

A unique sequence in the N-terminal regulatory region controls the nuclear localization of KLF8 by cooperating with the C-terminal zinc-fingers

Tina S Mehta¹, Heng Lu¹, Xianhui Wang¹, Alison M Urvalek¹, Kim-Hang H Nguyen¹, Farah Monzur¹, Jojo D Hammond¹, Jameson Q Ma¹, Jihe Zhao¹

¹Center for Cell Biology and Cancer Research, Albany Medical College, Albany, NY 12208, USA

Krüppel-like factor 8 (KLF8) transcription factor plays a critical role in cell cycle progression, oncogenic transformation, epithelial to mesenchymal transition and invasion. However, its nuclear localization signal(s) (NLS) has not been identified. KLF8 shares with other KLFs monopartite NLSs (mNLS) and C₂H₂ zinc fingers (ZFs), both of which have been shown to be the NLSs for some other KLFs. In this report, using PCR-directed mutagenesis and immunofluorescent microscopy, we show that disruption of the mNLSs, deletion of any single ZF, or mutation of the Zn²⁺-binding or DNA-contacting motifs did not affect the nuclear localization of KLF8. Deletion of >1.5 ZFs from C-terminus, however, caused cytoplasmic accumulation of KLF8. Surprisingly, deletion of amino acid (aa) 151-200 region almost eliminated KLF8 from the nucleus. S165A, K171E or K171R mutation, or treatment with PKC inhibitor led to partial cytoplasmic accumulation. Co-immunoprecipitation demonstrated that KLF8 interacted with importin-β and this interaction required the ZF motif. Deletion of aa 1-150 or 201-261 region alone did not alter the nuclear localization. BrdU incorporation and cyclin D1 promoter luciferase assays showed that the KLF8 mutants defective in nuclear localization could not promote DNA synthesis or cyclin D1 promoter activation as the wild-type KLF8 did. Taken together, these results suggest that KLF8 has two NLSs, one surrounding S165 and K171 and the other being two tandem ZFs, which are critical for the regulation of KLF8 nuclear localization and its cellular functions.

Keywords: KLF8, nuclear localization signal, importin, DNA synthesis, transcriptional activity

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Introduction

Krüppel-like factor 8 (KLF8) was initially described as a widely expressed transcription repressor [1] of the Krüppel-like family of C₂H₂-type zinc finger (ZF) transcription factors. Previously, we identified KLF8 as a FAK signaling effector that regulates cell cycle progression by activating cyclin D1 transcription [2]. We have further demonstrated that KLF8 is overexpressed in several types of human cancers, and plays a critical role in oncogenic transformation, epithelial to mesenchymal transition and cancer cell invasion, and identified KLF4

and E-cadherin tumor suppressors as KLF8-repressed targets [3, 4]. It has also been reported that KLF8 expression is important for glioblastoma progression [5]. KLF8 has thus emerged as one of the important oncogenic transcription factors. The expression, transcriptional activity and nuclear localization of such transcription factors are tightly regulated in normal cells and when dysregulation occurs the cells go awry. We have demonstrated that KLF8 expression is well regulated at the transcriptional level [2, 6] and sumoylation is a critical post-translational mechanism that limits KLF8's transcriptional activity and cellular function [7]. Like many transcription factors, KLF8 must first enter the nucleus in order to exert its function. How the nuclear localization of KLF8 is regulated, however, has not been investigated.

The nuclear localization of a protein is determined by outside-in (nuclear import) and inside-out (nuclear export) molecular trafficking across the nuclear membrane or

Correspondence: Jihe Zhao

Tel: 518 262-2305; Fax: 518 262-5669

E-mail: zhaojh@mail.amc.edu

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nuclear envelope through nuclear pore complexes that are composed of nucleoporins. The steady-state localization of the protein on either side of the nuclear envelope also depends on its interaction with the retention factor(s) in the cytoplasm or nucleus to which the protein anchors [8].

Nuclear import is regulated by mechanisms largely divided into two categories – free diffusion and non-free diffusion. Proteins of <30-50 kDa in size can usually pass through the nuclear pore by the free diffusion mechanism, whereas larger proteins use non-free diffusion importing mechanisms [8-10]. The latter mechanisms require the cargo protein to have nuclear localization signal (NLS(s)). Through the NLS(s), the cargo protein binds to the nuclear import receptor proteins called importins (importin- β 1 in most cases and importin- α / β 1 complex in other cases). Importin- β 1 then binds to the nucleoporins of the nuclear pore complexes that subsequently translocate the cargo protein to the nucleus. In the nucleus, importin- β 1 binds to RanGTP and this binding leads to the release of the cargo protein. The high level of RanGTP in the nucleus is maintained by its guanine nucleotide exchange factor RCC1. In rare cases, proteins without a conventional NLS, but with the nucleoporin-binding motif like that of importin- β 1, may directly bind the nucleoporins of the nuclear pore complexes and thus be imported to the nucleus in an importin-independent manner. On the other hand, if not anchored, proteins in the nucleus are subjected to nuclear export. Again, proteins of <30-50 kDa in size may freely diffuse out of the nucleus. For most of larger cargo proteins, their nuclear export requires them to have a nuclear export signal (NES(s)). Through the NES(s), they interact with exportin-1 (or CRM1), a critical member of the exportin family proteins, which recruits RanGTP. This cargo-CRM1-RanGTP complex is then translocated, with the help of the nuclear pore complexes, to the cytoplasm where RanGAP1-catalyzed RanGTP to RanGDP transition results in the release of the cargo protein. In some cases, a cargo protein without an NES can recruit an adapter protein that has an NES for CRM1 binding. In either case, the nuclear export is a CRM1-dependent transport process that is sensitive to inhibition by leptomycin B. In rare cases, nuclear export of NES-containing cargo proteins relies on other exportins such as calreticulin rather than CRM1.

NLSs are generally grouped into classical and non-classical types. The classical type NLSs include monopartite NLS (mNLS) (a single stretch of basic amino acid (aa) with a consensus sequence of (K/R)₄₋₆) and bipartite NLS (two small stretches of basic aa linked by 10-12 aa in between with a consensus sequence of (K/R)₂X₁₀₋₁₂(K/R)₃) [11]. Most of non-classical types of NLSs consist of a single long stretch of non-basic aa (20-40 aa)

as in hnRNP [12, 13], HuR [14], I κ B α [15] and Nab2p [16]. Some non-classical NLSs are a single short stretch containing one or more basic aa, but is distinct from the mNLS, as in Cdc6 [17], FGF2 [18], Mat2a [19, 20] and Sam68 [21]. NES sequences are short hydrophobic sequences rich in leucines and/or isoleucines with a consensus sequence of L/Ix₍₁₋₃₎Lx₍₂₋₄₎LxL [22]. Many studies have demonstrated that nuclear localization of proteins is strictly regulated through their post-translational modifications. These modifications include phosphorylation, ubiquitylation, sumoylation, acetylation, methylation and glycosylation [8, 23, 24].

