

*WHSC*2 cDNA encodes a highly hydrophilic protein of 528 amino acids (p*I* 9.8). Indeed, by in vitro transcription–translation, we could detect a single band with a molecular weight of 57 kDa, as predicted by computational analysis (Fig. 1).

*WHSC*2 was expressed in all the human tissues examined and was most abundant in the heart and placenta (Fig. 2A). *WHSC*2 was also detected in all the human cell lines examined (Fig. 2B).

3.2. WHSC2 expression in human EC exposed to angiogenic factors

The downregulation of *WHSC*2 by Tat detected by RNA fingerprinting was confirmed by Northern blot analysis. Tat (10 ng/ml) decreased *WHSC*2 RNA to almost undetectable levels within 4 h and up to 24 h (Fig. 3). Because Tat has an angiogenic activity, we evaluated whether prototypic angiogenic factors – such as VEGF, FGF-2 and HGF-modulated *WHSC*2 expression. While these three polypeptides stimulated endothelial proliferation, they did not modula-

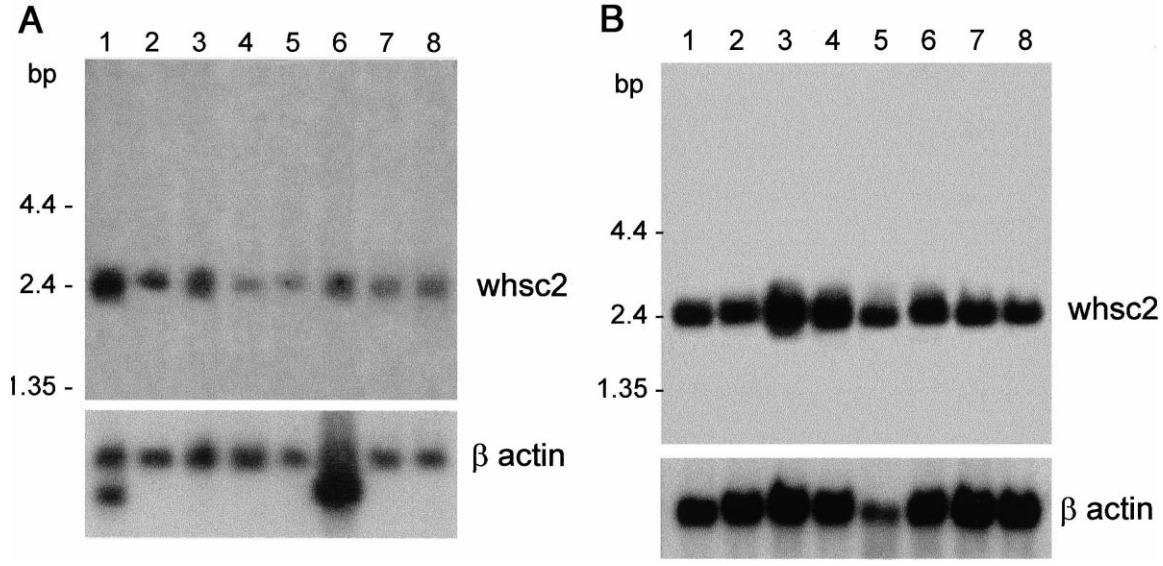

Fig. 2. Tissue distribution of *WHSC*2. A: Multiple human adult tissue Northern blot was purchased from Clontech. Northern blot was performed at high stringency using the human WHSC2 cDNA probe. Hybridization with β actin indicates that similar amounts of RNA were used per lane; as indicated by the manufacturers, heart and skeletal muscle contain two forms of β actin, 2.0 and 1.8 kb. Lanes: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, muscle; 7, kidney; 8, pancreas. B: Multiple human cancer cell lines Northern blot was from Clontech. Northern blot was performed as described above. Lanes: 1, promyelocytic leukemia HL-60; 2, HeLa; 3, chronic myelogenous leukemia K562; 4, lymphoblastic leukemia MOLT-4; 5, Burkitt's lymphoma Raji; 5, colorectal carcinoma SW480; 7, lung carcinoma A459; 8, melanoma G361.

