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Biochemical and Biophysical Research Communications 353 (2007) 47–53
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A putative transcriptional elongation factor hIws1 is essential for mammalian cell proliferation

Zhangguo Liu [a,c], Zhongwei Zhou [a], Guohong Chen [b], Shilai Bao [a,*]

[a] *Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101, PR China*
[b] *Animal Sci.-Tech. College, Yangzhou University, Yangzhou, Jiangsu 225005, PR China*
[c] *School of Animal Science, Zhejiang Forestry University, Hangzhou, Zhejiang 311300, PR China*

Received 16 November 2006
Available online 5 December 2006

Abstract

Iws1 has been implicated in transcriptional elongation by interaction with RNA polymerase II (RNAP II) and elongation factor Spt6 in budding yeast *Saccharomyces cerevisiae,* and association with transcription factor TFIIS in mammalian cells, but its role in controlling cell growth and proliferation remains unknown. Here we report that the human homolog of Iws1, hIws1, physically interacts with protein arginine methyltransferases PRMT5 which methylates elongation factor Spt5 and regulates its interaction with RNA polymerase II. Gene-specific silencing of hIws1 by RNA interference reveals that hIws1 is essential for cell viability. GFP fusion protein expression approaches demonstrate that the hIws1 protein is located in the nucleus, subsequently, two regions harbored within the hIws1 protein are demonstrated to contain nuclear localization signals (NLSs). In addition, mouse homolog of hiws1 is found to express ubiquitously in various tissues.

Keywords: hIws1; Immunoprecipitation; RT-PCR; Cell proliferation; NLSs; RNAi; PRMT5

During the transcription of eukaryotic genes performed by RNA polymerase II (RNAPII), transcription elongation is a complex phase, which needs a set of factors to regulate the catalytic activity of RNAP II. Up to date, lots of transcription elongation factors have been determined, while it is still required to probe into other unknown ones.

Yeast Iws1 (yIws1) encoded by an essential gene YPR133C [1] has initially been confirmed to interact with Spt6 [2], subsequently been found to physically associate with either Spt4 or Spt5 [3]. Both Spt4 and Spt5 are the subunits of a heterodimer complex, DSIF, required for RNAP II transcription elongation. Biochemical and genetic evidences support interactions between Spt4, Spt5 and RNAP II [4]. Early work has characterized a genetic interaction between Spt6 and DSIF (Spt4 and Spt5) [5]. Mutations in Spt6 gene lead to transcription initiation from cryptic start sites within the transcription active gene [6]. Furthermore, Spt6 also recruits on the coding region of Hsp70 gene after heat shock, as do Spt5 and RNAP II [7]. These results implicate that Spt6 serves as a modulator of RNAP II during transcriptional elongation. Chromatin immunoprecipitation (ChIP) on Spt6 or Iws1 demonstrates that both proteins cross-link throughout the transcription active gene [2]. Mutations in the gene encoding the Iws1 protein result in a Spt$^-$/-phenotypes, as do Spt4 and Spt5 [3]. And similar to Spt4, Spt5, and Spt6, Iws1 copurifies with RNAP II [2]. Additionally, in mammalian cells, Iws1 physically associates with the general transcription factor TF II S [8]. These collective results strongly imply that Iws1 functions as an RNAP II elongation factor *in vivo*, although very little mechanistic data have been provided.

The primary sequence of a protein is directed by the genetic code, but proper functions of which depend on various post-translational modifications. A variety of

* Corresponding author. Fax: +86 10 64889350.
E-mail address: slbao@genetics.ac.cn (S. Bao).

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doi:10.1016/j.bbrc.2006.11.133

posttranslational modifications are involved in the regulation of gene expression. Arginine methylation, which adds one or two methyl groups to the guanidine nitrogen atoms, is an important post-translational modification playing a regulatory role in gene expression [9]. PRMT5 is one major type II protein arginine methyltransferases (PRMTs) that generate monomethylarginines or symmetric dimethylarginines [10,11]. Methylation of histones H3 and H4 arginine residues by PRMT5 has been shown to result in transcriptional repression [12,13]. More recently, it is shown that arginine methylations of Spt5 by PRMT5 would reduce its association with RNAP II, so decrease Spt5-mediated transcriptional stimulatory properties [14].

