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Determination of Nuclear Localization Signal Sequences for Krüppel-Like Factor 8

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Abstract

Transcription factor proteins function in the nucleus to regulate gene expression. Many transcription factors are critical regulators of tumor progression. Conversely, many oncogenic and tumor suppressor proteins are transcription factors or other types of nuclear proteins. Because of their critical physiological and pathological roles, these tumor regulators are tightly regulated not only in the protein expression but also in their subcellular localization. This chapter is focused on experimental strategies and method details for the identification and characterization of nuclear localization signal sequences for nuclear proteins using the Krüppel-like transcription factor 8 as an example.

Keywords

Krüppel-like factor; KLF8; Nuclear localization; Site-directed mutagenesis; Overlapping PCR; Transfection; Western blotting; Fluorescent microscopy

1. Introduction

KLF8 belongs to the Krüppel-like C₂H₂ zinc-finger transcription factor family proteins. Several KLF family members, including KLF4, KLF5, and KLF6, have been identified as either oncogenes or tumor suppressors (1–3). KLF8 is a relatively new member of this family and is emerging as a critical regulator of cancer progression. KLF8 was initially identified as a transcriptional repressor (4). Later we found KLF8 to be a dual transcriptional factor that can both repress and activate transcription of target genes including cyclin D1, KLF4, and E-cadherin (5–7). We and others identified KLF8 as a downstream effector of FAK (7) and a crucial regulator of oncogenic transformation, epithelial to mesenchymal transition and tumor cell invasion (7–11). Nuclear oncogenic or tumor suppressor proteins are tightly regulated in nuclear localization in addition to protein expression. Nuclear localization signal (NLS) sequences have been identified for a few KLFs (12–14). Importantly, we have now identified the NLS sequences for KLF8. While the results of this study are submitted to elsewhere, we describe here in details about the experimental design strategies and methods which hopefully apply to nuclear localization studies for other nuclear proteins.

2. Materials

2.1. PCR and DNA Agarose Gel Electrophoresis (DAGE)

1. Deep Vent DNA polymerase (New England Biolabs).
2. T4 DNA ligase (New England Biolabs).
3. dNTP (New England Biolabs).
4. Ethidium bromide (Bio-Rad).
5. Certified Molecular Biology Agarose (Bio-Rad).
6. Aurum Plasmid Mini Kit (Bio-Rad).
7. Powerpac Basic Power Supply (Bio-Rad).
8. Powerpac HC Power Supply (Bio-Rad).
9. Qiaquick Gel Extraction Kit (Qiagen).
10. QIAprep Spin Mini-Prep Kit (Qiagen).
11. iCycler Thermal Cycler (Bio-Rad).
12. Mini Sub-Cell GT System (Bio-Rad).
13. Kodak GL440 Imaging Documentation System (Kodak).
14. 1 kb Plus DNA Ladder (Invitrogen).
15. Ampicillin (Fisher).
16. Microcentrifuge (Eppendorf).

2.2. Cell Culture and Transfection

1. Lipofectamine 2000 Transfection Reagent (Invitrogen).
2. DMEM (Invitrogen).
3. Falcon 12-well tissue culture plate (Fisher).

2.3. SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

1. 40% Acrylamide:Bisacrylamide Solution (Bio-Rad).
2. TEMED (Bio-Rad).
3. Precision Plus Standard Dual Color protein molecular weight markers (Bio-Rad).
4. Mini-PROTEAN 3 CELL (Bio-Rad).
5. HRP-Donkey Anti-mouse IgG (H + L) (Jackson Immuno-Research Lab).
6. HRP-Donkey Anti-rabbit IgG (H + L) (Jackson Immuno-Research Lab).
7. Normal goat serum (Jackson ImmunoResearch Lab).
8. Protran BA83 Nitrocellulose Membrane (Whatman).

9. Nestle Carnation Instant Nonfat Dry Milk (Nestle USA).
10. Microcentrifuge (Eppendorf).
11. Isotemp Heating Block (Fisher).
12. Pierce* Enhanced Chemiluminescent (ECL) Substrate (Fisher).
13. Automatic X-ray Film Processor (Hisapdebu JP33).
14. Fuji Film LAS-3000 Imager (Fuji Film Global).