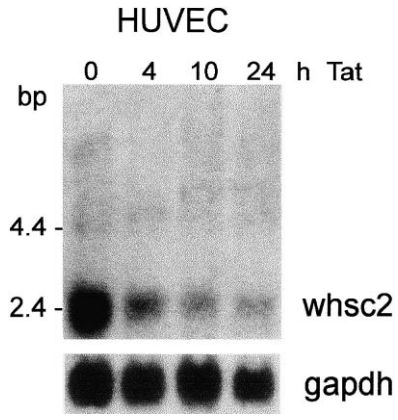

Fig. 3. Modulation of *WHSC*2 expression by Tat in human EC. Confluent cultures of HUVEC were exposed to 10 ng/ml Tat for 1, 4 and 24 h. Total RNA (10 μg/lane) was analyzed by Northern blot as described. The blot was rehybridized to GAPDH to verify equal amounts of RNA loading among the lanes.

te*WHSC*2 RNA (Fig. 4A). We then asked whether *WHSC*2 could be modulated in the differentiation of EC [23]. Since interleukin 1 and the phorbol ester TPA promote endothelial differentiation [23], we treated HUVEC with IL-1 and TPA for different times. Fig. 4B shows that no modulation of *WHSC*2 RNA occurs under these experimental conditions.

3.3. *WHSC2 is a nuclear protein*

By computational analysis, we found that WHSC2 possesses two potential nuclear localization sequences (NLS). The NLSs start at amino acid 166 and 272, and are composed by a basic amino acid cluster which is homologous to the NLS of simian virus 40-encoded large tumor antigen [24]. To evaluate whether the NLSs drive WHSC2 to the nucleus, we fused WHSC2 in frame with GFP and transiently transfected the construct in NIH-3T3 cells. As shown in Fig. 5A, the fusion protein is localized in the nucleus of transfected cells, while GFP alone is distributed throughout the cytoplasm. We also examined the cellular distribution of WHSC2 in HUVEC after cell fractionation. Western blot analysis using anti-WHSC2 IgGs showed that WHSC2 was localized in the nucleus (Fig. 5B). Controls on the cytosolic and nuclear fractions were performed with anti-LDH and anti-SP1, respectively (not shown).

Prediction of the secondary structure by the Chou–Fasman method has shown that WHSC2 has a putative helix-loop-helix (HLH) structural motif between amino acid 130 and 185 (not shown). This motif has been aligned and compared with the HLH domain of other members of the family, among which c-myc, MyoD and Id (Fig. 6). Fig. 6 also reports the putative HLH domain, where conserved amino acids are boxed and apolar residues indicated with an asterisk.

4. Discussion

*WHSC*2 has been recently isolated within the WHS critical region and hypothesized to play a role in the phenotype of the WHS, a syndrome characterized by a constellation of symptoms including growth deficiency, severe mental retardation, facial, skeletal and heart defects [19]. Here we show that *WHSC*2 is expressed in all the human tissues and cell lines tested. The ubiquitous expression of *WHSC*2 may explain, at least in part, the severe phenotype observed in patients with a deletion in the short arm of chromosome 4 as described in WHS [19]. The development of mouse strains with the *WHSC*2 deletion may identify a role for this gene in WHS.

Human EC express rather high levels of *WHSC*2 RNA. Tat, which transactivates both viral and host genes [1,5–12], downregulates *WHSC*2, while angiogenic factors and pro-differentiative molecules have no effect on *WHSC*2 RNA levels. The different response to VEGF and Tat is surprising since Tat has been shown to specifically bind and activate the VEGF-A receptor, namely Flk-1/kdr [13]. Since the extracellular matrix plays an important role in presenting growth factors to their cognate receptors [25], it is conceivable that a particular microenvironment is necessary for Tat to interact with Flk-1/kdr. Alternatively, since it is known that Tat is taken up by the cells and then transported to the nucleus

