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Nuclear localization of Hif-3α requires two redundant NLS motifs in its unique C-terminal region

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Hif-3α, a member of the hypoxia-inducible factor (HIF) family, enters the nucleus and regulates gene expression in response to hypoxia. The molecular basis of its nuclear localization is not clear. HIF-1α and HIF-2α use a bipartite nuclear localization signal (NLS) to enter the nucleus. This motif is not conserved in Hif-3α. Although there is a conserved Arg/Lys rich motif in the Hif-3α N-terminal region, deletion of this region has minimal effect on Hif-3α nuclear localization. Here, we mapped the functional NLS to the unique C-terminal region of Hif-3α and identified two clusters of basic residues critical for its nuclear localization. The two NLS motifs are functionally redundant. Our results, thus, suggest that Hif-3α nuclear localization is mediated through two redundant NLS motifs located in its unique C-terminal region.

Keywords: hypoxia; hypoxia-inducible factor; nuclear localization signal; zebrafish

Hypoxia-inducible factors (HIFs) are evolutionarily ancient transcription factors that play key roles in coordinating the transcriptional response to hypoxia [1]. HIFs are made by an oxygen-regulated α subunit and a stable β subunit (also known as ARNT) [2]. In human and other vertebrates, there are three HIF-α genes [3]. While HIF-1α and HIF-2α have similar domain structure and share high sequence identities, HIF-3α has a distinct C-terminal region and its sequence identities to HIF-1α and HIF-2α are relatively low [3,4]. To date, our knowledge of HIF biology is mainly derived from studies on HIF-1α and HIF-2α. Under normal oxygen tension, HIF-1α/2α is hydroxylated at conserved proline residues by prolyl hydroxylases. The hydroxylated HIF-1α/2α in turn are recognized by von Hippel–Lindau protein (pVHL) and targeted for proteasome degradation [3]. Under hypoxic conditions, HIF-1α/2α are stabilized and enter the nucleus *via* a bipartite nuclear localization sequence (NLS) motif in the C-terminal region [5,6]. They form dimers with the common HIF-β subunit and regulate target gene expression by binding to hypoxia response elements (HRE) in promoter regions of target genes [3].

HIF-3α is unique in that it lacks the C-transactivation domain, but contains a unique leucine zipper (LZIP) domain in the C-terminal region [7,8]. Earlier studies suggested that HIF-3α has weak or no transcriptional activity or even acts as a negative regulator of HIF-1/2α [8–11]. We now understand that the HIF-3α gene gives the rise to many variants, due to alternative splicing, alternative promoters, and alternative translation initiation codons [4,12]. These different HIF-3α isoforms have distinct and sometimes even opposite biological actions [4]. We have recently reported that hypoxia increased the nuclear presence

Abbreviations
HIF, hypoxia-inducible factor; HRE, hypoxia response elements; LZIP, leucine zipper; NLS, nuclear localization signal; pVHL, von Hippel–Lindau protein.

of zebrafish Hif-3α1 (the full-length protein, referred as Hif-3α hereafter) and that Hif-3α binds to specific region(s) in its target genes and stimulates their expression *in vivo* [13]. Importantly, this function is conserved in full-length human HIF-3α [13,14]. How Hif-3α/HIF-3α enters the nucleus is still unclear. The objective of this study was to determine the structural motif(s) in Hif-3α responsible its nuclear localization.

Materials and methods

Chemicals and reagents

PCR primers, Oligo(DT)18, and routine chemicals were obtained from Sangon Biotech (Shanghai, China). Vent DNA polymerase and restriction enzymes were purchased from New England BioLabs (Ipswich, MA, USA). The KOD kit was purchased from TOYOBO (Shanghai, China). Dulbecco's modified Eagle's medium, McCoy's 5A medium, OPTI-MEM, fetal bovine serum, and antibiotics were purchased from Gibco (Grand Island, NY). Lipofectamine was bought from Invitrogen Life Technologies (Carlsbad, CA, USA).