2.4. Immuno-fluorescent Staining and Microscopy

1. FITC-Donkey Anti-Mouse IgG (H + L) (Jackson Immuno-Research Lab).
2. FITC-Donkey Anti-rabbit IgG (H + L) (Jackson Immuno-Research Lab).
3. TexasRed-Donkey Anti-Mouse IgG (H + L) (Jackson Immuno-Research Lab).
4. TexasRed-Donkey Anti-Rabbit IgG (H + L) (Jackson Immuno-Research Lab).
5. SlowFad Gold Antifade Reagent (Invitrogen).
6. Microscope Slides (Fisher).
7. Microscope Cover Glass (coverslip) (Fisher).
8. Microscope (Olympus BMX-60 microscope equipped with a cooled charge-coupled device (CCD) sensi-camera (Cooke, Auburn Hills, MI) and Slidebook software (Intelligent Imaging Innovations, Denver, CO)).

3. Methods

3.1. Overall Research Flow (Fig. 1)

As shown in Fig. 1, the general procedure involves the identification of suspected NLS sequences in KLF8 and determination of their requirement and sufficiency for the nuclear localization of KLF8 by disrupting the sequences and fusing the sequences to GFP, respectively, followed by transfection and fluorescent microscopy.

3.2. NLS Sequence Search (Fig. 2)

There are in general two strategies to search for suspected NLS sequences: computer programs and amino acid sequence homology search (see Note 1). There are a number of free internet computer programs that are widely used for this purpose. Essentially, these programs perform the automatic search for potential NLS sequences, particularly the classical type NLSs including monopartite NLS (mNLS, a single stretch of basic 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) (15). We used the PSORT program (<http://psort.ims.u-tokyo.ac.jp/form2.html>) to predict potential NLSs in

¹Although the classical NLS sequences dominate the nuclear localization of nuclear proteins, nonclassical NLS sequences also play an important role in regulating many nuclear proteins and these types of NLS sequences may not be identified by the computer programs or sequence homology analysis. Therefore, step-wise terminal truncation in combination of internal deletion strategy is necessary to identify nonclassical, in most cases novel, NLS sequences from the amino terminal region of KLF8 as one example (Fig. 2).

KLF8. With this program, all that needs to be done was to enter the amino acid sequence or access number of SWISS-PROT and then click the “Submit” button. The program would then immediately predict the probability of nuclear localization and the potential NLS sequences and positions. This program identified two mNLSs (269KRRR and 352HRRR or 353RRRH) in KLF8 flanking the zinc-finger region (see Fig. 2). Homology search predicted the zinc-finger region as suspected NLS sequence as well, given that previous studies demonstrated that the conserved zinc-finger regions (as well as the leftmost mNLS) are functional NLSs for both KLF1 (12, 13) and KLF4 (14).

3.3. PCR-Based Mutagenesis and DAGE to Disrupt the Predicted NLS Sequences (Fig. 3)

Disruption of the suspected NLS sequences can be achieved by site-directed mutagenesis using PCR to generate either point mutations (see Note 2) or deletion mutations. Because it is relatively easy to make terminal truncation mutants by a simple onestep PCR, no method details are discussed here. Overlapping PCR is one of the many convenient techniques used to generate point or internal deletion mutations. The main steps of the procedure are described below:

3.3.1. Mutant Primer Design—The pair of the mutant primers must match the cDNA template except for the nucleotides encoding the residues to be mutated or deleted. The nonmutated nucleotides in the primers should be at least 8-base longer at both ends. G/C content in the primers should be 50% or greater with a preferable 5-prime A/T and a 3-prime C/G placement. Master primers are usually 16–20-base long (see Note 3) and are immediately upstream (forward primer) or downstream (reverse primer) to the multiple cloning sites in the plasmid vector. Many companies can provide good quality service for primer synthesis and we usually use the services provided by Invitrogen.

3.3.2. Overlapping PCR—This procedure consists of two rounds of PCR. The first-round PCR uses plasmid cDNA as template and the forward master primer (Master-F) paired with the reverse mutant primer (Mutant-R) or the forward mutant primer (Mutant-F) paired with the reverse master primer (Master-R). The products are two fragments with the targeted site either mutated or deleted. The second-round PCR uses a mixture of these two fragments purified from gel (see below) as template (see Note 4) and the master primer pair. The PCR reaction in 50 µl (see Note 5) are prepared in the following order: 39.5 µl of nanopure water, 5 µl of 10× buffer, 1 µl of 10 mM dNTP, 1 µl of 50 mM MgCl₂, 1 µl of 1 Mg/µl primer-F, 1 µl of 1 Mg/µl primer-R, 1 µl of 0.2 Mg/µl template DNA, and 1 µl of DNA polymerase (see Note 6). The PCR program consists of 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 30 s, 55–65°C (determined by the primers used) for 45 s and 72°C for 1 min per kb in length of product, and 1 cycle of 72°C for 15 min.