Iws1 is an evolutionally conserved protein, which has varieties of metazoan homolog. In addition to yIws1, few of these putative proteins have previously been characterized. FLJ10006 (GenBank Accession No. CAD38875) is a putative human Iws1 homolog (hIws1). In addition to C-terminus Iws1 domain (residues 117–349), hIws1 has another acidic N-terminal MDN1 domain (residues 558–819) corresponding to the AAA ATPase containing von Willebrand factor type A (vWA) domain [8]. Alignment analysis demonstrates that human hIws1 and yeast yIws1 share 32% amino acid identity, and hIws1 and which *Drosophila* homolog share 48% identity [3]. In the present work, we have characterized a putative protein hIws1 and probed into the relation between hIws1 and PRMT5 *in vivo*.

Materials and methods

Cell culture. HCT116 (human colorectal carcinoma) cells are cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL) supplemented with 9% fetal bovine serum and penicillin/streptomycin (100 U/ml) at 37 °C with 5% CO_2 in air. Cells are routinely subcultured with trypsin (0.25%) and EDTA (1 mM) when they reach 80–90% confluence.

Semi-quantitative RT-PCR. Total RNA is extracted with SV Total RNA Isolation System (Promega) from various organ tissues of mouse, including cerebra, heart, liver, lung, spleen, stomach, kidney, prostate, testicle, uterus, and large and small intestine. Then about 1 μg of total RNA is reverse transcribed into first strand cDNA using oligo(dT)-Adaptor primer of TaKaRa RNA La PCR TM Kit (AMV) (TaKaRa).

Then PCR using Ex *Taq* polymerase (TaKaRa) is performed with prepared first strand cDNA as a template and with β-actin as a reference gene. To amplificate miws1, the primers used are 5′-gattctgatgataacataaagagagg-3′ (forward primer) and 5′-aactcgagggctgcctgaaacctggatgactcc-3′ (reverse primer). The primers for β-actin amplification comprise of forward primer: 5′-ctacaatgagctgcgtgtggctc-3′ and reverse primer: 5′-tcctgcttgctgatccacatctg-3′.

The PCR for miws1 and β-actin is carried out with Ex *Taq* polymerase (TaKaRa) and consists of an initial denaturation at 94 °C for 2 min, and 27 cycles of 94 °C for 30 s, 60 °C for 35 s, 72 °C for 55 s, and a final extension of 8 min at 72 °C. The mRNA signals detected by RT-PCR are primarily normalized by the use of uniform amounts of cDNA preparations indicated by reference gene β-actin.

The PCR products are separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. Semi-quantitative analysis is performed with the Dolphin 1D software. The expression intensity of miws1 is denoted with the ratio of the photodensity of the RT-PCR products of miws1 and β-actin.

Fluorescence microscopy assay. cDNA fragments encoding the wild-type and various truncations of hIws1 (showed as Fig. 2A) are cloned between the *Eco*RI and *Bam*HI restriction sites of vector pEGFP-C1 (Clontech). For transfection, various construct plasmids are purified by plasmid mini kit (Qiagen) following the manual instructions.

For fluorescence microscopy, 2×10^5 HCT116 cells are seeded in 35 mm dishes with glass coverslips 1 day before transfection. Then cells are transfected with about 1 μg various GFP fusion constructs using Fugene6 transfection Kit (Invitrogen) as described by the manufacturer. Thirty hours later, the cells are fixed with 4% paraformaldehyde and washed with PBS, then stained with DAPI (VECTOR). Finally, the fluorescence images are observed with an Olympus Fluoview 300 laser-scanning confocal microscope.

Western blot assays. For immunoblotting analysis, cells are lysed in RIPA buffer (150 mM NaCl, 20 mM Na_2HPO_4, 1.0% Triton X-100, pH 7.4) containing inhibitors (1% aprotinin, 5 mM PMSF, 10 μg/ul Leupeptin, 100 mM NaF, and 2 mM Na_3VO_4) on ice for 30 min. Cell lysates are separated by SDS–PAGE on 10% polyacrylamide gels. Then the proteins are transferred onto a nitrocellulose membrane (Millipore), and blocked with TBS-T containing 5% nonfat milk. The following specific primary antibodies, anti-hIws1 polyclonal antibody, anti-PRMT5 polyclonal antibody, and anti-γ-tublin monoclonal antibody are independently used to detect hIws1, PRMT5 or γ-tublin. The result of immunoblotting is visualized with ECL enhancer Kit (Amersham Pharmacia Biotech).