Plasmid construction

The construction of zebrafish Hif-3α-EGFP has been reported previously [7]. Several Hif-3α truncation mutants, Δ1–346, Δ1–456, Δ391–626, Δ509–626, were generated by PCR and subcloned into the pCS2-EGFP plasmid using the primers shown in Table S1. To generate the β-Gal-EGFP dual tag plasmid, the open reading frame of β-Gal was amplified by PCR using pSV40-LacZ plasmid as template. The primers are 5′-CCGCTCGAGGCCACCATGG TCGTTTTACAACGTCGTGAC-3′ and 5′-CCCAAGCTT TTTTTGACACCAGACCAACTGGT-3′. The amplified PCR product was digested with XhoI and HindIII and subcloned pEGFP-N1, resulted in the β-Gal-EGFP plasmid. DNA fragment corresponding to various regions of Hif-3α1, including amino acid (aa) 457–626, 457–517, 518–626, 518–567, and 568–626 were amplified by PCR using primers shown in Table S2. The PCR products were inserted into the 3′s end of β-Gal in the β-Gal-EGFP plasmid. Site-directed mutagenesis was used to change specific residue(s) in pCS2-Hif-3α-EGFP and β-Gal-Hif-3α-EGFP as previously reported [15] using the primers shown in Table S3.

Cell culture, transfection, and subcellular localization

Hela cells were cultured as previously reported [7]. For transfection, cells were seeded into 35-mm tissue culture dishes. Plasmid DNA was transiently transfected into cells as described previously [7]. Thirty-six hours later, cells were washed and counterstained with DAPI. After rinsing in 1× PBS, images were recorded with a Nikon ECLIPSE 80i microscope equipped with a digital camera (Melville, NY, USA) with appropriate filters and a digital camera.

Results and Discussion

Human HIF-1α and HIF-2α use a bipartite NLS motif located in the C-terminal region to enter the nucleus [5,6]. HIF-3α/Hif-3α C-terminal region differs considerably from HIF-1α and -2α [7,8]. Sequence analysis

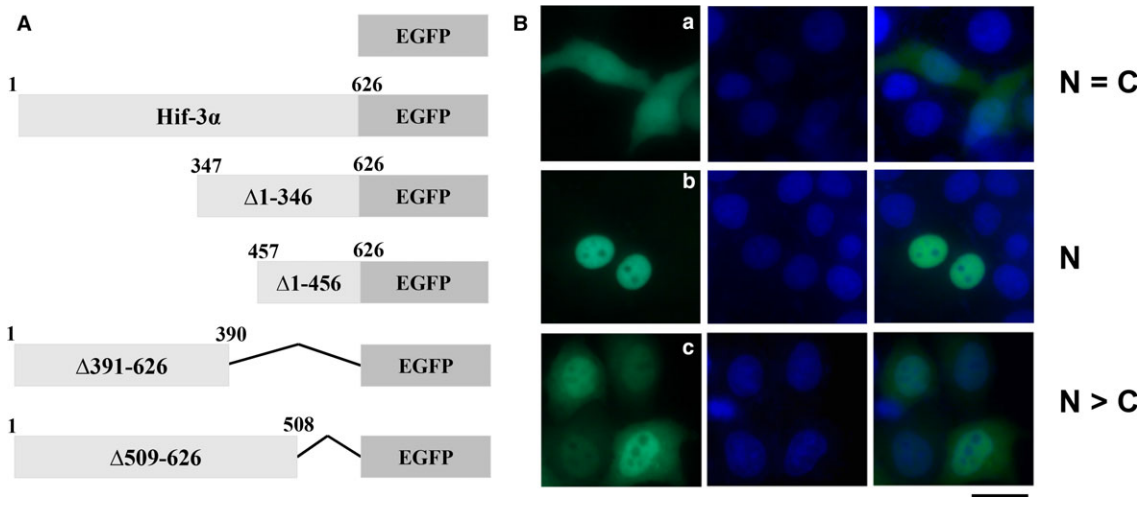

Fig. 1. Hif-3α C-terminal region is important for nuclear localization. (A) Schematic diagrams showing Hif-3α-EGFP plasmids engineered and tested. (B) Representative views of three categories of subcellular distribution of GFP signal. N = C, GFP signal is distributed in both nuclear and cytosol; N, complete nuclear; N > C, stronger GFP signal in the nucleus than the cytoplasm. Scale bar = 20 μm.