We have demonstrated that KLF8 is localized in the nucleus in both cultured cells and tumor tissue specimens [2-4]. Similar to most other members of the KLF family, KLF8 contains, in the C-terminus, three C₂H₂-type ZF motifs in tandem flanked by two mNLSs [25, 26]. Previous studies have demonstrated that the conserved mNLS and ZF regions are functional NLSs for both KLF1 [27, 28] and KLF4 [29].

In this study, we identified two NLSs in KLF8. One is located in the first two ZF regions in the C-terminus of KLF8. The other is located in the aa 151-200 region within the N-terminal regulatory region. The latter one is novel and unique to KLF8 and its nuclear import function is probably regulated by post-translational modifications at the S165 and K171 residues. Both of the NLSs belong to non-classical type of NLSs. The efficient nuclear localization of KLF8 requires both of the NLSs to mediate its nuclear import and is essential for its cellular functions.

Results

The 1st and 2nd ZFs (but not the 3rd), but not the classical mNLSs, are required for KLF8 nuclear localization.

We previously demonstrated that the wild-type full-length KLF8 protein is exclusively localized in the nucleus [2, 3] and identified two potential classical mNLSs (Figure 1A) [2] that could be responsible for the nuclear localization of KLF8. Both the mNLS1 and the ZFs are well conserved among all the KLF members [30, 31] and have been demonstrated to control the nuclear localization of KLF1 and KLF4 [27-29], suggesting a possibility that these regions may also be required for the nuclear localization of KLF8. To test this possibility, we deleted each of the mNLS sequences and the ZFs from the C-terminus (Figure 1B, dC8 to dC96). After their protein expression was confirmed by western blotting (Figure 1C), we transfected each of these mutants and examined the subcellular localization of these mutants by indirect immunofluorescent microscopy (Figure 1D). Deletion of

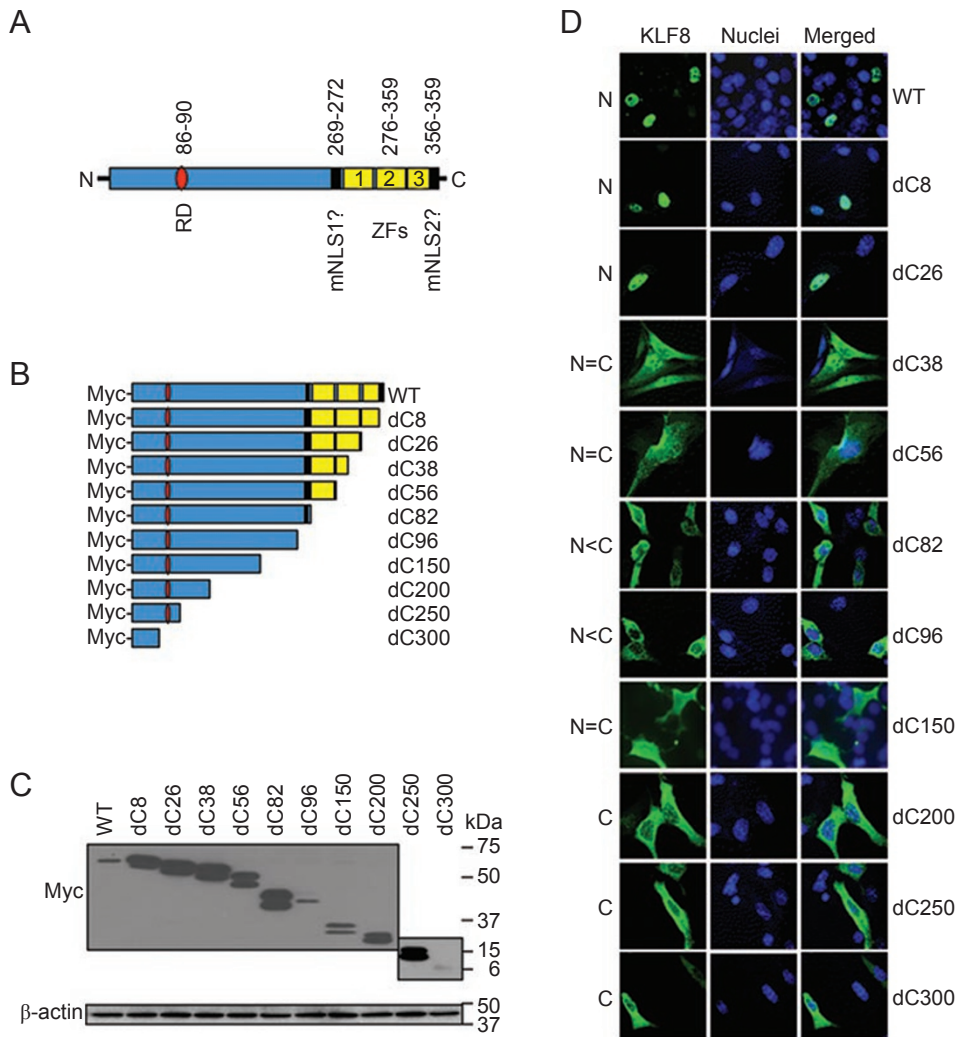


Figure 1 Zinc finger 2 is required for the KLF8 nuclear localization. **(A)** A diagram of KLF8 protein with the aa positions numbered on the top. The CtBP-binding repression domain (RD), two classical mNLS consensus sequences (mNLS1: KRRR and mNLS2: HRRR or RRRH) and the three zinc fingers (ZF) are indicated. **(B)** Diagram of KLF8 wild-type (WT) and C-terminal deletion (dC) mutants. These mutants were made by PCR-directed mutagenesis. **(C)** Western blotting confirmation of protein expression of the KLF8 constructs. Whole cell lysates prepared from NIH3T3 cells transfected with the constructs were used for anti-Myc or β -actin blotting. The dC250 and dC300 did not bind well to the cellulose membrane with standard size of pores, and thus were transferred onto membrane with smallest size of pores (0.2 μ m) in a separate blot. However, due to the small molecular size, only a small fraction of the dC300 mutant proteins could be captured even by this type of membrane during transfer. **(D)** Protein localization of the KLF8 constructs. Transfected NIH3T3 cells were analyzed by anti-Myc staining of the KLF8 proteins (green) and Hoechst staining of the nuclei (blue) followed by fluorescent microscopy. Patterns of localization are summarized on the left by observing 100 transfected cells from 10 to 15 independent fields. N, exclusively nuclear; C, exclusively cytoplasmic; N=C, most cells show both nuclear and cytoplasmic localization with similar staining intensity in the nucleus and cytoplasm; N<C, most cells show both nuclear and cytoplasmic localization with brighter staining intensity in the cytoplasm; N>C, most cells show both nuclear and cytoplasmic localization with brighter staining intensity in the nucleus.

the mNLS2 (dC8) or the whole ZF3 (dC26) had no effect on the nuclear localization of KLF8. However, when ZF3 and half of the ZF2 were deleted from the C-terminus (dC38), the mutant protein became diffusively present throughout the cell. Complete removal of both the ZF2 and ZF3 (dC56) resulted in more proteins in the cytoplasm than in the nucleus. Deletion of whole ZF region

(dC82) or the whole ZF region along with the mNLS1 (dC96) led to the cytoplasmic presence of the mutant proteins in the majority of the cells examined. Interestingly, the dC150 mutant partially regained the nuclear localization. Further stepwise deletion towards the N-terminus caused a complete loss of the nuclear presence and exclusive cytoplasmic localization of the proteins

(dC200 – dC300). These results suggest that (1) neither ZF3 nor mNLS2 plays a role in the nuclear localization of KLF8; (2) ZF1, ZF2 and possibly mNLS1 are required for the nuclear localization of KLF8 and (3) the mNLS1 alone or its combination with ZF1 is not sufficient to keep KLF8 in the nucleus.