RNA interference vector construction. RNA interference plasmids, small hairpin RNAs (shRNAs) plasmid for shRNAs specific for firefly luciferase (siLuc) are previously prepared by our laboratory. Three target sequences of the hiws1 cDNA (GenBank Accession No. AL834178) are selected and designed as shRNAs, named as siIws1-1, siIws1-2, and siIws1-3, respectively. These sequences independently target the 273–291, 5′-ggacagcgactctgaatct-3′; 1261–1281, 5′-gacagtgatgctgtatcagac-3′; 1701–1719, 5′-gaatgaagctgctgaggaa-3′ nucleotides of the hiws1 cDNA. The chemically synthesized oligonucleotides are annealed and cloned into the vector pNeoU6+1. For transfection, various construct plasmids are purified by plasmid mini kit (Qiagen) following the manual instructions.

Cell colony formation assay. HCT116 cells (6×10^5) are plated on 60 mm dishes 1 day prior to transfection at 80–90% confluence, 6 μg of various shRNAs for hiws1 or control plasmid siLuc is transfected into cells with Fugene6 reagent. Twenty-four hours later, each group is trypsinized, counted, and seeded in triplicate for the colony forming assay in 100 mm dishes at 0.8×10^4 cells per dish. While the rest of transfected cells are seeded into other dishes for further analysis. Stable transfectant is selected by using 500 μg/ml G418. About 10 days after transfection, colonies are fixed and stained with 2% crystal violet. At the same time, the cells prepared for further analysis are collected and used for Western blot assay.

Immunoprecipitation analysis. For immunoprecipitation, the prepared HCT116 cell lysate containing about 500 μg total proteins is incubated with about 8 μg of preimmune serum, anti-hIws1 polyclonal antiserum, anti-PRMT5 polyclonal antiserum or 8 μl RIPA buffer for 2 h at 4 °C. Thereafter, 15 μl 50% protein A–Sepharose suspension (Pharmacia) is added and incubation continues for another 2 h at 4 °C. Then the Sepharose beads are collected and washed with RIPA buffer for two times and with PBS for another two times. Antigen–antibody complexes are separated by 10% SDS–PAGE, and the Western blot assay is performed with anti-hIws1 polyclonal antiserum or anti-PRMT5 polyclonal antiserum as aforementioned.

Results

Iws1 is ubiquitously expressed in various mouse tissues

To assess the hiws1 gene transcript distribution, and since hIws1 (GenBank Accession No. CAD38875) and which mouse homolog (mIws1, GenBank Accession No. NP-775617) share more highly similarity at 84%, we use mouse organ tissues to investigate the tissue specificity of iws1 by RT-PCR. Miws1 mRNA is found to be expressed

in all of the studied organ types (Fig. 1). And the level of mRNA expression differs to a great extent in different organs. It appears to be expressed at the highest level in kidney, then testicle, large intestine, small intestine, spleen, and prostate, whereas the lowest level is detected in heart (Fig. 1).

Nuclear localization of the hIws1 protein

To determine the subcellular localization of the hIws1 protein, fusion protein comprising green fluorescent protein (GFP) (~27 kDa) at NH_2-terminus of the hIws1-WT (~90 kDa) is transiently expressed in HCT116 cells. The result shows that at the subcellular level, the GFP-hIws1 protein is just localized in the nucleus (Fig. 2B), which means hIws1 is a nuclear protein.

The exchange of molecules between the nucleus and the cytoplasm occurs through nuclear pore complexes (NPCs) [15,16]. These complexes span both membranes of the nuclear envelope (NE) and form aqueous channels that can accommodate the passive diffusion of small molecules up to 9 nm in diameter and the active (energy consuming) transport of molecules and complexes with diameters up to 25 nm. Thus, proteins with masses that exceed 50 kDa, as well as many smaller proteins, are unable to traverse the NE passively and are dependent on active, signal-mediated pathways for translocation.