showed that the bipartite NLS motif is not conserved in Hif-3α C-terminal region. Instead, there is an Arg/Lys rich sequence conserved in the N-terminal regions in all three HIF-αs (Fig. S1). To test whether the N-terminal region is functionally important, we engineered Hif-3α Δ1–346-EGFP and Hif-3αΔ1–456-EGFP, 2 Hif-3α deletion mutants tagged with EGFP (Fig. 1A). When transfected into Hela cells, full-length Hif-3α-EGFP signal was detected in the nucleus in 96% transfected cells. In 4% transfected cells, its signal in the nucleus was stronger than that in the cytoplasm (Fig. 1B; Table 1). In comparison, GFP signal was distributed both in the nucleus and cytoplasm in 100% cells transfected with the EGFP vector (Fig. 1B; Table 1). Deletion of aa 1–346 did not change the subcellular distribution of Hif-3α-EGFP (Table 1). The Hif-3αΔ1–456-EGFP signal was detected in the nucleus in 85% transfected cells. In 15% transfected cells, the Hif-3αΔ1–456-EGFP signal was greater in the nucleus (Table 1). These results suggested that the N-terminal region Hif-3α is largely dispensable, although it may be involved in the nuclear localization of Hif-3α to some degree. These data are in good agreement with a previous study on zebrafish Hif-3α2 [15]. Hif-3α2, an alternatively spliced zebrafish Hif-3α variant that lacks the N-terminal region (including belchbHLH, Per/ATNT/Sim domain, PAC, and part of ODD domain), is able to enter the nucleus and has HRE-dependent activity [15].

Table 1. Subcellular distribution of various Hif-3α-EGFP fusion proteins.

	Subcellular distribution (% transfected cells)		
Construct name	N	N > C	N = C
EGFP	0	0	100
Hif-3α-EGFP	96	4	0
Hif-3α Δ1–346-EGFP	97	3	0
Hif-3α Δ1–456-EGFP	85	15	0
Hif-3αΔ391–626-EGFP	13	79	8
Hif-3αΔ509–626-EGFP	0	98	2

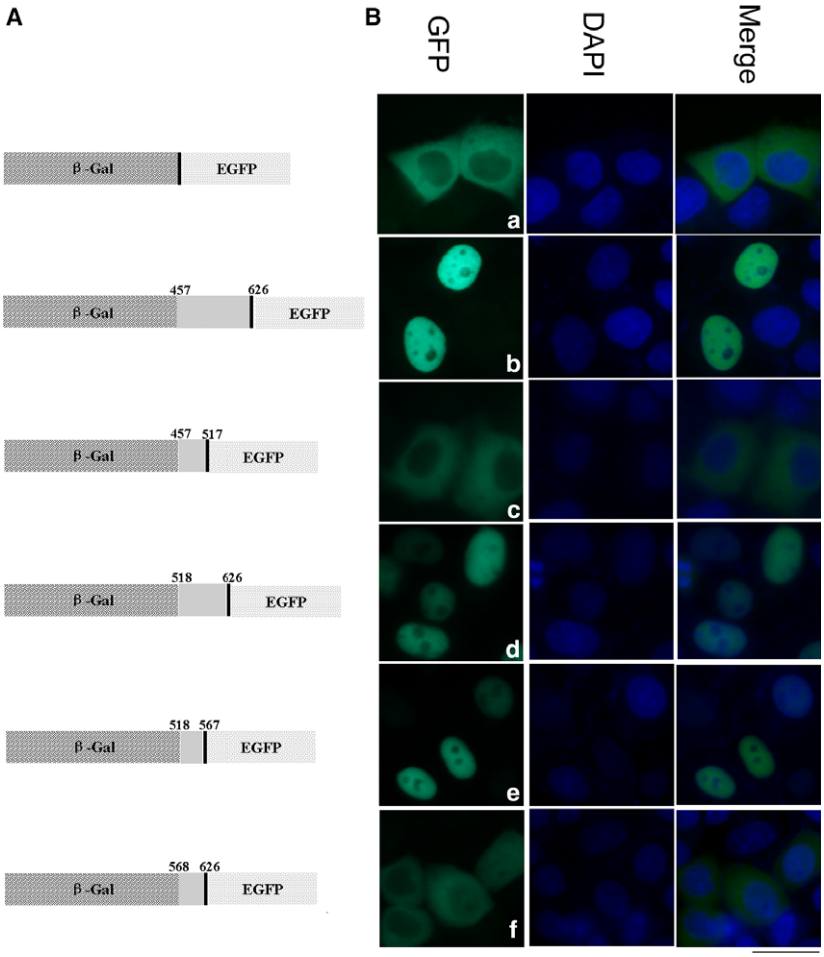

Fig. 2. Mapping the functional NLS in Hif-3α C-terminal region. (A) Schematic diagrams showing β-Gal-Hif-3α-EGFP plasmids engineered and tested. (B) Representative views. Panels a–f are cells transfected with the plasmids shown in (A). Scale bar = 20 μm.