²To disrupt the mNLS sequences, point mutation of K or R to A or M is usually performed to disrupt the basic feature of the key residues. To disrupt the zinc fingers, the zinc-binding residues and/or DNA contact residues (16, 17) are often point-mutated.

³Longer primers may be designed to meet the requirement for the G/C content or the 5c-A/T or 3c-G/C criteria.

⁴Up to a half of the total amount of the purified fragments can be included in the second PCR reaction.

⁵PCR reaction can be scaled up to 100 µl if necessary.

⁶Add DNA polymerase to the reaction mixture last.

3.4. DAGE and DNA Purification

3.4.1. Making and Running Agarose Gel

1. Prepare a small tray for agarose pouring. Make sure the tray is level. Insert comb.
2. Pour 50 ml of the agarose gel TAE solution pre-warmed in the 55°C water bath into a 50 ml centrifuge tube. Add 5 ml ethidium bromide (10 mg/ml) into the solution. Invert a few times to mix.
3. Pour the gel solution slowly into the tray (see Note 7). Wait for about 30 min at room temperature.
4. Pour about 220 ml of 1× TAE running buffer (for a 500 ml stock solution, combine 93.05 g EDTA-Na₂ and 400 ml water and adjust the pH to 8 with NaOH, then top up with water to a final volume of 500 ml) into the running chamber.
5. When the gel is solid, release by untwisting the gel from the big white chamber slowly, take comb out by slowly pulling up the comb up while pushing the glass tray down.
6. Place the glass tray into the running chamber. The side with the wells should be facing the negative (black) side.
7. Add 22 ml of the ethidium bromide stock to the running buffer on the positive (red) side of the running chamber.
8. Take 10 ml of 6× loading buffer and aliquot it into eight dots on a piece of parafilm (1 ml/dot).
9. Write down in notebook the positions of where each DNA sample is going to be loaded.
10. Load the gel (see Note 8) starting with the marker. For each DNA sample, mix 5 ml of DNA solution with the dye dot by slowly pipetting up and down a couple of times.
11. Put the cover with the electrodes red-to-red and black-to-black.
12. Run at 120 V for 30 min (see Note 9). When done, carefully remove the gel with the tray.
13. Take a picture of the gel using the Kodak Image Documentation System.

3.4.2. Purification of DNA Fragments from DNA Agarose Gels

1. Place the gel on top of the long-beam UV light box with plastic shield in the chamber of the Kodak Imaging Documentation System.
2. Cut desired DNA bands from gel (see Note 10).

⁷Pour slowly to avoid bubbles but quickly enough to avoid gel polymerization in the tube. Do not move the gel until it is solid.

⁸Load the sample all the way down to the bottom of the well. Try to be stable. Put the pipette tip into the well closely to, but do not puncture, the bottom.

⁹Check if the gel starts to run by looking at the electrophoresis-generated bubbles from the wires in the tank.

3. Transfer the gel slices into a 1.5-ml Eppendorf tube by using pipette tips.
4. Spin down the gel slice to the bottom of the tube for 15 s for easy estimation of the gel volume. Add 3 volumes of Buffer QG (see Note 11).
5. Put the Epp tube(s) in the 55°C water bath for 3–10 min until the gel is melted.
6. Add 1 volume of isopropanol to the solution. After pipetting up and down a few times to mix the solution, transfer 700 μ l of the solution to a Qiaquick spin column. Spin at 13,000 rpm for 30 s (see Note 12). Discard flow-through.
7. Add 0.5 ml Buffer QG to each spin column. Spin for 30 s at 13,000 rpm. Discard flow-through.
8. Add 0.75 ml Buffer PE. Spin for 30 s at 13,000 rpm. Discard flow-through.
9. Spin at 13 krpm for 1 min without adding any solution. Discard flow-through.
10. Transfer the top part of the spin column into a 1.5 ml Eppendorf tube.
11. Add 35 μ l sterile Buffer EB pre-warmed in the 55°C water bath. Let the solution sit at room temperature for 1 min. Spin down at 13,000 rpm for 1 min. Repeat once to collect a final volume of 70 μ l of DNA solution.
12. Run an agarose gel with a little (1–5 μ l) of the DNA to confirm the recovery.
13. After determining the DNA concentrations by spectrometry, keep the remaining at –20°C for future use.