Generally, the import of nuclear protein into the nucleus is directed by relatively short basic sequences called nuclear localization signals (NLSs) [17]. Given the fact that nuclear pore limits the passive transportation of any protein greater than 50 kDa, the uptake of the GFP-WT fusion protein (~117 kDa) is most likely via NLSs harbored within the hIws1 protein. Therefore, hIws1 protein sequence is first analyzed by computer program (http://cubic.bioc.columbia.edu/predictNLS) to predict the NLSs. The software fails to reveal any typical NLS, while on looking at the amino acid sequence of the hIws1 protein, we find several clusters of basic amino acids, RKK (residues 357–359), KKQK (residues 371–374), KRK (residues 391–393) and KRGK (residues 518–521), within the boundary (residues 350–557) between the MDN1 and IWS1-C domains, which suggests that the boundary sequence might target to the nucleus.

To test our hypothesis, fusion proteins, GFP tagged various truncated mutants of the hIws1 protein (Fig. 2A), are independently transfected into HCT116 cells. The fluorescence microscopy assays show that GFP-N391 is nuclear (Fig. 2B), while GFP-N355 distributes in both cytoplasm and the nucleus (Fig. 2B), which implicates some NLSs exist within the region residues 356–391 Aa. Similarly, expression of GFP-C389 is accumulated in the nucleus (Fig. 2B), while GFP-△WT(including both N391 and C524 of hIws1) just displays as GFP-N355, distributing in both nucleus and the cytoplasm (Fig. 2B), which indicate the region residues 389–524 Aa may harbor some another motifs acting as NLSs.

Interestingly, fusion protein GFP-C389 locates in the nucleus with some concentrated dots (Fig. 2B), while GFP-C297 just locates in the nucleus without dots (Fig. 2B). So at least the region residues 297–388 Aa might affect the distribution of C-terminus truncation of hIws1 in nucleus.

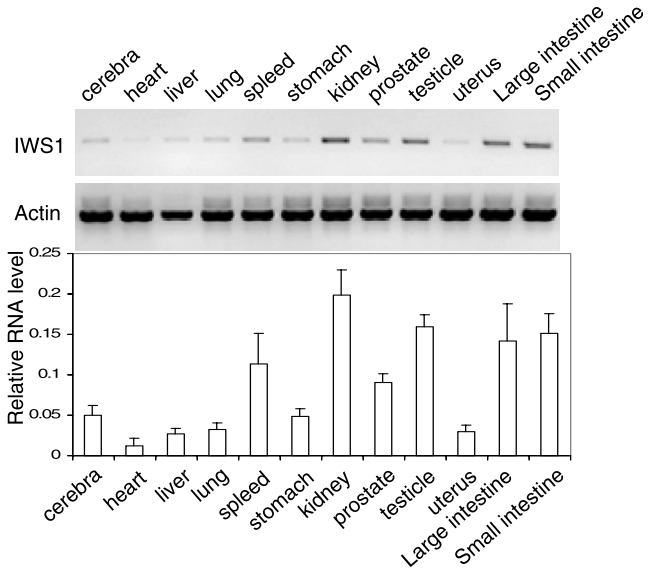

Fig. 1. Expression pattern of iws1 in mouse. Tissue distribution specificity of miws1 is determined by RT-PCR. Upper panel illustrates PCR products of miws1 transcript in various mouse organ tissues after electrophoresis on 1.2% agarose gel. Lower panel represents densiometrically determined respective relative mRNA levels normalized to β-actin. Data represent means ± SE of at least three independent experiments.

HIws1 is essential for cell viability

Recently, RNA interference has provided a practical tool to silence gene expression in mammalian cells, opening new possibilities for studying the functions of interesting gene or protein.