The major biochemical function of the ZFs in the nucleus is to contact the target gene promoter DNA in a Zn^{2+} -binding-dependent manner [32, 33]. To determine whether these bindings and/or the mNLS1 are required for the nuclear localization of KLF8, we individually disrupted each of the DNA contact (dc) motifs (mZF1dc and mZF2dc) or the Zn^{2+} -binding motifs (H294/8L and H324/8L) in ZF1 and ZF2 or mutated the mNLS1 (K296M) in the full-length context, and examined the localization of the mutants in the cells (Figure 2). Surprisingly, none of these mutations affected the nuclear localization of KLF8. These results suggest that (1) the ZFs regulate KLF8 nuclear localization through a mechanism other than the Zn^{2+} -dependent DNA binding and (2) the mNLS1 does not play a significant role.

The aa 151-200 region in the N-terminal regulatory domain is essential for targeting KLF8 to the nucleus

It has been shown that the ZF region is both required and sufficient for KLF1 nuclear localization [27, 28]. To

test if the ZF region itself is sufficient to localize in the nucleus, we transfected this fragment alone (Figure 3, ZF) and examined its localization. We found that almost all of the transfected cells showed diffuse localization of this fragment in both the nucleus and cytoplasm (Figure 3C, ZF). This finding suggests that the deleted N-terminal regulatory region is also required for the exclusive nuclear localization of KLF8. To further explore this, we made a series of N-terminal deletion mutants and examined their localization. We found that removal of up to 152 residues (Figure 3C, dN50 – dN152) from the N-terminus did not cause any change in the nuclear localization. Surprisingly, when 200 residues were deleted from the N-terminus, the mutant protein became exclusively cytoplasmic in all the cells observed (Figure 3C, dN200). Interestingly, further deletion to aa 248 resulted in partial restoration of the nuclear localization (Figure 3C, dN248). These results strongly suggest that besides the ZF1 and ZF2 region, some residues within the aa 151-200 region are essential for the exclusive nuclear localization of KLF8.

To further verify this notion, we made an internal deletion of the aa 151-200 region (Figure 4, d151-200) and found that indeed all of the cells showed cytoplasmic localization of the mutant protein, although some of the cells showed a light staining of the protein in the nucleus

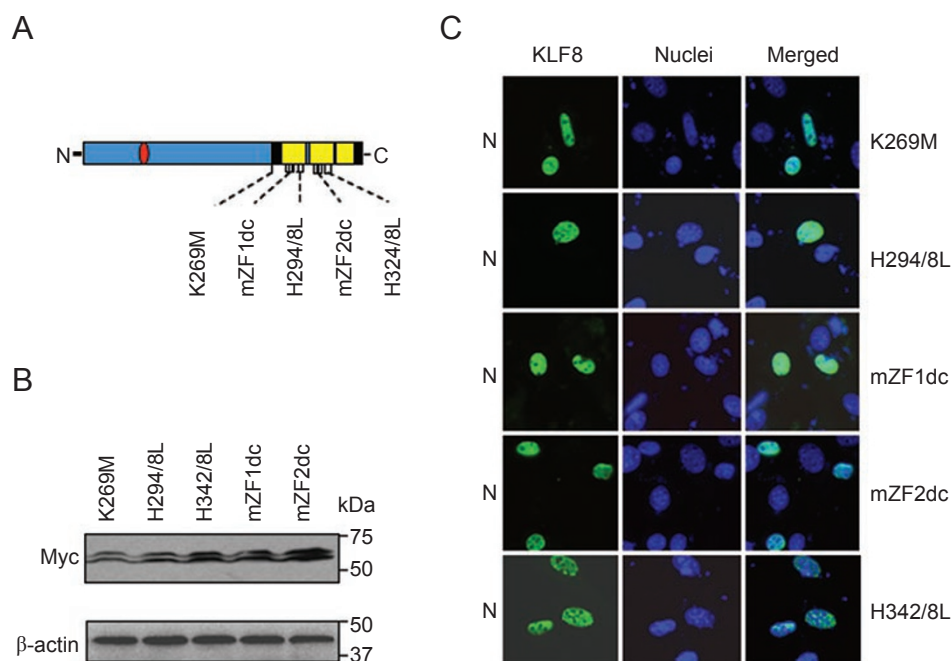


Figure 2 The mNLS1 or the binding to DNA or zinc ions by ZF1 or ZF2 is not required for KLF8 nuclear localization. **(A)** Diagram of KLF8 mutants in which disabling of the mNLS1 (K269M), the DNA contact (dc) motifs of ZF1 (mZF1dc) or ZF2 (mZF2dc), and the zinc-binding motifs of ZF1 (H294/8L) or ZF2 (H324/8L) was shown. These mutants were made by overlap PCR-directed point mutagenesis. The protein expression **(B)** and localization **(C)** were analyzed similarly as in Figure 1. Patterns of localization are summarized on the left.

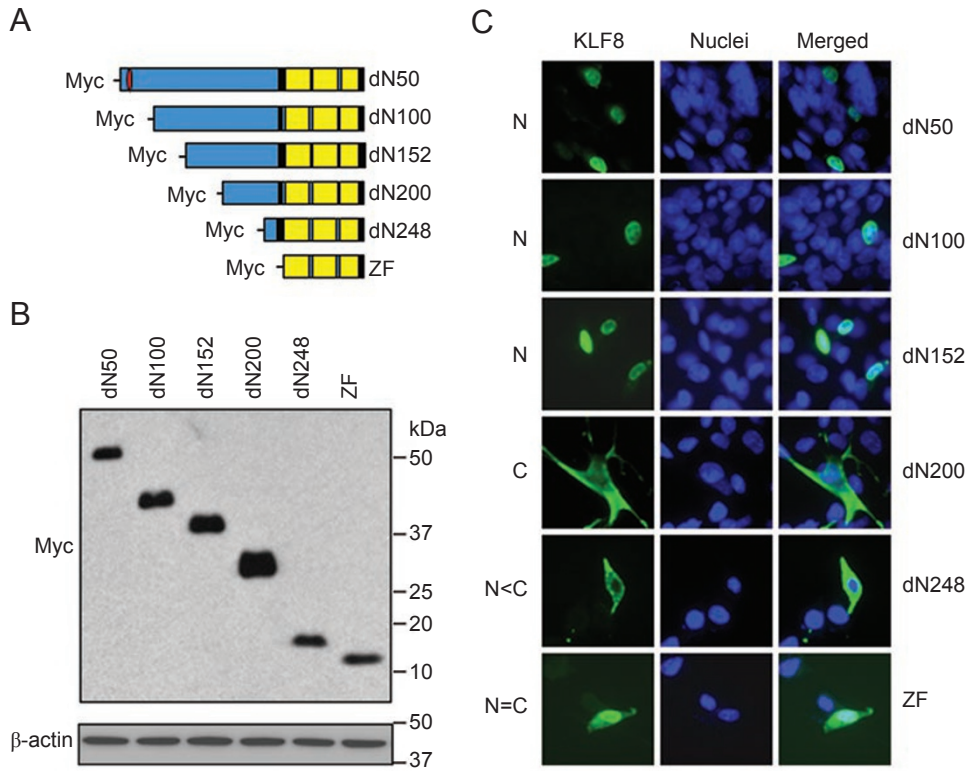


Figure 3 The aa 151-200 region is required for KLF8 nuclear localization. **(A)** Diagram of KLF8 N-terminal deletion (dN) mutants. These mutants were made by PCR-directed mutagenesis. The protein expression **(B)** and localization **(C)** were analyzed similarly as in Figure 1. Patterns of localization are summarized on the left.