3.4.3. Restriction Digestion

1. Prepare 20 μ l of reaction mixture in an Eppendorf tube containing 0.5–2 μ g of DNA, 2 μ l of 10 $\times$ Buffer, 0.2 μ l of 100 $\times$ BSA, 0.5 μ l of enzyme, and nanopure water that adds up to 20 μ l.
2. Mix well by stirring with the pipette tip. Centrifuge briefly at 13,000 rpm to spin down all the contents in the tube.
3. Start reaction at the temperature and duration specific for the enzyme (see Note 13).
4. Add 4 μ l of 6 $\times$ loading dye to the reacted mixture to stop the reaction. Run an agarose gel to confirm the presence and size of the digested DNA.

¹⁰Be sure to cut the gel into little pieces so that it is easier to remove for purification. A little piece on the gel should be left for further reference by imaging. Make sure not to touch neighbor bands in order to avoid cross contamination. Thoroughly wash and clean the blade between different band cuttings.

¹¹The QG Buffer can be added a little more than 3 volumes to get both tubes to the same level so that they are balanced for centrifugation.

¹²If gel slices from the same DNA band had to be transferred into more than one Eppendorf tubes in step 1, combine them now into the same column by repeating the centrifugation.

¹³Most restriction enzymes digest DNA well at 37°C for 2 h.

3.4.4. Ligation

1. Prepare 20 µl of reaction mixture in a Eppendorf tube containing 1:1 molecular ratio of gel purified vector and insert DNA fragments (up to 2 µg in total), 5 µl of 5× Buffer and nanopure water. Use the vector mixture alone as a control.
2. Mix well by stirring with the pipette tip. Centrifuge briefly at 13,000 rpm to spin down all the contents in the tube.
3. Put the tube in 16°C water bath (in 4°C fridge or room) and let the reaction take place overnight.

3.4.5. Transformation

1. Set the temperature on the heating block (Isotemp) to 42°C (see Note 14).
2. Take new Eppendorf tube and place on ice.
3. Thaw DH5A competent E. coli taken from the –80°C fridge on ice for a few minutes (see Note 15).
4. Pipette 100 µl DH5A into new Eppendorf tubes containing the 20 µl of ligation mixture pre-cooled on ice. Mix well by stirring with the pipette tip. Leave on ice for 20 min.
5. Heat the tubes in 42°C for 60 s. Immediately put on ice.
6. Add 900 µl of 4°C-stored sterile liquid broth (LB) (see Note 16).
7. Incubate in a 37°C shaker for 1 h.
8. In the meantime, prepare for streaking. Take out 2 clean LB + ampicillin (see Note 17) agar plates and let them dry under the fume hood for ~30 min.
9. Spin down the transformed E. coli at 5,000 rpm for 2 min. Take out ~900 µl of supernatant. Resuspend the bacteria in the remaining supernatant by tapping the tube.
10. Transfer the bacteria suspension with pipette and streak it evenly to the LB + Amp agar plate.
11. Incubate at 37°C overnight with the plates inverted. During the time the bacterial colonies containing the plasmid DNA should form on the agar surface.

3.4.6. Plasmid DNA Preparation

1. Pick up a single colony of transformed E. coli by scraping the single spot with an autoclaved culture tip.

¹⁴Never trust the mechanical or electronic temperature indicator. Be sure to check for the accurate temperature using a mercury or ethanol thermometer.

¹⁵Never return thawed competent E. coli back into freezer because the thawing/freezing operation dramatically reduces transformation efficiency.

¹⁶Make sure sterile measures are taken during transformation experiments to avoid cross contamination by other bacteria and contamination of the culture medium.

¹⁷Depending on vector's feature, an antibiotic other than ampicillin (e.g., tetracycline) may be used.

2. Drop the colony along with the tip into a 15 ml bacteria culture tube containing 2 ml of LB with 50 Mg/ml of ampicillin.
3. Incubate in a 37°C shaker overnight.
4. Place 1.5 ml of the bacteria growth in a 1.5 ml Eppendorf tube. Centrifuge for 1 min at 13 krpm. Decant supernatant.
5. Add 250 μ l of resuspension solution and vortex.
6. Add 250 μ l of lysis solution and invert 5–6 times.
7. Add 350 μ l of neutralization solution and invert 5–6 times.
8. Centrifuge for 5 min at 13 krpm.
9. Transfer supernatant to a spin column in another 1.5 ml tube with.
10. Centrifuge for 5 min at 13 krpm. Discard filtrate.
11. Add 750 μ l of wash solution and flash-spin for 1 min.
12. Discard filtrate and spin again without adding any solution.
13. Transfer column to a clean 1.5 ml tube.
14. Elute, quantify, and keep the DNA for future use as described above in c.2.
15. Pick two clones for each mutant plasmid and send for sequencing to confirm the mutation by following the instructions from the service providers. We use Fisher's sequencing service.