There are several clusters of charged aa in the C-terminal regions of HIF-3α (Fig. S1). To determine whether any of them acts as the functional NLS motif (s), two truncation Hif-3α mutants were generated. Deletion of the C-terminal sequence (i.e., Hif-3αΔ391–626-EGFP and Hif-3αΔ509–626-EGFP) greatly reduced the nuclear presence (Fig. 1; Table 1). In cells transfected with Hif-3αΔ509–626-EGFP, 0% cells showed nuclear signal. These data suggest that C-terminal region of Hif-3α is critical. A caveat of the above experiment is the size of the fusion proteins. It is known that proteins smaller than 50 kDa can diffuse across the nuclear pore [16]. To overcome this problem, we engineered a β-Gal-EGFP dual reporter plasmid (Fig. 2A). β-Gal has a molecular size of 190 kDa and it does not contain any known NLS [17]. We inserted Hif-3α457–626 to test whether the C-terminal region contains a functional NLS motif (s) (Fig. 2A). While β-Gal-EGFP was exclusively present in the cytoplasm, the β-Gal-Hif-3α457–626-EGFP signal was detected only in the nucleus (Fig. 2B; Table 2). We divided Hif-3α457–626 into two fragments, Hif-3α457–517 and Hif-3α518–626 (Fig. 2A). When tested in Hela cells, β-Gal-Hif-3α 457–517-EGFP was distributed in the cytoplasm. In comparison, β-Gal-Hif-3α518–626-EGFP signal was completely nuclear (Fig. 2B; Table 2), suggesting there is a functional NLS motif(s) in this region. The Hif-3α518–626 region was further divided into two smaller fragments and tested. While β-Gal-Hif-3α518–567-EGFP signal was nuclear in 100% transfected cells, the β-Gal-Hif-3α568–626-EGFP signal was detected more in the nucleus (N > C) in 9% of the cells transfected (Fig. 2B; Table 2). In 66% of the cells transfected, the β-Gal-Hif-3α568–626-EGFP signal was detected in both the nucleus and cytosol. In the remaining 25% transfected cells, the signal was mainly distributed in the cytoplasm (Fig. 2; Table 2). These data suggested that there are likely two functional NLS motifs. One is located within aa 518–567. This appears to have strong activity. The other is located between aa 568 and 626 and its activity is weaker. Sequence analysis identified two clusters of basic residues in Hif-3α aa 518–567, i.e., R523K524R525 and K544K545 (Fig. S1). To test whether any of these residues are critical, they were changed into Ala and the impact on nuclear localization was tested. When R523, K524, and R525 were all mutated, the signal was detected only in the cytoplasm (Fig. 3A, Table 2). Next, these three residues was mutated individually. While mutation of 523R had no effect, change either K524 or R525 abolished the nuclear localization of β-Gal-Hif-3α518–567-EGFP. In comparison, mutation of K544 and K545 had little effect (Fig. 3A, Table 2). These data suggest that K524 and R525 are critical. We next investigated the importance of the basic residues in Hif-3α 568–626 region. The results showed that mutation of K578, R579, and/or R586 into Ala into in the β-Gal-Hif-3α568–626-EGFP background abolished the nuclear localization (Fig. 3B, Table 2). In contrast, mutation of R610, R615, and R616 did not change the subcellular localization of β-Gal-Hif-3α568–626-EGFP (Fig. 3B, Table 2). These data suggest that the K578 and R579 motif are critical for the weak nuclear localization activity in Hif-3α568–626-EGFP.

To determine the relationship between these two functional NLS motifs, mutations were introduced into β-Gal-Hif-3α457–626-EGFP, which contains both NLS motifs. Mutation of K524 and R525 did not change the nuclear localization of β-Gal-Hif-3α457–626-EGFP (Fig. 3C, Table 2), suggesting that in the presence of the K578 and R579 motif, mutation of K524 and R525 alone is not enough to reduce the nuclear localization. Likewise, mutation of K578 and R579 in β-Gal-Hif-3α457–626-EGFP had little effect (Fig. 3C, Table 2).

Table 2. Subcellular distribution of various β-Gal-EGFP fusion proteins.