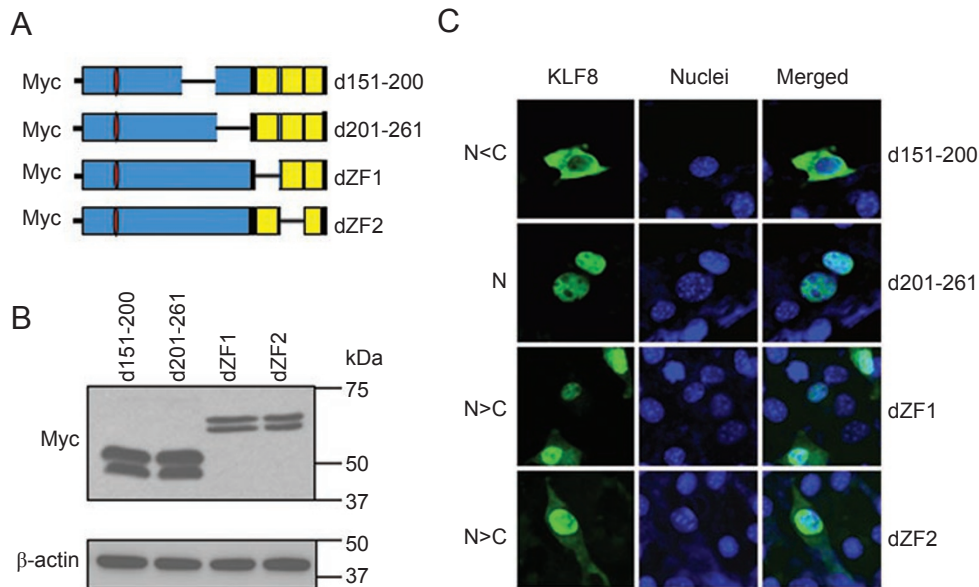


Figure 4 The aa 151-200 region and at least two ZFs in tandem are, but the aa 201-261 region is not, required for KLF8 nuclear localization. **(A)** Diagram of the KLF8 mutants in which the indicated regions were deleted by overlap PCR-directed mutagenesis. The protein expression **(B)** and localization **(C)** were analyzed similarly as in Figure 1. Patterns of localization are summarized on the left.

(Figure 4C, d151-200). To test another possibility that the region between aa 201 and the mNLS1 could contain additional residues required for KLF8 nuclear localization, we also generated an internal deletion of this region (Figure 4, d201-261) and found that this mutant protein remained exclusively nuclear (Figure 4C, d201-261). This result suggests that no NLS is present within the aa 201-261 region. To further distinguish the requirement of ZF1 from ZF2 for KLF8 nuclear localization, we individually deleted these two ZFs (Figure 4, dZF1 and dZF2). Surprisingly, removal of either of the ZFs only slightly reduced the nuclear presence of the proteins (Figure 4C, dZF1 and dZF2). This finding suggests that it is the simultaneous presence of two tandem ZFs immediately adjacent to the regulatory domain rather than the presence of either the ZF1 or ZF2 that is critical for KLF8 nuclear localization.

The serine 165 and lysine 171 residues within the aa 151-200 region are critical for the nuclear localization of KLF8

To identify critical residues within the aa 151-200 region, we first made a series of small deletions from within this region (Figure 5) and tested nuclear localization. We found that deletion of aa 161-170 or 171-180 region caused substantial cytoplasmic localization of the proteins (Figure 5C, d161-170 and d170-180), whereas deletion of the remaining small segments within the aa 151-

200 region had no effect on nuclear localization (Figure 5C, d151-160, d181-190 and d191-200). To further narrow down the responsible residues, we mutated the serine, threonine or lysine residues within this region (Figure 6), since these residues were potential targets for post-translational modifications or interaction with importins that would contribute to nuclear transport [9, 10, 23]. We tested cellular localization of the mutants and found that mutation of serine 165 to an alanine (S165A) or lysine 171 to either a glutamic acid (K171E) or arginine (K171R) caused a dramatic reduction of the proteins in the nucleus, whereas the nuclear localization of the other mutants did not change (Figure 6C). Interestingly, both of these two residues fall into the 161-180 core in the aa 151-200 region as shown in Figure 5. Importantly, both of the residues are completely conserved across the species (Figure 6D). These results suggest that post-translational modification of and/or importin binding to these residues may play an important role in KLF8 nuclear localization.

The C-terminal ZF regions are critical for KLF8 interaction with importin- β

Transcription factors interact with the importin proteins through their NLS(s) and are then shuttled into the nucleus. To test if the S165 and/or the K171 in the aa 151-200 region and/or the ZF region are important for KLF8 interaction with importin- β , indicated Myc-tagged

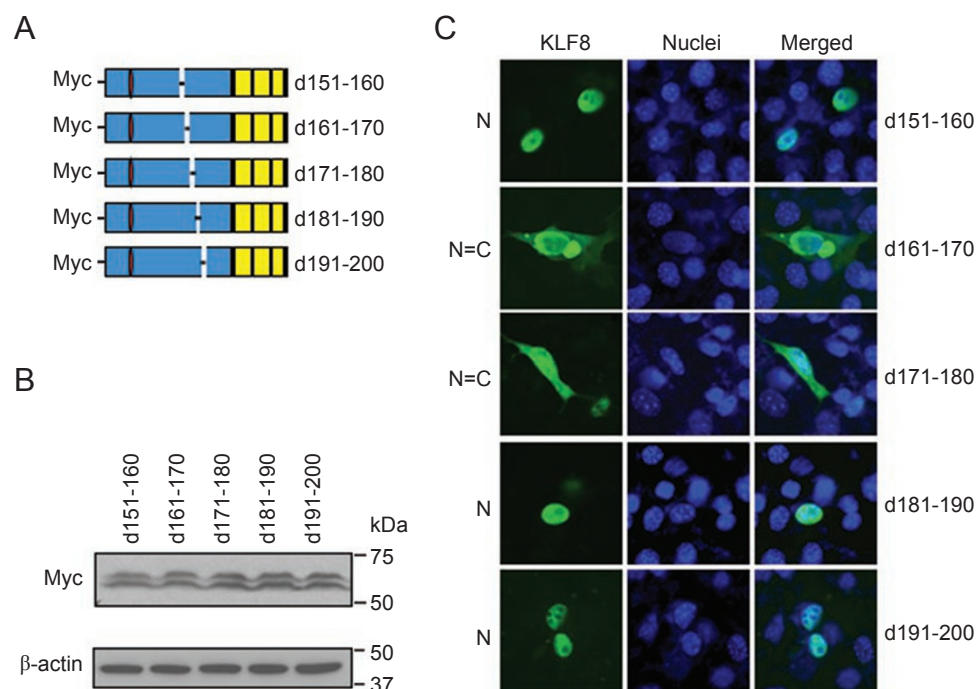


Figure 5 The aa 161-180 region is the minimal region required for KLF8 nuclear localization. **(A)** Diagram of the KLF8 mutants with small deletions within the aa 151-200 region as indicated. The protein expression **(B)** and localization **(C)** were analyzed similarly as in Figure 1. Patterns of localization are summarized on the left.