To investigate the functional effect of the down-regulation of the hIws1 expression, a colony forming assay is performed. After transfected with the prepared shRNAs, siIws1-1, siIws1-2, siIws1-3, and control plasmid siLuc, cells in the siIws1-2 group show a little decrease in the number of cell colonies compared with the control siLuc group, while a significant decrease in the number of colonies is observed either in siIws1-1 or siIws1-3 group (Fig. 3B). To determine if the decreased colony formation is a direct consequence of shRNAs-mediated deletion of the hIws1 protein, the Western blot analysis on the transfected cells extract is performed. The result shows that the extent of protein knockdown is in good agreement with changes in colony conformation. As shown in Fig. 3A, in four independent experiments, siIws1-1 and siIws1-3 exhibit highly potent effect, which result in a significant reduction in endogenous hIws1 expression compared to the cells treated with siLuc. While siIws1-2 knocks down hIws1 protein

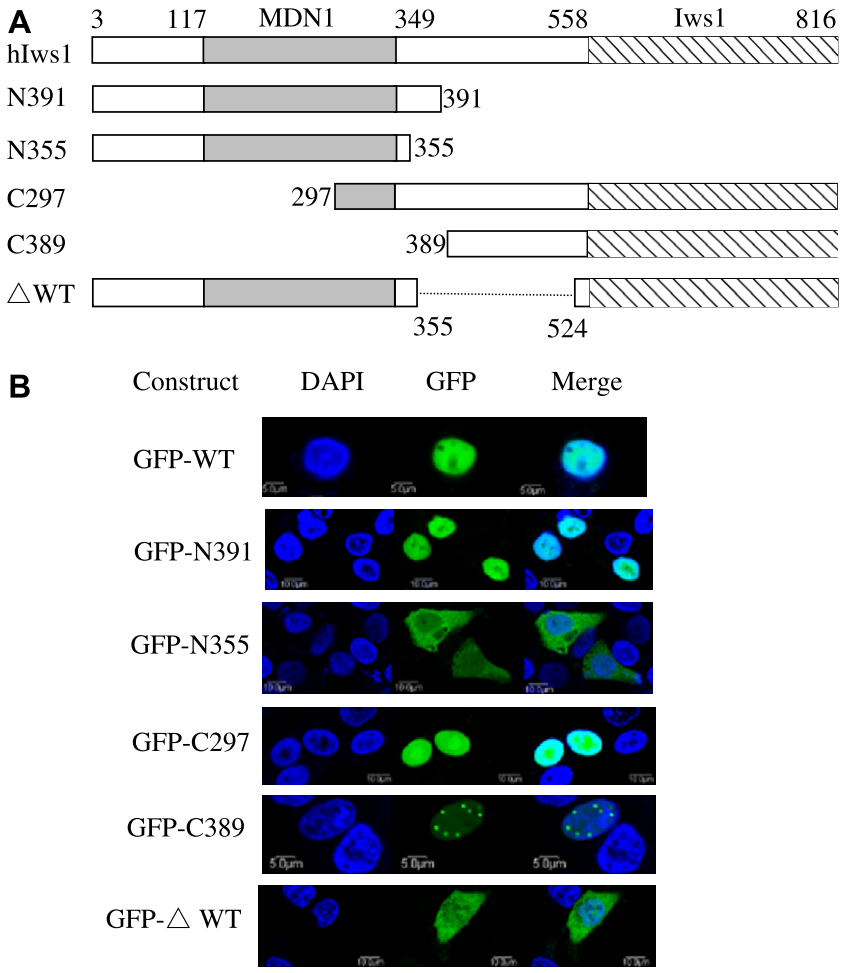

Fig. 2. Nuclear localization of hIws1. (A)The schematic of the wild type (WT) and various truncations of the hIws1 protein. These proteins are analyzed for their subcellular localization by N-terminally fused GFP tag. (B) The constructs are transiently transfected into HCT116 cells. Thirty hours later, cells are fixed and stained with DAPI, then observed with laser-scanning confocal microscope. GFP-WT, GFP-N391, and GFP-C297 are localized in the nucleus, and GFP-C389 appears some concentrated dots in the nucleus, while GFP-N355 or GFP-△WT distributes both in the nucleus and cytoplasm.

only modestly. These results demonstrate that the depletion of the hIws1 protein impairs cell viability, suggesting that hIws1 is essential for the growth and survival of cells.