3.5. Transfection and Western Blotting to Confirm the Expression of the Mutant Proteins

3.5.1. Transfection—Correct DNA sequencing results do not always guarantee that the plasmids are capable of driving the protein expression.

1. Split the subconfluent 293 cells (see Note 18) by 1:4 or 1:5 into a 12-well dish and incubate overnight.
2. When the cells are 70–80% confluent, continue with the transfection.
3. Everything is to be done under the tissue culture hood under sterile condition.
4. Prepare transfection reaction mixture in the order of diluting 1 μ g DNA in 50 μ l of serum-free medium, diluting 3 μ l of Lipofectamine 2000 in 50 μ l of serum-free medium, and combine these two dilutions by gentle pipetting. Incubate at room temperature for 20 min.
5. Transfer the mixture to a tube containing 0.4 μ l of serum-free medium, gently pipette and add to the cells pre-washed with serum-free medium. Incubate for 3–5 h.
6. Replace the medium with growth medium (see Note 19) and incubate overnight.

¹⁸293 and Cos7 cells are the most frequently used cells to quickly test for expression due to their high transfection efficiency and protein expression capacity.

7. Carefully decant the medium and wash the cells with ice-cold PBS (see Note 20).
8. Add 100 ml of 1× SDS Sample Buffer to the cells. Swirl the plate on the rocker for 5–10 min.
9. Transfer the lysates to 1.5 ml Eppendorf tubes and label these tubes.
10. Boil the tubes for 3–5 min in the Isotemp heat block. The lysates are now ready for western blotting or can be kept at –20°C for future use.

3.5.2. SDS-PAGE

1. Place 2 glass plates on napkins. Wipe the inner sides with 70% ethanol and Kimwipes. Wait a few seconds for the ethanol to evaporate.
2. Set up the gel cassette and make sure the glass plates are in level with the ground and there is no leakage.
3. Make a 1.5-mm 10% separating gel: mix 4 ml of nanopure water, 2 ml of 4× separating buffer (combine 91 g of Tris with 450 ml of nanopure water, adjust pH to 8.8 with 10–15 ml of concentrated HCl, add 10 ml of 20% SDS and nanopure water to 500 ml), 2 ml of 40% acrylamide:bisacrylamide solution, 40 ml of APS and 8 ml of TEMED. Pour the gel mix into the cassette. Gently add 0.5 ml of water on the top of the gel solution. Wait 20–30 min until the gel polymerizes (when a sharp line between the gel and water is visible).
4. While waiting, make the stacking gel: Mix 2.6 ml of nanopure water, 1 ml of 4× stacking buffer (combine 30.25 g of Tris with 450 ml of nanopure water, adjust pH to 6.8 with 10–15 ml of concentrated HCl, add 10 ml of 20% SDS and nanopure water to 500 ml), 0.4 ml of 40% acrylamide: bisacrylamide solution, 20 ml of APS and 4 ml of TEMED. Pour the gel mix into the cassette after removing the 0.5 ml water from it. Gently insert the comb into the gel solution (see Note 21). Wait for 20–30 min for the gel to polymerize.
5. Move the gel cassette into the running tank with the short glass plate facing the center.
6. Pour 800 ml of 1× SDS running buffer diluted from the 5× stock (combine 320 g of NaCl, 8 g of KCl, 57.6 g of Na₂HPO₄, and 330 ml of water, adjust pH to 7.4 with 9.6 g of KH₂PO₄, 5 N NaOH or HCl, and add water to 4 L) into the tank, starting from the middle of the apparatus. Let it spill into the outside compartment of the tank until it is under the white part of the apparatus that juts out. Carefully remove the comb (see Note 22) and bubbles from the wells on the top and from the bottom of the gel using a syringe.

¹⁹Before moving on, make sure that lipofecamine particles are present in the medium by microscopy.

²⁰Vacuum remove the PBS residue from the wells to prevent the buffer to be added from over-diluting.

²¹Avoid bubbles during comb inserting.