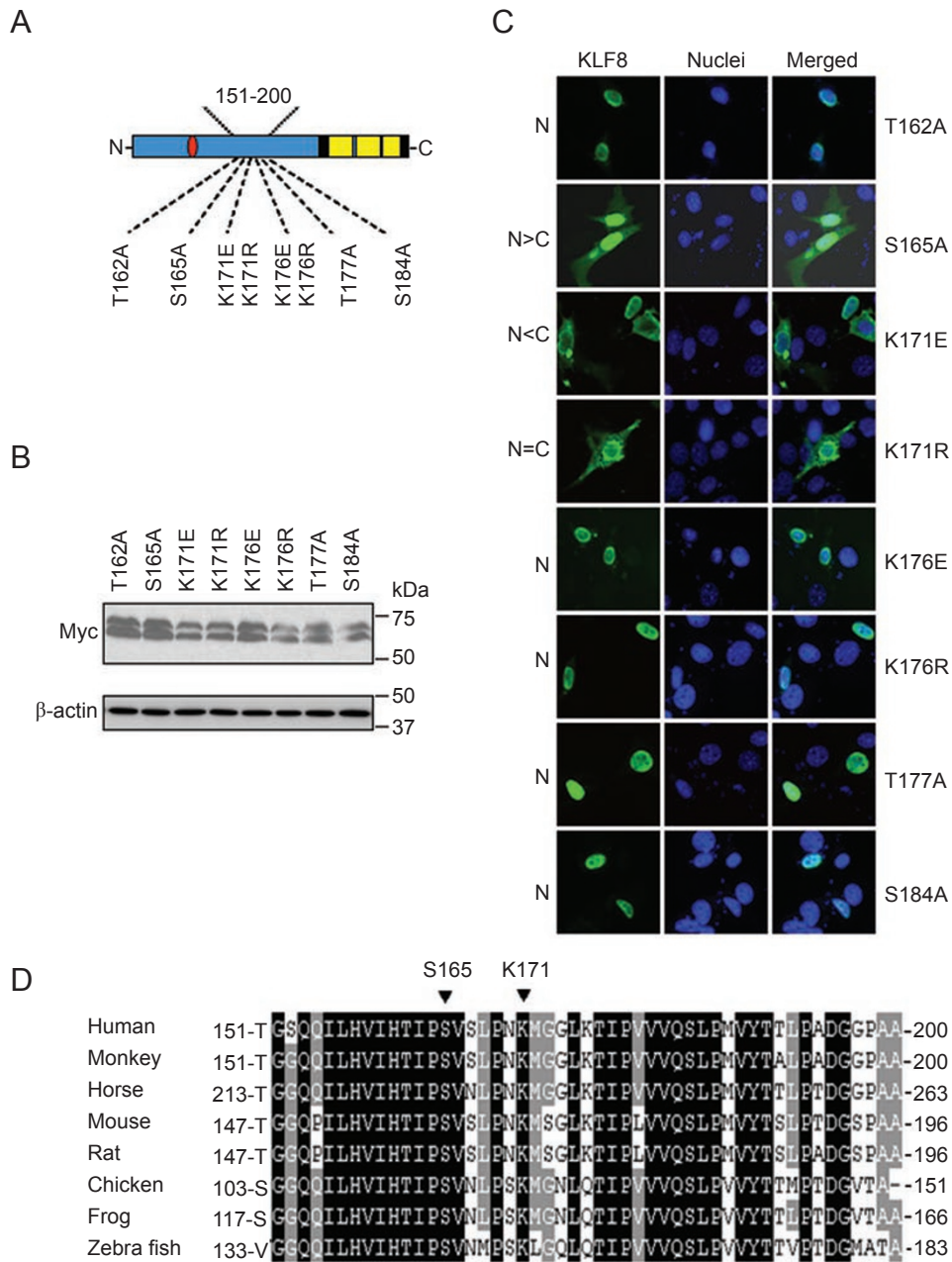


Figure 6 The serine 165 (S165) and lysine 171 (K171) residues within the aa 151-200 region are critical for KLF8 nuclear localization. **(A)** Diagram of the KLF8 mutants with point mutation of the serines (S), threonines (T) or lysines (K) to alanines (A), glutamic acids (E) or arginines (R) within the aa 151-200 region as indicated. The protein expression **(B)** and localization **(C)** were analyzed similarly as in Figure 1. Patterns of localization are summarized on the left. **(D)** Conservation of the S165 and K171 of KLF8 among the species.

KLF8 proteins were transiently expressed in 293 cells and co-IPed with importin-β (Figure 7A and 7B). Clearly, KLF8 (WT) interacted with importin-β, whereas the negative control IgG did not. This interaction was completely abolished when the ZF region was deleted (dC82 and dN/dC), whereas mutation of S165 or K171 did not affect the association. These results suggest that the C-terminal ZF region is critical for KLF8 interaction with

importin-β during its nuclear translocation, and the S165 and K171 residues in the aa 151-200 region may use a different mechanism to regulate KLF8 nuclear localization. Interestingly, a search for phosphorylation sites in KLF8 using software such as NetPhosK identified the S165 as a potential PKC phosphorylation site. This raises a possibility that PKC-mediated phosphorylation at S165 could play a role in KLF8 nuclear translocation. To test