Construct name	Subcellular distribution (% transfected cells)			
	N	N > C	N = C	C
β-Gal-EGFP	0	0	0	100
β-Gal-457–626-EGFP	100	0	0	0
β-Gal-457–517-EGFP	0	0	0	100
β-Gal-518–626-EGFP	100	0	0	0
β-Gal-518–567-EGFP	100	0	0	0
β-Gal-518–567/R523A/K524A/R525A-EGFP	0	0	0	100
β-Gal-518–567/R523A-EGFP	100	0	0	0
β-Gal-518–567/K524A-EGFP	0	0	0	100
β-Gal-518–567/R525A-EGFP	0	0	0	100
β-Gal-518–567/K544A/K545A-EGFP	100	0	0	0
β-Gal-568–626-EGFP	0	9	66	25
β-Gal-568–626/K578A/R579A-EGFP	0	0	0	100
β-Gal-568–626/K578A/R579A/R586A-EGFP	0	0	0	100
β-Gal-568–626/R610A/R615A/R616A-EGFP	0	8	63	29
β-Gal-457–626/K524A/R525A-EGFP	100	0	0	0
β-Gal-457–626/K578A/R579A -EGFP	100	0	0	0
β-Gal-457–626/K524A/R525A/K578A/R579A-EGFP	0	0	0	100
β-Gal-457–626/K524A/R525A/R610A/R615A/R616A-EGFP	100	0	0	0