²²Do not take out the comb too early before the gel becomes solid to avoid formation of tortured wells.

7. Pipette up to 20 ML of boiled lysates into each well (see Note 23). For the molecular size marker, combine 5 ML of the marker and 15 ML of the 1× SDS sample buffer to make a final volume of 20 ML.
8. Put the tank lid on and run for 10–20 min at 50 V and then for 1 h at 150 V.
9. Prepare the transfer sandwich while waiting for the running to complete: per gel, get 2 sponges, 2 filter papers, and 1 nitrocellulose membrane. Cut a small top-right corner off the membrane and write on it: initials, date, name of antibody (across the top), and the name of the samples on the gel (on the side). Put the sponges on the pan. Submerge them with 1× transfer buffer diluted from 5× stock (combine 58 g of glycine and 116 g of Trizma base, and add water to 4 L; to make 1× buffer, mix 1 volume of the 5× stock, 3 volumes of water, and 1 volume of methanol). Remove bubbles from the sponges by rubbing them out through the sides. Put one piece of 3MM filter paper on top of one of the sponges. Put the membrane on top of the filter. Put the second filter paper on top and then the second sponge. Let the sandwich soak in the transfer buffer while the gel is running.
10. Once the gel has completed running, take out the gel and cut the top right corner of the gel. Rinse the gel a few times in the transfer buffer. Place the gel facing down on one of the filter paper in the sandwich. Place the membrane on top of the gel with the cut corner aligned together. Assemble the sandwich in the black/white holder (see Note 24) and slide it into the transfer tank.
11. Put an ice block from the –20°C fridge in the transfer tank next to the black side.
12. Pour 1× transfer buffer into the tank until the tank juts out (~800 mL). Run at 100 V for 1 h (see Note 25).

3.5.3. Western Blotting

1. After the transfer is complete, use tweezers to remove the membrane and place it facing up in 20 ml of blocking buffer (5% carnation skim milk in PBS-T. To make PBS-T, mix 100 ml of 10× PBS, 900 ml of nanopure water and 1 ml of Tween-20). Rock slowly for 1 h.
2. Dump the blocking buffer into the sink. Rinse the membrane twice with 20 ml of PBS-T.
3. Apply 10 ml of the primary antibody solution in PBS-T (see Note 26). Rock at 4°C overnight (see Note 27).
4. Recover the antibody solution and keep at 4°C for future reuse.

²³Put the pipette tip against the tall glass plate and slide into the well and stop right before the tip reaches the bottom of the well. Apply slowly to prevent leak and bubbles. Load same volume of the 1× sample buffer alone into unused wells to avoid asymmetrical protein resolution such as “smiling lanes.”

²⁴It is critical to rub out all bubbles from the sandwich.

²⁵Alternatively, the transfer can be done overnight at 15 V.

²⁶For most primary and secondary antibodies, 1:500–1:2,000 and 1:5,000–1:10,000 dilution are recommended, respectively.

²⁷Pros and cons: the overnight strategy makes the antibody solution more reusable (more than five times on average) but takes longer, whereas the room temperature strategy is quick but makes the antibody less reusable. It is the matter of choice between saving time and saving reagent. Reuse of secondary antibodies is not recommended.

5. Wash the membrane with 20 ml of PBS-T for five times (rinse twice, rock once for 10 min and twice for 5 min).
6. Apply 10 ml of the second antibody solution in PBS-T. Repeat incubating and washing as in steps 3 and 5 above.
7. Treat the membrane with 3 ml of ECL Reagents for 1 min. Put saran wrap on top of the membrane. Use Kimwipes to smooth out the membrane and rub out bubbles.
8. Expose the membrane to an X-ray film and develop the film: Fold the top right corner of the X-ray film to match the cut corner of the membrane. Make four exposures of one membrane on a single film for 1 s, 15 s, 30 s, and 1 min. Develop the film.
9. Retrieve the X-ray film and record the results.

3.6. IF Staining and Microscopy to Determine the Requirement of the Predicted NLS Sequences for the Nuclear Localization (Fig. 4)

3.6.1. Set up Cells for Transfection

1. Using tweezers put a sterile coverslip into the bottom of each well of a 12-well plate. Rinse the coverslips with 1 ml of 1× PBS.
2. Re-plate NIH3T3 cells to the coverslips at a density of 1/4 or 1/5 confluence. Incubate overnight.
3. Transfect the cells as described above in 3.5.1.