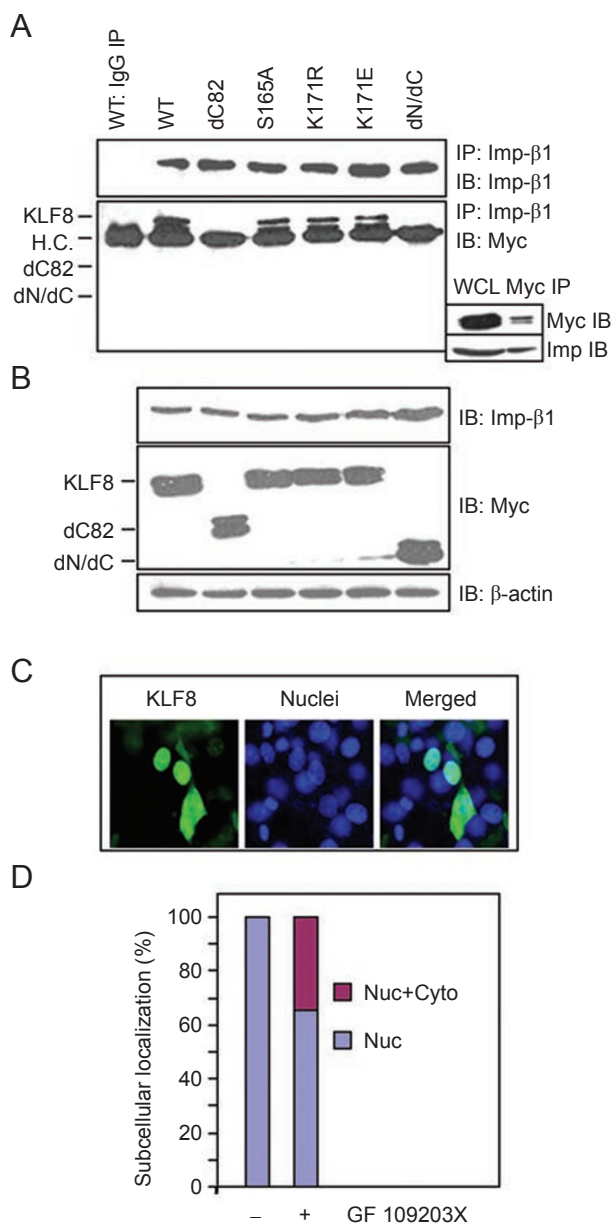


Figure 7 The ZF regions are essential for KLF8 binding to importin-β1 and PKC is required for fully nuclear presence of KLF8. **(A and B)** Myc-tagged KLF8 and mutants were transiently expressed and co-IPed with importin-β1 antibody and blotted with anti-importin-β1 or anti-Myc, the attached to A shows an inverse Co-IP for Wt KLF8 **(A)**. Whole cell lysates were blotted similarly **(B)**. Positions of the KLF8 proteins are marked on the left side of the blots (H.C., IgG heavy chain). *Note*, dN/dC is a double deletion mutant lacking both the aa 151-200 and C82 ZF regions. For some unknown reasons, this mutant expresses to a level that is ~40% compared to the other KLF8 proteins, thus 2.5 times of the whole cell lysate for this mutant was used for the IP. **(C and D)**, NIH3T3 cells expressing the Myc-tagged KLF8 were treated with 3 mM GF 109203X for 3 h prior to anti-Myc staining **(C)**. KLF8 localization from 100 transfected cells for both the PKC inhibitor treated and untreated groups was summarized **(D)**.

this possibility, we blocked PKC activity using the pan-PKC inhibitor GF 109203X in the cells expressing KLF8 (Figure 7C and 7D) and found that in ~35% of the cells KLF8 failed to exclusively localize in the nuclei. This result suggests that PKC regulation at S165 may contribute to the regulation of KLF8 nuclear localization, albeit not by promoting its interaction with importin-β.

Both the N-terminal aa 151-200 and C-terminal ZF regions are essential for KLF8 nuclear and cellular functions

We next examined the effect of KLF8 nuclear localization on the transcriptional activity of KLF8 toward the cyclin

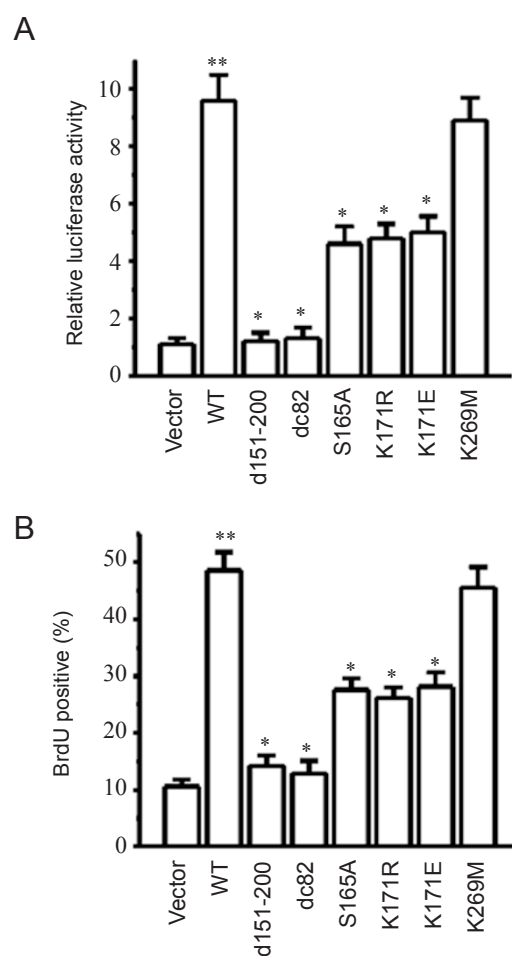


Figure 8 KLF8 localization in the nucleus is essential for its regulation of transcriptional targets and DNA synthesis. **(A)** NIH3T3 cells were co-transfected with KLF8 or its mutant constructs along with cyclin D1 promoter reporter, and luciferase activities were measured after 16 h. **(B)** Myc-KLF8 (WT) or its mutants as indicated were transiently expressed in the NIH3T3 cells, after 24 h of serum deprivation, and cells were stimulated with serum containing BrdU for 14 h before they were prepared for immunofluorescent staining with antibodies against HA and BrdU. 50 HA-positive cells were counted for BrdU-positive cells. Shown was the mean + S.E. of at least three independent experiments. ** $P < 0.01$ compared to Vector and * $P < 0.01$ compared to WT.

D1 promoter. Using promoter luciferase reporter assays, we found that the wild-type KLF8 enhanced the cyclin D1 promoter activity by ~10-fold (Figure 8A, compare WT and Vector). In contrast, when the aa 151-200 or ZF region was missing, the KLF8 mutants totally failed to activate the cyclin D1 promoter (Figure 8A, compare d151-200 or dC82 with WT and Vector). The S165A, K171R or K171E KLF8 mutants only maintained approximately 50% of the activity and the K269M KLF8 mutant showed no change in its transactivation function (Figure 8A, compare S165A, K171R, K171E or K269M with WT).

We then tested if the aa 151-200 and ZF regions are required for the cellular functions of KLF8. We previously showed that KLF8 promoted the G1 phase progression of the cell cycle [2, 6, 7]. BrdU incorporation assays indicated that the wild-type KLF8 increased the DNA synthesis rate by fivefold as expected (Figure 8B, compare WT with Vector); in contrast, when the aa 151-200 or ZF region was missing, the KLF8 mutants totally failed to promote DNA synthesis (Figure 8B, compare d151-200 or dC82 with WT and Vector). The S165A, K171R or K171E KLF8 mutants only maintained approximately 50% of the promoting activity and the K269M KLF8 mutant again did not show any change in its function (Figure 8B, compare S165A, K171R, K171E or K269M with WT).

Taken together, these results suggest that aa 151-200 and ZF regions that control the nuclear localization of KLF8 are essential for KLF8 regulation of its transcriptional target genes as well as its cellular functions and that the classical-type mNLS upstream to the ZF does not play a critical role.

