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Characterization of the nuclear localization signal of the mouse TET3 protein

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ABSTRACT

DNA demethylation is associated with gene activation and is mediated by a family of ten-eleven translocation (TET) dioxygenase. The TET3 protein is a 1668-amino-acid DNA demethylase that is predicted to possess five nuclear localization signals (NLSs). In this paper, we used a series of green fluorescent protein-tagged and mutation constructs to identify a conserved NLS (KKRK) embedded between amino acid 1615 and 1618 of mouse TET3. The KKRK sequence facilitates the cytoplasmic protein's translocation into the nucleus. Additionally TET3 may be imported into the nucleus by importin-α and importin-β.

1. Introduction

Nuclear proteins, imported into the nucleus through nuclear pore complexes (NPCs), have important roles in biological events, including transcription and DNA demethylation [1,2]. Small molecules can be imported into NPCs by passive diffusion, such as ions and proteins of up to 20–40 kDa, whereas large proteins are imported by active translocation, a selective process using proteins belonging to the importin family [3]. This selective and active nuclear protein transport is mediated by a nuclear localization signal (NLS) [4].

NLSs are classified into canonical and non-canonical types. The first canonical type consists of a single cluster of basic amino acids such as SV40 large T antigen [5]. The second canonical type contains bipartite sequences that carry two clusters of positively charged amino acid residues separated by a spacer region, such as the NLS of *Xenopus laevis* nucleoplasmin [6]. Canonical types, import of which is mediated by importin-α and importin-β, can be predicted computationally [7–9]. Import of a portion of the non-canonical type is directly mediated by importin-β2, and is termed PY-NLS [10]. Other non-canonical types are still unknown.

Recent studies have demonstrated that the TET family of 5mC hydroxylases can convert 5mC to 5-hydroxymethylcytosine (5hmC) [11], and further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5CaC) [12,13]. These studies also suggest that additional modification of 5mC converted by TET enzymes may regulate the dynamics of 5mC and its mediated gene regulation [14]. Analysis of the TET3 amino acid sequence has revealed that TET3 contains several conserved domains, including a CXXC domain and a catalytic domain that is typical of Fe(II)- and 2-oxoglutarate (2OG)-dependent dioxygenase [15]. TET3 also acts as a transcription factor located in the nucleus [16]. Although TET3 is known to be targeted to the nucleus, the NLS responsible for TET3 has not yet been identified. In this report, we characterize a traditional NLS embedded in TET3.

2. Materials and methods

2.1. Plasmid construction

Fragments encoding the KKRK peptide and various mouse TET3 (GenBank: NM_183138) fragments were generated by polymerase chain reaction(PCR) from total cDNA from adult ovaries. Fragment primer pairs contained *Eco*RI and *Bgl*II restriction sites. The mouse glutathione S-transferase (GST, GenBank: X65021.1) and pig growth hormone (GH) were PCR-amplified from total cDNA from adult ovaries and plasmid pTRE-Tight-BI-GH-rtTA [17], respectively. Fragment primer pairs contained *Bsp*EI and *Bgl*II restriction sites. The fragment encoding KKRK or the TET3 fragment excised from the PCR product by *Eco*RI and *Bgl*II was ligated into the pEGFP vector (Clontech, USA) that had been previously digested with *Eco*RI and *Bgl*II. The fragment encoding GST or GH excised from the PCR product by digestion with *Bsp*EI and *Bgl*II was ligated into pEGFP, pEGFP-TET3 1310–1668 or pEGFP-KKRK, previously

Abbreviations: TET, ten-eleven translocation dioxygenase; TET1, ten-eleven translocation dioxygenase 1; TET3, ten-eleven translocation dioxygenase 3; PCR, polymerase chain reaction; GH, growth hormone; GST, glutathione S-transferase; NLS, nuclear localization signal; EGFP, enhanced green fluorescent protein.

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digested with *Bsp*EI and *Bgl*II. Site-directed mutagenesis were carried out by CloneMutation (Vazyme, China). All constructs were confirmed by DNA sequencing. Primers used for PCR are shown in Table S1 in the supplementary material.

2.2. Cell culture and transfection

NIH 3T3 cells were cultured in growth medium containing GB11995 (Gibco, USA), 10% fetal bovine serum (FBS; Gibco, USA) and penicillin/streptomycin (Gibco, USA). Transfection was carried out using the Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's procedure.

2.3. Microscopy

NIH 3T3 cells were seeded onto glass over slides, grown at 37 °C, and transiently transfected with each construct. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, washed twice with phosphate-buffered saline (PBS) and permeabilized with methanol for 10 min at −20 °C. Cells were then washed with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI) for 15 min at room temperature. After washing with PBS, cells were visualized by confocal laser scanning microscopy.

3. Results

3.1. The C-terminus is required for TET3 nuclear localization

TET3 catalyzes 5mC into 5hmC in the nucleus. Numerous reports show that TET3 is localized in the nucleus [1,2,16,18]. However, TET3 (~180 kDa) cannot diffuse into the nucleus. One or more functional NLSs are thought to be embedded in TET3.