3.6.2. Immunostaining for Fluorescent Microscopy

1. Rinse cells twice with ice-cold 1× PBS plus 100 mM CaCl₂ and 100 mM MgCl₂.
2. Add 1 ml of the fixative solution (i.e., 4% paraformaldehyde in PBS: in fume hood, combine 36 g of paraformaldehyde, 600 ml of water, and 2 ml of 10 N NaOH and stir on 45°C hot plate until dissolved. Add 90 ml of 10× PBS and water to 900 ml. Store 50–100 ml aliquots at –20°C. Thaw at 37°C water bath until turbidity disappears before use) to each well. Incubate for 15 min at room temperature (see Note 28).
3. Rinse the cells twice with PBS.
4. Apply 30–60 µl of primary antibody solution (1:100–1:300 in 1× PBS). Incubate at 37°C for 30 min. Rinse as in step 3.
5. Apply 30–60 µl of the FITC-conjugated secondary antibody and repeat steps 3 and 4.
6. Add 0.2 ml of 0.5 µg/ml Hoechst 33258 in PBS to the cells. Incubate at room temperature for 10 min. Rinse twice with 1× PBS and once with water.

²⁸Fixed cells can be kept overnight or longer at 4°C.

7. Place a drop of mounting solution on a slide. Carefully transfer the coverslip upside down to the drop with curved edge tweezers (see Note 29).
8. Gently press down on the coverslip to get rid of any excess mounting solution. Use a pipette tip and the vacuum to remove excess mounting solution from the coverslip edges (see Note 30).
9. Glue the edge of the coverslip to the microscope slide with clear nail polish.
10. Take cell images under a fluorescent microscope (green image for the protein stained and blue images for the nuclei in the cells) (see Fig. 4).

3.6.3. GFP-based Fluorescent Microscopy to Determine the Sufficiency of the Predicted NLS Sequences for the Nuclear Localization—

A sequence required for the nuclear localization may be only a part of the intact NLS sequence (see Note 31). Therefore, such a fusion approach is essential to determine the entire NLS sequence that can sufficiently target a recombinant protein to the nucleus. The suspected NLS sequence can be fused to either the amino (using the pEGFP-N vector series) or carboxyl (using the pEGFP-C vector series) terminus of GFP. Inclusion of both types of the fusions can enhance the result of the interpretation. Vector construction, transfection, and fluorescent microscopy are the same as described above except that the antibody staining is not necessary and the protein localization can be observed in live cells.

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References

1. Ghaleb AM, Nandan MO, Chanchevalap S, Dalton WB, Hisamuddin IM, Yang VW. Kruppel-like factors 4 and 5: the yin and yang regulators of cellular proliferation. *Cell Res.* 2005; 15:92–96. [PubMed: 15740636]
2. Narla G, DiFeo A, Fernandez Y, et al. KLF6-SV1 overexpression accelerates human and mouse prostate cancer progression and metastasis. *J Clin Invest.* 2008; 118:2711–2721. [PubMed: 18596922]
3. Narla G, Heath KE, Reeves HL, et al. KLF6, a candidate tumor suppressor gene mutated in prostate cancer. *Science.* 2001; 294:2563–2566. [PubMed: 11752579]
4. van Vliet J, Turner J, Crossley M. Human Kruppel-like factor 8: a CACCC-box binding protein that associates with CtBP and represses transcription. *Nucleic Acids Res.* 2000; 28:1955–1962. [PubMed: 10756197]
5. Wang X, Zheng M, Liu G, et al. Kruppel-like factor 8 induces epithelial to mesenchymal transition and epithelial cell invasion. *Cancer Res.* 2007; 67:7184–7193. [PubMed: 17671186]
6. Wei H, Wang X, Gan B, et al. Sumoylation delimits KLF8 transcriptional activity associated with the cell cycle regulation. *J Biol Chem.* 2006; 281:16664–16671. [PubMed: 16617055]

²⁹Make sure not to drop or break the coverslips while transferring.

³⁰No bubbles should be present between the coverslips and the microscopic slide.

³¹A sequence that is required but not sufficient for nuclear localization may represent just a part of the NLS sequence, a non-NLS sequence that plays a critical role in maintaining the NLS function or inhibiting the nuclear export of the protein. Additional experiments are helpful to test the effect of the sequence on the protein interaction with nuclear importin and exportin proteins and on the exportin dependent (e.g., Leptomycin B-sensitive) nuclear export.