Discussion

In this paper, we determined the previously unidentified NLSs responsible for the nuclear localization of KLF8. Our surprising findings include that (1) KLF8 contains two functional non-classical type of NLSs located within the aa 151-200 region of the N-terminal regulatory area (NLS1) and the first two ZFs at the C-terminus (NLS2); (2) simultaneous presence of both the NLSs is essential for efficient nuclear localization of KLF8; (3) the NLS2 plays a critical role in mediating KLF8 interaction with importin- β ; (4) in addition to mediating nuclear localization, the NLSs are also essential for KLF8 regulation of target gene expression and cell cycle progression; (5) the NLS1 is novel and unique to KLF8 and its function may be regulated through modification at S165 (possibly by PKC) and at K171; and (6) the classical mNLSs do not play a critical role in the nuclear localization or

functions of KLF8.

It appears that KLF8 needs both of the NLSs to work together for its maximal nuclear localization. Although we do not know at present the underlying molecular mechanisms, enhanced nuclear import, inhibited nuclear export and/or nuclear retention mediated by the collaboration of the two NLSs could be involved.

The significance of our identification of the novel NLS1 of KLF8 is twofold. First, the NLS1 is unique to KLF8, since the aa sequence in general and the core residues S165 and K171 are very well conserved among all the KLF8 orthologs (see Figure 6D), but not to any other KLF members, suggesting that the nuclear localization of KLF8 is more strictly regulated through the NLS1 compared to other KLFs. Second, it is likely that the NLS1 is regulated by post-translational modifications including PKC regulation at S165 and other modifications such as ubiquitylation or acetylation at K171.

Phosphorylation is the best understood post-translational mechanism that regulates the nuclear localization of large amounts of nuclear proteins [8, 23]. Our data that S165 (and PKC) is required for KLF8 nuclear import but not for KLF8-importin- β interaction suggest an interesting possibility that PKC regulation at S165 may be required for unmasking the NLS1 from a cytoplasmic retention factor, for the NLS1 interaction with an importin protein other than importin- β , or for masking the intramolecular NES. Whether PKC family proteins can directly phosphorylate the S165 site remains unclear and requires additional future experiments to determine. The other potential phosphorylation sites tested, including T162, T177 and S184, although also conserved in KLF8 orthologs, did not play a significant role in KLF8 nuclear localization. However, we cannot rule out other potential roles these residues may play for KLF8.

The basic aa lysine (K) serves as a core residue in all types of NLSs. Since mutation of K171 to another basic aa arginine (R) resulted in cytoplasmic localization equally as the K171E (acidic glutamic acid) did (see Figure 6C), the basic or positively charged nature of the K171 residue does not seem to be functionally relevant. Lysines also serve as modification target sites by a variety of mechanisms such as ubiquitylation, sumoylation and acetylation. It is clear that this lysine is not a sumoylation target site, since we have recently identified K67 as the sole sumoylation site in KLF8 [7]. In addition to mediating proteasome-dependent protein degradation, ubiquitylation also regulates protein localization in the cells [24]. Similarly, acetylation at a lysine within an NLS has been shown to facilitate the NLS-importin binding and thus the nuclear import of proteins such as SRY [34]. It will be interesting to determine whether K171 is

the target of ubiquitylation or acetylation and whether such a modification(s) is critical to the nuclear import of KLF8.

The point mutants (S165A, K171E and K171R) and the small deletion mutants (d161-170 and d171-180) are less cytoplasmic than the dN200 and d151-200 mutants are (see Figure 3, 5 and 6), suggesting that these two residues (S165 and K171) play a partially independent role. It is worth investigating how these two residues and their modifications cooperate to regulate the nuclear localization of KLF8.

Our finding that the ZF region is also critical for the nuclear localization of KLF8 is consistent with previous reports that the ZFs of many ZF-containing transcription factors play an additional role as NLSs [11]. Among these transcription factors are the C₂H₂-type ZF transcription factors including KLF1 [27, 28], KLF4 [29] and Zif268 [35]. Our results suggest that the presence of two consecutive ZFs is required for the nuclear localization of KLF8, since removal of more than one and a half ZFs inhibited the nuclear localization of KLF8 and removal of any of the ZFs individually did not affect the nuclear localization of KLF8. This property is novel to KLF8, as all three ZFs are required for the nuclear localization of KLF1 [27, 28], although the whole ZF region is well conserved among all of the KLFs [25, 26]. It is also true for Zif268 that all the three ZFs are required for its nuclear localization [35]. Within the two required ZFs, however, neither the zinc-binding motifs nor the DNA-contact motifs [32, 33] are essential, suggesting that the ZF-mediated nuclear localization of KLF8 does not require zinc-dependent DNA binding, which is consistent with the case of KLF1 [27], but is in contrast with the case of Zif268 whose nuclear localization depends on the Zn²⁺-binding [35]. The ZF region of KLF8 plays an NLS role, although it cannot effectively target KLF8 to the nucleus in the absence of the NLS1 at the N-terminus. Indeed, the ZFs have been shown to mediate the interaction of KLF1 with importin- α and importin- β , although whether this interaction requires the zinc-binding motifs or the DNA-contact motifs has not been examined [28]. Nevertheless, our results suggest that the first two ZFs of KLF8 play a role in both the nuclear localization and DNA binding, whereas the major role for the last ZF may be DNA binding.

We failed to detect the requirement of the classical mNLSs for the nuclear localization and functions of KLF8, although a similar mNLS has been demonstrated to be important in the nuclear localization of KLF1 as well as KLF4 [27-29]. Interestingly, a close look at the mNLSs reveals that the mNLS sequences in KLF8 are well conserved in KLF3 and KLF12, whereas the mNLS

identified in KLF1 and KLF4 (also conserved in KLF2) is somewhat distinct. We therefore suggest that the lack of function of the mNLSs in KLF8 may also apply to KLF3 and KLF12 as well.

In summary, this study identified two likely NLSs for KLF8. Both the NLSs could be regulated by intracellular signaling-mediated post-translational modification of KLF8. Expression at the right level, at the right time and at the right subcellular location is critical to key regulating proteins, particularly nuclear oncogenes and tumor suppressors. Given the emerging important role of KLF8 in cancer progression, KLF8 may join the group of short-lived oncogenic transcription factors such as c-Myc, HIF-1 and Snail whose tightly regulated subcellular localization in addition to expression is critical in the control of cellular homeostasis.