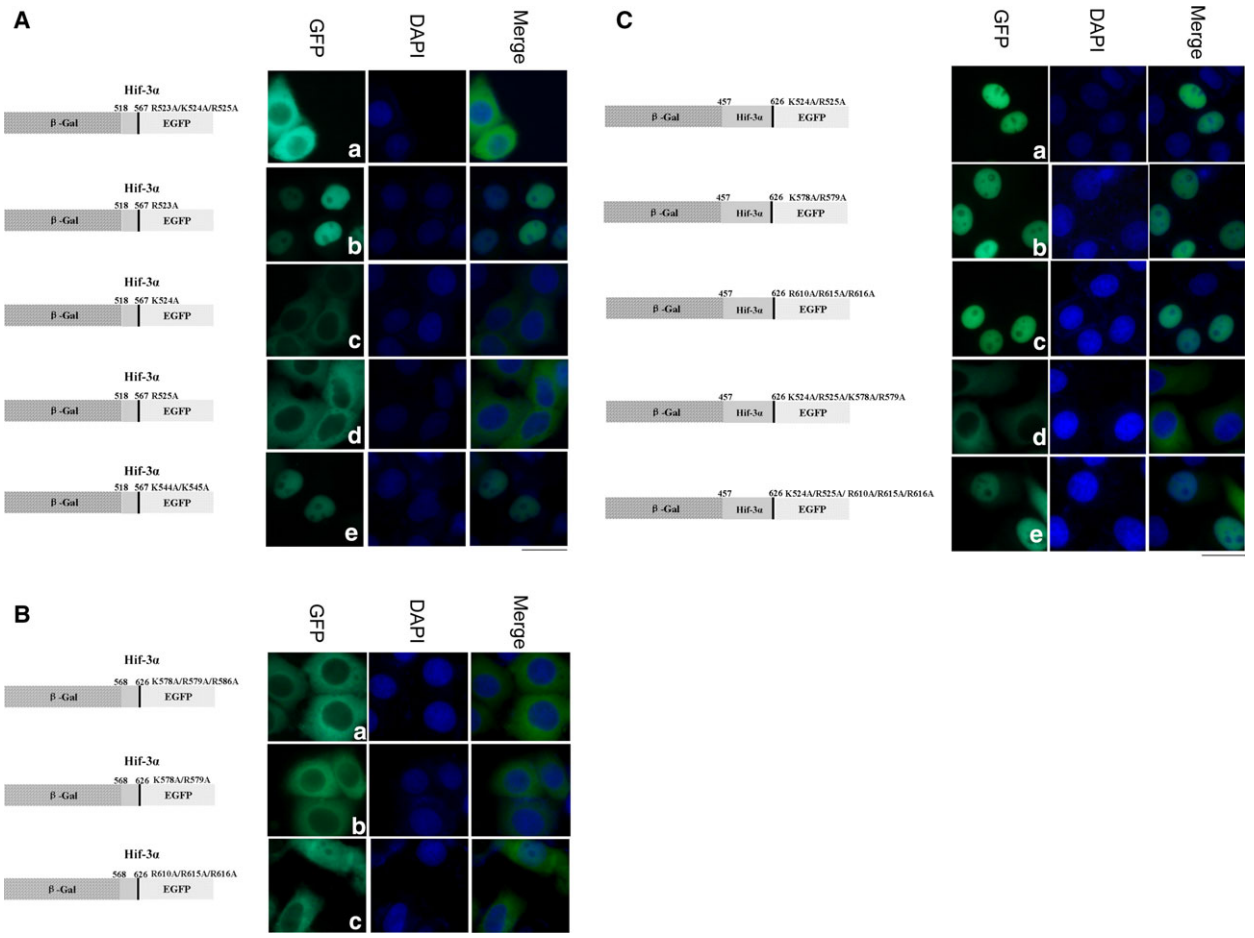

Fig. 3. Mapping key residues critical for Hif-3α nuclear localization. (A) Schematic diagrams showing Hif-3α-518–567 point mutants engineered and tested. A representative result is shown in the right panel. (B) Schematic diagrams showing Hif-3α-568–626 point mutants engineered and tested. A representative result is shown in the right panel. (C) Schematic diagrams showing β-Gal-457–626-EGFP point mutants engineered and tested. A representative result is shown in the right panel. Scale bar = 20 μm.

When all these four residues are mutated into Ala, the nuclear location was abolished. This interaction is specific because mutation of R610/R615/R616 with K524A/R525A did not alter the nuclear location of β-Gal-Hif-3α457–626-EGFP (Fig. 3C, Table 2).

The above findings suggest that there are two functional NLS motifs located in the unique C-terminal region of Hif-3α. One contains K524 and R525 and this motif has stronger activity and can be considered the primary site. Another, containing K578 and R579, has weaker activity. Importantly, these two motifs are functionally redundant. In the absence of the K578/R579 motif (i.e., in the β-Gal-Hif-3α518–567-EGFP background), mutation of K524 and R525 abolished the nuclear localization. In the presence of the K578/R579 motif (i.e., in the β-Gal-Hif-3α457–626-EGFP background), however, mutation of K524 and R525 had little effect. Likewise, mutation of the K578/R579 motif only affects the nuclear localization of Hif-3α in the absence of the K524/R525 motif. When both NLS motifs were mutated, the nuclear signal was abolished (Fig. 3C, Table 2). These findings suggest that the nuclear localization mechanism of Hif-3α is distinct from those previously reported for HIF-1α and -2α. It should be mentioned that our conclusion is based on observations using the C-terminal fragments. While the N-terminal sequence is largely dispensable (as indicated by Hif-3α-1–346-EGFP and Hif-3α-1–456-EGFP data), it appears to play a minor role in the nuclear localization of Hif-3α. Future studies are needed to further evaluate the interplay between the N-terminal region and the two NLS motifs located in the C-terminal region in the full-length Hif-3α context and to perform functional assays to examine the effects of these mutations in altering the downstream target gene expression.

Much less is known about HIF-3α biology compared to that of HIF-1α and -2α. This is partially due to the existence of multiple HIF-3α variants. At least eight distinct HIF-3α protein products have been documented [4]. These HIF-3α variants are expressed in different tissues and/or different developmental stages. The full-length human HIF-3a, HIF-3α1, has HRE-dependent transcriptional activity [8,13]. Human HIF-3α2 and 3α4 isoforms, which lacks the C-terminal LZIP domain, have been shown to inhibit HIF-1/2α action by competing for the common HIF-β subunit [18,19]. Likewise, overexpression of mouse IPAS, a short HIF-3α variant that lacks the LZIP domain, in HeLa cells inhibits hypoxia-induced HRE-dependent reporter gene activity and the mRNA levels of VEGF and PGK1, two HIF-1α target genes [9]. Although a previous study showed that mouse IPAS uses a bipartite type NLS in the N-terminal region to enter the nucleus [20], this sequence is not present in the full-length HIF-3α and other HIF-3α isoforms. Until this study, there is no report on the nuclear localization mechanism of full-length and longer human HIF-3α isoforms. Sequence analysis suggests that these two functional NLS motifs identified in this study are conserved in the full-length human HIF-3α (HIF-3α1) and several long HIF-3α variants including HIF-3α isoform 8 and HIF-3α isoform 9, but is absent in short HIF-3α isoforms, such as HIF-3α isoforms 2–5. Future studies are needed to find out whether these conserved motifs act in a similar way in the nuclear localization of the full-length and longer human HIF-3α isoforms.

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Authors' contributions

CD, QY, and PZ designed the research; QY and PZ conducted the research; CD and QY wrote the manuscript. QY, PZ, LL, YL, YL, and CD analyzed and interpreted the data; all authors revised the manuscript, and gave final approval for publication.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Sequence alignment of the N-terminal region and C-terminal region of the indicated proteins.

Table S1. Primers used in generating the Hif-3α deletion mutants.

Table S2. Primers used in generating Hif-3α N- and C-terminal fragments.

Table S3. Primers used in generating Hif-3α point mutants.