The in vivo physical association between hIws1 and PRMT5

PRMT5 is an important mammalian type II PRMTs, which has been implicated to methylate Spt5 and Histone H3 resulting in transcriptional repression of active genes [12,14]. To determine the existence of functional interaction between hIws1 and PRMT5 *in vivo*, coimmunoprecipitation assays are performed on HCT116 cell extraction with a PRMT5-specific antiserum and an anti-hIws1 serum. The hIws1 protein could be coprecipitated by anti-PRMT5 antibody as anti-hIws1 antibody do. Similarly, the PRMT5 protein may be pull down by anti-hIws1 antibody as anti-PRMT5 antibody do. These reactions are specific, since no signals are present when the preimmune serum or protein A–Sepharose alone is used for coprecipitation (Fig. 4). These results indicate physical association between both proteins *in vivo*.

Discussion

Although a strict consensus sequence for NLSs has not been found among many nuclear proteins, NLSs are typically simple sequences of 3–5 basic amino acid residues, often preceded by an acidic amino acid or proline, sometimes associated with a glycine residue (D, E, P, G) [18,19] and a subset of effective NLSs fit the tetrapeptide consensus sequence Lys-Arg/Lys-X-Arg/Lys. where X may be substituted by the amino acids K, R, V, P, and A but not by N [20]. However, a NLS may also consist of bipartite clusters of basic amino acids separated by a spacer region of approximately 10–12 amino acids, often flanked by a neutral or acidic amino acid (D, E, W, Y, S, C, M, N, Q, T) [18].

Furthermore, there are nonclassical NLSs with relaxed sequence conservation [20], which varies from short stretches of residues to large protein domains. Among these nonclassical NLSs, the best characterized is the NLS of hnRNP A1 and related proteins. Called the M9 domain, the sequence consists of a glycine-rich sequence

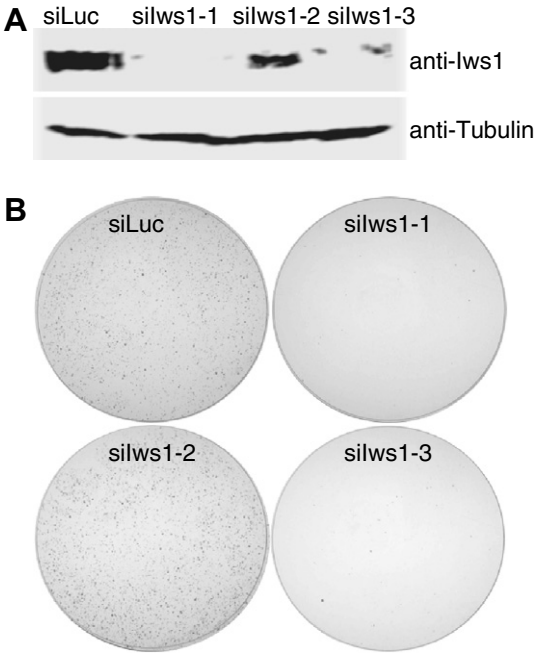

Fig. 3. Effect of hIws1 depletion on the cell viability. A subset of hct116 cells treated with three hiws1-specific shRNAs and the control siLuc are used for cell colony-forming assay, and the rest of cells are cultured and expanded for further Western blot analysis. (A) Ten days later, proteins (30 μg/lane) are separated by 10% SDS–PAGE, transferred to a nitrocellulose membrane, and probed with anti-hIws1 and γ-tublin-specific antibodies. (B) Ten days later, the colonies are fixed by 4% paraformaldehyde and stained with 2% crystal violet.

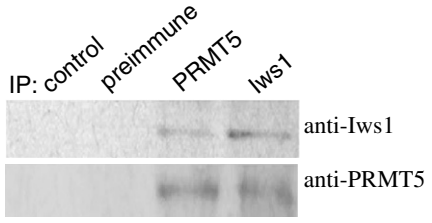

Fig. 4. Western blot analysis of physical association between hIws1 and PRMT5 in HCT116 cells after immunoprecipitation with hIws1-specific antibody, PRMT5-specific antibody, preimmune serum or control RIPA buffer. Antigen–antibody complexes are separated by 10% SDS–PAGE, and the Western blot assay is performed with anti-hIws1 polyclonal antiserum or anti-PRMT5 polyclonal antiserum as indicated.

of 38 amino acids and is required for both import and export of A1 [21]. Although considerably different in structure, NLSs generally play the same role: recognized by soluble factors, such as importinα and importinβ that mediate the protein to import into nucleus [16,22–24].