Materials and Methods

Plasmid DNA Construction

Mammalian expression plasmids pKH3-KLF8 and pHAN have been described previously [2, 7, 36]. pHAN-KLF8 was generated by transferring the KLF8 cDNA insert between Sma I and Eco RI sites from the pKH3-KLF8 into the same sites in the pHAN vector. The C-terminal and N-terminal deletion mutants were generated by PCR-directed truncation. The point mutations and internal deletions were carried out using site-directed mutagenesis by overlapping PCR. These mutants were inserted in the pHAN vector in order to encode Myc-tagged proteins. The reverse primers (5' to 3') used to generate the KLF8 C-terminal deletion mutants are as follows. dC8: cgg aat tct aga ggg aca ggt ggt cag; dC26: cgg aat tct aga gct tga tgc ctg; dC38: cgg aat tct aga gct cat ctg agc gag c; dC56: cgg aat tct aga cat gta aga tct gct ggc; dC82: cgg aat tct aga cac att ggt gaa tcc gtc; dC96: cgg aat tct aga gcg act ctc cct cca ttt ggg c; dC150: cgg aat tct aga cat ctc ctc caa tga g; dC200: cgg aat tct aga tga cat gta tct gct ggc; dC250: cgg aat tct aga gac gta ttg gag c; dC300: cgg aat tct aga ggt tct cca tgg gg. A common master forward primer to the pHAN vector (pKH3-F primer: cccaagcttctcaggtcg) was used to pair with the above primers. The forward primers (5' to 3') used to generate the KLF8 N-terminal deletion mutants are as follows. dN50: atc ccc cgg gac tcc tgg atg cca acc cc; dN100: atc ccc cgg gtg cta gca tgc tac aag c; dN152: atc ccc cgg gta gcc agc aga tct tac; dN200: atc ccc cgg gca tta cag tcc cac tc; dN248: atc ccc cgg gac tac agc aag aac cag cag; ZF (dN273): cgg gat ccc cac caa tgt gac ttg cag g. A master reverse primer to the pHAN vector (pKH3-R primer: gga caa acc aca act aga atg cag) was used to pair with the above primers. The primer pairs (forward/reverse) used to generate the point and internal deletion mutants are as follows. K269M: gct tga ctt gat gag aag acg g/ccg tct tct cat caa gtc aag c; F1dc: tgt aca cct ata gcg ctc tcc tga aag ttc acc gca g/tct gcg gtg aac ttt cag gag agc gct tat ggt gta cac; H294/8L: acc tga aag ctc tcc gca gaa tcc tta cag gag ag/ttc tct cct gta agg att ctg cgg aga gct ttc agg; ZF2dc: tgg aaa ttt gct cac tca ggt ggg ctc act cac cat ttc cgc/tgc gga aat ggt gag tga gcc cac ctg agt gag caa att tcc; H342/8L: tca ctc gcc ttt tcc gca agc tca cag gca tc/atg cct gtg agc ttg cgg aaa agg cga gtg ag; T162A: tgt cat tca cgc tat ccc ctc agt cag tct gcc/tgg cag act gac tga ggg gat

agc gtg aat gac; T165A: atc atc ccc gca gtc agt ctg cc/tgg cag act gac tgc ggg gat agt g; K171R: tgc caa ata gga tgg gtc gcc/agg cca ccc atc cta ttt ggc ag; K171E: tgc caa atg aga tgg gtc gcc/agg cca ccc atc tca ttt ggc ag; K176R: atg ggt ggc ctg agg acc atc cca g/tgg gat ggt cct cag gcc acc c; 176E: atg ggt ggc ctg gag acc atc cca g/tgg gat ggt ctc cag gcc acc c; T177A: tgg cct gaa ggc cat ccc agt gg/act ggg atg gcc ttc agg cc; T184A: tag tgc agg ctg tgc cca tg/atg ggc aga gcc tgc act acc; d151-200: tca gag cat tac agt ccc act c/act gta atg ctc tga gag gag g; d201-261: tgc agc cca ggg agg aga gtc g/tcc tcc ctg ggc tgc agg gcc ccc; dZF1: tca cca aac agg aga gaa gcc/tct cct gtt tgg tga ata agt c; dZF2: tta taa aac agg cat caa gcc/tgc ctg ttt tat aag gct tct c; d151-160: tca gag cca cac tat ccc ctg/tag tgt ggc tct gag agg agg; d161-170: tgt cat taa gat ggg tgg cct g/acc cat ctt aat gac atg taa gat c; d171-180: tgc caa atg tag tgc agt ctc tg/tgc act aca ttt ggc aga ctg ac; d181-190: tcc cag tga ctt tgc ctg cag atg/agg caa agt cac tgg gat ggt ctt c; d191-200: tgt ata cta tta cag tcc cac tc/act gta ata gta tac acc atg gg. All the constructs were verified by DNA sequencing.

Cell culture and transient transfection

293T or NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum or calf serum, respectively. For the transfection, cells were seeded on tissue culture plates or coverslips at the density to give about 80% confluent monolayer after overnight culture. Transfections of the plasmid DNAs were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In some experiments, the transfected cells were treated with BrdU or GF109203X, a specific inhibitor of PKC family proteins.

Western blotting and co-immunoprecipitation (Co-IP)

Western blotting was performed essentially as described previously [2]. 293T or NIH3T3 cells were transfected with the KLF8 constructs for 16 to 24 h, washed with ice-cold PBS and lysed with 1× SDS sample buffer. The lysates were used for blotting. The primary antibody used was mouse anti-Myc monoclonal antibody (9E10, 1:2 000, Santa Cruz, CA, USA) and the secondary antibody was HRP-donkey-anti-mouse antibody (1:5 000, Jackson ImmunoResearch Laboratory, West Grove, PA, USA). For Co-IP, 500 µg of whole cell lysate was pre-cleared with 5 µl of Protein A/G agarose beads (Santa Cruz) for 30 min at 4°C with rotation. Importin-β1 was IPed from the lysate using 2.0 µg of antibody (Karyopherin-β, BD Transduction Laboratories) or mouse IgG for 2 h and 25 µl of Protein A/G beads overnight at 4°C. The beads were then washed, boiled in sample buffer and subject to SDS-PAGE gel electrophoresis and western blotting with either the Myc or importin antibody.

Fluorescent microscopy

NIH3T3 cells were transfected with the KLF8 constructs for 24 h and processed for indirect immunofluorescence staining as previously described [37]. The primary antibody used was the 9E10 anti-Myc antibody (1:200). The secondary antibodies used were FITC-conjugated goat-anti mouse antibody (1:200, Jackson ImmunoResearch Laboratory, West Grove, PA). The nuclei were stained with Hoechst 33258 dye. Images were acquired with an Olympus BMX-60 microscope equipped with a cooled charge-coupled device sensi-camera (Cooke, Auburn Hills, MI, USA) and Slidebook software (Intelligent Imaging Innovations, Denver,

CO, USA). At least fifty positively transfected cells for each of the KLF8 plasmids from 10-15 independent fields were examined for each of multiple experiments. Exposure conditions were set unchanged within each of the experiments.

Promoter reporter assays

Luciferase reporter assays were performed essentially as described previously [7]. Briefly, NIH3T3 cells were co-transfected with KLF8 or its mutants along with cyclin D1 promoter reporter construct. Control reporter expressing *Renilla luciferase* was used to normalize transfection efficiencies. Luciferase activity was determined using the dual luciferase reporter assay system (Promega) and 20/20n luminometer (Turner BioSystems).

5-Bromodeoxyuridine (BrdUrd) incorporation assays

BrdUrd incorporation assays were essentially as described previously [37]. NIH3T3 cells were transfected with KLF8 or its mutants. After serum deprivation and stimulation, BrdU incorporation rate was analyzed for the transfected cells.

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