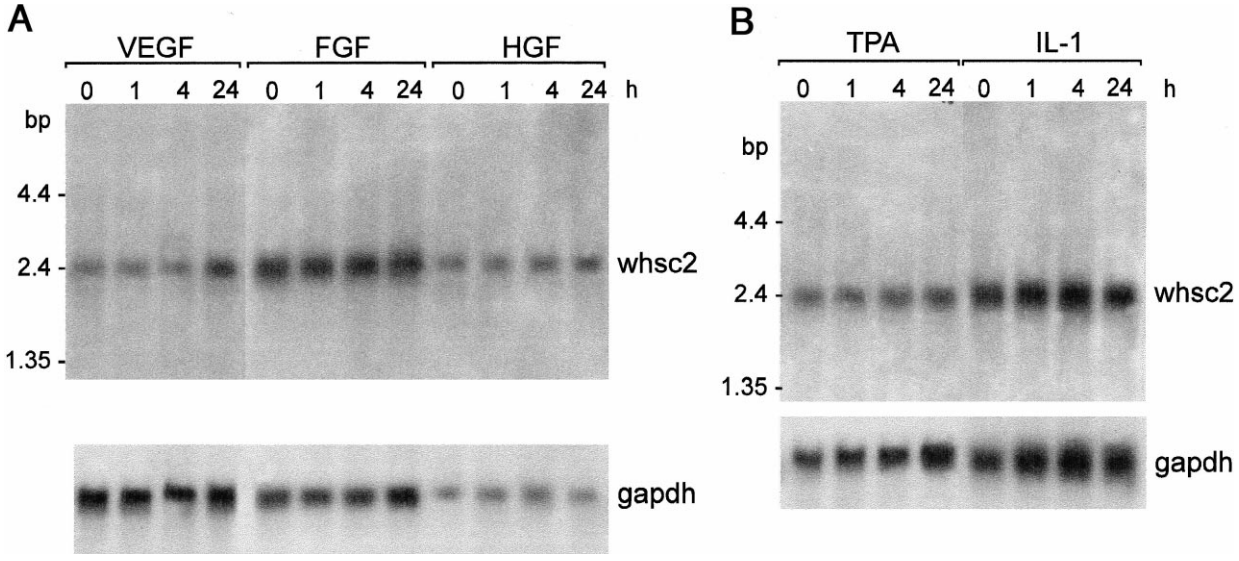

Fig. 4. Modulation of *WHSC*2 expression in human EC. Confluent cultures of HUVEC were exposed to 10 ng/ml HGF, FGF-2 and VEGF (A) or with TPA (10 nM) and IL-1 (10 ng/ml)(B) for 1, 4 and 24 h. Total RNA (10 μg/lane) was analyzed by Northern blot as described. The blot was rehybridized to GAPDH to verify equal amounts of RNA loading among the lanes.