In this study, computer software fails to reveal any signals of NLSs, while we have determined two regions (residues 356–391 Aa, and 389–524 Aa) within the boundary between the two domains of the hIws1 protein, which could independently direct protein into nucleus. The first region harbors the following short stretches of basic residues, RKK (residues 357–359) and KKQK (residues 371–374). Each of them is flanked by aspartic acid (D) and methylnine (M), respectively, and is spaced by 12 residues, so it is possible that these two residue clusters serve as a typical bipartite NLS, while it could not exclude either of them serves as a monopartite NLS. Among the second region, there are another two basic residue clusters, KRK (residues 391–393) and KRGK (residues 518–521), it is possible either of which is capable of directing cargo protein into the nucleus.

There are some composite nuclear targeting motifs comprising of a canonical basic NLS and a CK II phosphorylation site such as SSDDE and EDESSEED. The classical NLS is sufficient for nuclear import, while phosphorylation of the CK II site increase the rate of nuclear import [25–27]. Putative composite targeting motifs, composed of a classic or nonclassic basic NLS and an acidic CK II site, are found in a variety of proteins, they may cooperate in mediating rapid nuclear import [25,28,29]. In hIws1, all of the four aforementioned putative NLSs motifs are preceded or followed by CK II site-like motifs. For example, RKK (residues 357–359) is followed by SSDSE, KKQK (residues 371–374) is followed by DSDEDE, KRK (residues 391–393) is followed by SDSEDE and KRGK (residues 518–521) is preceded by EDSDSD. It is possible that some of these four motifs may function as the composite NLSs, and the CK II site would promote the nuclear import.

Interestingly, it is found that the GFP-C389 fusion protein localizes throughout the nucleus with some concentrated dots. While GFP-WT and GFP-C297, both of which containing hIws1-C389, all localize in the nucleus without dots. It implicates that the acidic N-terminal truncation residues 3–388, even residues 297–388 play a crucial role in the normal interactions between hIws1-WT and involved components in nucleus.

Immunoprecipitation assays show that hIws1 protein is capable of physical interaction with the type II methyltransferase PRMT5 *in vivo*. It has long been known that proteins that harbor glycine and arginine-rich (GAR) motifs are often targets for PRMTs [30]. While the type II enzymes, PRMT5 and PRMT7 modify the isolated arginines or arginines within GAR motifs. It is now known that lots of factors, such as Coilin, Histone H2A, H3, H4, and Spt5 are substrates of PRMT5, and more substrates are likely to be identified from the GAR motif. There is no canonical GAR motif within hIws1 exclusive of KRGK (residues 518–521), GIGRA (residues 656–660) and GIGRL (residues 784–788). It is possible that some of these three motifs or other isolated arginine residues are targets of PRMT5. However, there is recent evidence demonstrating that based on the larger multisubunits complex, some subunits could interact with PRMT5 but not subject to methyl modification [31]. It could not exclude that both hIws1 and PRMT5 may be components of some larger complex.

Double-stranded small interfering RNAs (siRNAs) or small hairpin RNAs-mediated RNA interfering (RNAi) provides the approach to degrade specific mRNAs of any

endogenous genes in mammals, which allows to investigate the function of a target gene. In yeast, yIws1 and Spt6 are all essential for cell growth [1,32]. To explore the functional role of hIws1 *in vivo*, hiws1-specific small hairpin RNAs are used to knock down target gene expression. Significant inhibition of hiws1 gene expression is observed in HCT116 cells by using of shRNAs. The number of colony formation is decreasing according to the depletion level of the hIws1 protein. This means that RNAi dramatically reduces the hIws1 protein production, which, in turn, translates into a range of growth inhibitory effects. In other words, hIws1 is functionally unredundant and essential for cell viability.

Acknowledgments

This work was supported by President Fund of Chinese Academy of Sciences, the National Basic Research Program of China (2005CB522400), National Natural Science Foundation of China (30670479).

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