7. Zhao J, Bian ZC, Yee K, Chen BP, Chien S, Guan JL. Identification of transcription factor KLF8 as a downstream target of focal adhesion kinase in its regulation of cyclin D1 and cell cycle progression. *Mol Cell*. 2003; 11:1503–1515. [PubMed: 12820964]
8. Wang Z, Spittau B, Behrendt M, Peters B, Kriegstein K. Human TIEG2/KLF11 induces oligodendroglial cell death by down-regulation of Bcl-X(L) expression. *J Neural Transm*. 2007; 114:867–875. [PubMed: 17308981]
9. Wang X, Urvalek AM, Liu J, Zhao J. Activation of KLF8 transcription by focal adhesion kinase in human ovarian epithelial and cancer cells. *J Biol Chem*. 2008; 283:13934–13942. [PubMed: 18353772]
10. Wang X, Zhao J. KLF8 transcription factor participates in oncogenic transformation. *Oncogene*. 2007; 26:456–461. [PubMed: 16832343]
11. Ding Q, Grammer JR, Nelson MA, Guan JL, Stewart JE Jr, Gladson CL. p27Kip1 and cyclin D1 are necessary for focal adhesion kinase regulation of cell cycle progression in glioblastoma cells propagated in vitro and in vivo in the scid mouse brain. *J Biol Chem*. 2005; 280:6802–6815. [PubMed: 15557280]
12. Pandya K, Townes TM. Basic residues within the Kruppel zinc finger DNA binding domains are the critical nuclear localization determinants of EKLF/KLF-1. *J Biol Chem*. 2002; 277:16304–16312. [PubMed: 11844803]
13. Quadrini KJ, Bieker JJ. Kruppel-like zinc fingers bind to nuclear import proteins and are required for efficient nuclear localization of erythroid Kruppel-like factor. *J Biol Chem*. 2002; 277:32243–32252. [PubMed: 12072445]
14. Shields JM, Yang VW. Two potent nuclear localization signals in the gut-enriched Kruppel-like factor define a subfamily of closely related Kruppel proteins. *J Biol Chem*. 1997; 272:18504–18507. [PubMed: 9218496]
15. LaCasse EC, Lefebvre YA. Nuclear localization signals overlap DNA- or RNA-binding domains in nucleic acid-binding proteins. *Nucleic Acids Res*. 1995; 23:1647–1656. [PubMed: 7540284]
16. Corbi N, Libri V, Onori A, Passananti C. Synthetic zinc finger peptides: old and novel applications. *Biochem Cell Biol*. 2004; 82:428–436. [PubMed: 15284895]
17. Pabo CO, Peisach E, Grant RA. Design and selection of novel Cys2His2 zinc finger proteins. *Annu Rev Biochem*. 2001; 70:313–340. [PubMed: 11395410]

1. Predicting NLS sequences by computer programs and sequence similarity to known NLS in paralogs or orthologs.
▼
2. Disrupting the predicted NLS sequences by site-directed mutagenesis by PCR.
▼
3. Confirming mutant protein expression by transfection and western blotting.
▼
4. Determining requirement of the predicted NLS for the nuclear localization by transfection and immunofluorescent (IF) microscopy.
▼
5. Determining sufficiency of the predicted NLS for the nuclear localization by GFP-fusion strategy and fluorescent microscopy.

Fig. 1.

Flowchart of the procedure. Shown are general strategic sequences of the experiments to be done.

Fig. 2.

NLS prediction. The diagram of KLF8 protein structure is shown. The PSORT computer program predicts two classical monopartite nuclear localization signal (mNLS) sequences, the first one is located at the amino terminus of the first zinc finger and the second one is located within the carboxyl terminus of the third zinc finger. Both the first mNLS and the zinc fingers are conserved among the KLF family proteins and have been suggested to be the functional NLS sequences for KLF1 and KLF4. Neither the computer program nor the homology search has predicted any NLS sequences within the amino terminal regulatory region marked with the question mark.

Fig. 3.

Construction of NLS mutants by PCR. Shown are Schematic illustrations of the PCR strategies to be used. The double strand cDNA template and primers are shown in lines and the PCR products are illustrated as protein peptide diagrams shown as white and black boxes. Terminal truncation mutations are generated by a one-step PCR. Point mutations and internal deletion mutations involve two-step overlapping PCR.

Fig. 4. Immunofluorescent microscopic examination of protein localization. Shown is immunofluorescent microscopic images for KLF8 (top row, stained with anti-Myc tag antibody) and the nuclei (middle row, stained with Hoechst 33258).