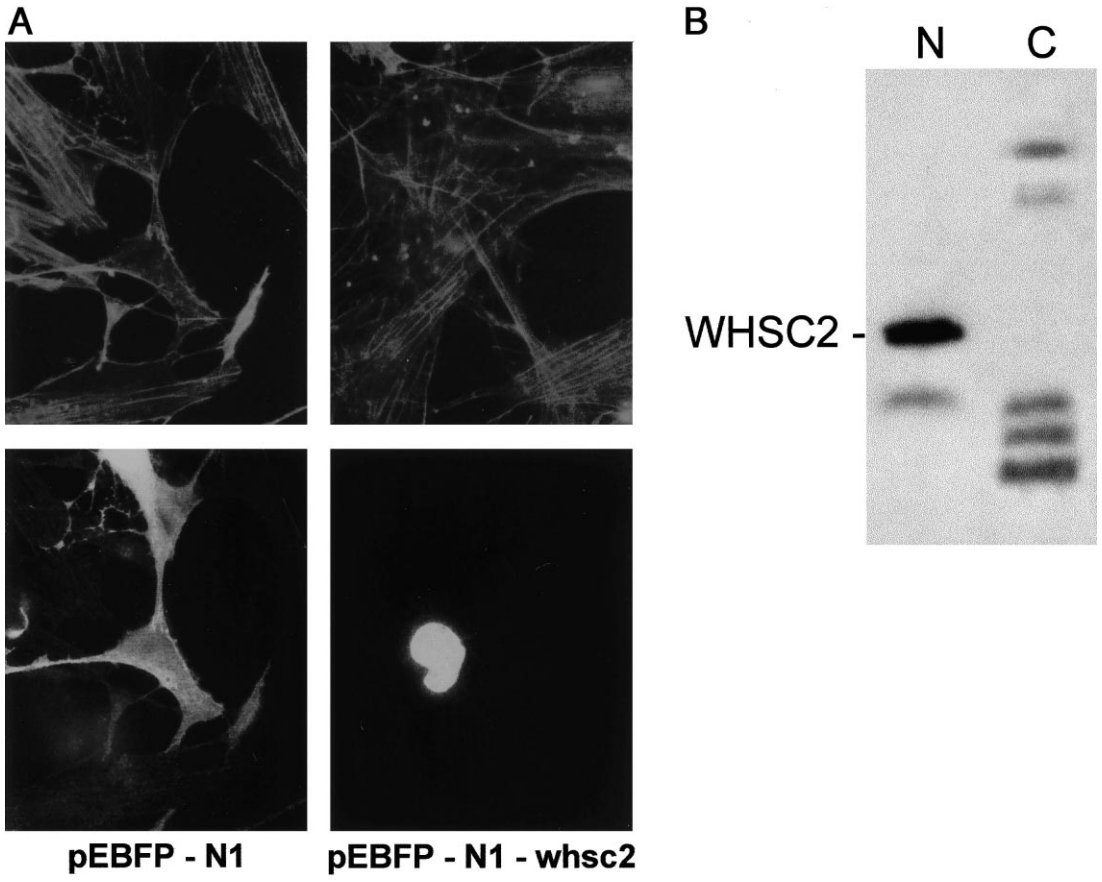

Fig. 5. Nuclear localization of WHSC2. A: NIH-3T3 were transfected with GFP (pEBFP-N1) or *WHSC*2 fused to GFP (pEBFP-N1-whsc2). After labeling the cytoskeleton with rhodamine-conjugated phalloidin, cells were observed with a fluorescence microscope (60×) and photographed. B: Western blot analysis was performed on the cytosolic and nuclear fractions of HUVEC utilizing anti-WHSC2 IgGs. C: cytosol; N: nucleus.

[26], it is reasonable to propose that Tat regulates *WHSC*2 expression by directly acting in the nucleus.

*WHSC*2 encodes a 528 amino acid protein which was in vitro translated and migrated with a molecular mass of 57 kDa. Interestingly, WHSC2 possesses two putative NLSs. These basic sequences are recognized by NLS-binding proteins that function as adapter molecules of the nuclear transport machinery [24]. The NLSs we have identified in WHSC2 are similar to the prototypical NLS found in simian virus 40 large tumor antigen in their high content of basic amino acids [24] and are able to translocate GFP to the nucleus of transiently transfected 3T3. Moreover, after cell fractionation, we detected WHSC2 mainly in the nuclear fraction of HUVEC. We therefore conclude that WHSC2 is a nuclear protein.

On the basis of a careful sequence analysis, we propose that WHSC2 contains an HLH motif in the N-terminus region. Basic HLH transcription factors regulate cell-type-specific gene expression and the expression of cell cycle regulatory genes [27]. Proteins lacking the basic DNA binding domain upstream from the HLH – such as Id – dimerize with basic HLH [28], thus behaving as transcriptional repressors by preventing the basic HLH proteins from binding DNA [28]. However, an alternative mechanisms of action of WHSC2 can be proposed. Since WHSC2 is a member of the negative elongation factor (NELF) protein complex [29], which

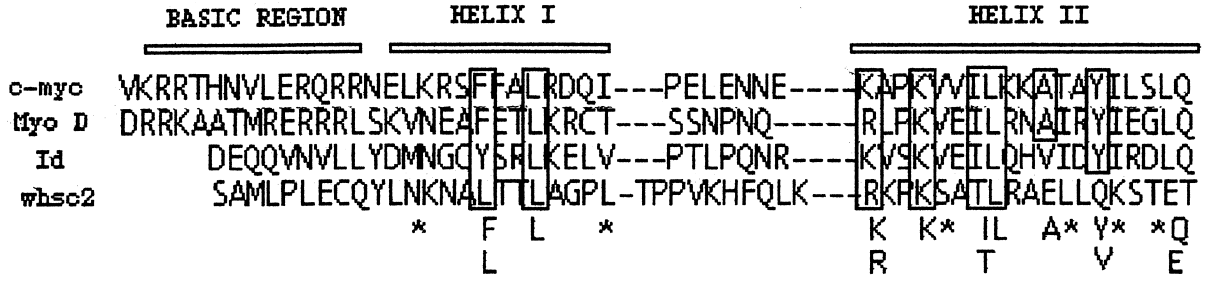

Fig. 6. Sequence comparison between the putative HLH domain of WHSC2 and other members of the HLH family. The putative HLH motif of WHSC2 was compared with MyoD, c-myc and Id. Conserved residues are boxed and reported below together with apolar amino acids, which are indicated with an asterisk, to identify the HLH region.

strongly represses polII elongation, WHSC2 may exert its control on gene expression by modulating the elongation step of transcription.

Further experiments are required to elucidate whether WHSC2 represses transcription and which mechanisms are involved.

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