Structurally, TET3 contains an entire catalytic domain from amino acid 697 to 1668 [2,15]. Therefore, we fused a fragment from amino acid 664 to 1668 in the C-terminus of TET3 to the C-terminus of enhanced green fluorescent protein (EGFP) and examined the subcellular distribution of this fusion protein in NIH 3T3 cells. As shown in Fig. 1C, the C-terminal fragment (pEGFP-C1-TET3 664–1668) of TET3 was able to direct EGFP to the nucleus. We also fused a fragment from amino acid 1 to 339 and amino acid 333 to 672 in the N-terminus of TET3 to the C-terminus of EGFP and examined the subcellular distribution of these two fusion proteins in NIH 3T3 cells. As shown in Fig. 1C, the N-terminal fragments (pEGFP-C1-TET3 1–339 and pEGFP-C1-TET3 333–672) of TET3 did not direct EGFP to the nucleus. To determine the precise position of the NLS, we constructed the pEGFP-C1-TET3 995–1668 and pEGFP-C1-TET3 1310–1668. As shown in Fig. 1C, these fragments directed EGFP to the nucleus. To confirm that the NLS is embedded within the fragment between amino acid 1310 and 1668, we also constructed pEGFP-C1-TET3 664–1323. As shown in Fig. 1C, the fragment (pEGFP-C1-TET3 664–1323) did not direct EGFP to the nucleus. Therefore, the NLS located in TET3 is embedded within the fragment from amino acid 1310 to 1668. All fusion proteins were too large for nuclear pore complex (NPC) transport of small molecules through passive diffusion.

3.2. Prediction and mutation of the NLS in TET3

We used the web-based computer software, PSORT (available from the PSORT web site), to search for the NLS within TET3. There were five putative NLS regions containing multiple positively charged amino acid residues located at amino acids 439–445, 442–445, 443–446, 1615–1618 and 1617–1633, designated NLS1, NLS2, NLS3, NLS4 and NLS5, respectively (Fig. 2A). Because NLS1, NLS2 and NLS3 lie outside the region mapped by the truncation experiment, we focused on two NLSs within the C-terminus, NLS4 and NLS5.

To determine whether these two NLSs were functional, site-directed mutagenesis was performed to generate three mutant constructs. As shown in Fig. 2C, the mutant K1630 N/E1631 N/K1632 N/K1633 N did not impair the nuclear localization of pEGFP-C1-TET3 1310–1668. However, the mutant K1615 N/K1616 N/R1617 N/K1618 N disrupted the nuclear localization of pEGFP-C1-TET3 1310–1668. The double mutation of KEKK and KKRK showed the same distribution pattern as the KKRK mutation. Thus, KKRK is crucial in mediating nuclear localization of pEGFP-C1-TET3

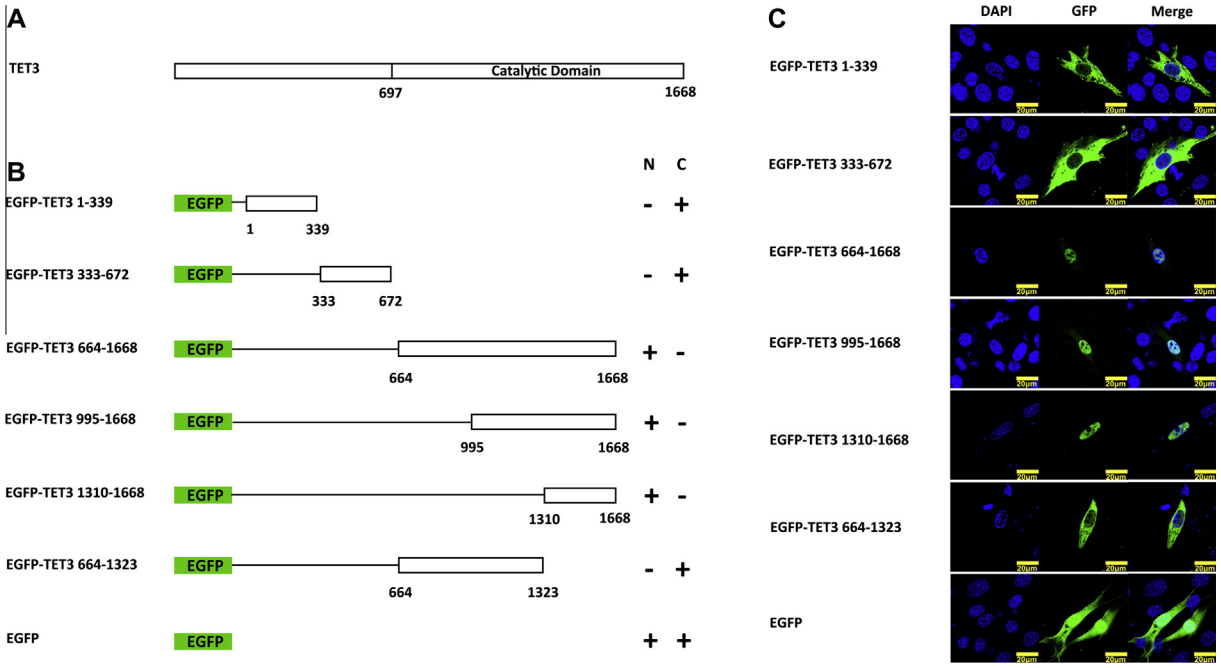

Fig. 1. The C-terminus of TET3 is sufficient for nuclear localization of EGFP. (A) Structure of TET3 protein. (B) Schematic diagram for the EGFP-TET3 fragment fusion proteins and their respective subcellular localizations. (C) EGFP and six deletion constructs were generated and transiently transfected into NIH 3T3 cells. Forty-eight hours after transfection, the subcellular localizations of EGFP and EGFP-tagged proteins were visualized by fluorescence microscopy.