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Identification of a putative nuclear export signal motif in human NANOG homeobox domain

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ABSTRACT

NANOG is a homeobox-containing transcription factor that plays an important role in pluripotent stem cells and tumorigenic cells. To understand how nuclear localization of human NANOG is regulated, the NANOG sequence was examined and a leucine-rich nuclear export signal (NES) motif (^{125}MQELSNILNL^{134}) was found in the homeodomain (HD). To functionally validate the putative NES motif, deletion and site-directed mutants were fused to an EGFP expression vector and transfected into COS-7 cells, and the localization of the proteins was examined. While hNANOG HD exclusively localized to the nucleus, a mutant with both NLSs deleted and only the putative NES motif contained (hNANOG HD-ΔNLSs) was predominantly cytoplasmic, as observed by nucleo/cytoplasmic fractionation and Western blot analysis as well as confocal microscopy. Furthermore, site-directed mutagenesis of the putative NES motif in a partial hNANOG HD only containing either one of the two NLS motifs led to localization in the nucleus, suggesting that the NES motif may play a functional role in nuclear export. Furthermore, CRM1-specific nuclear export inhibitor LMB blocked the hNANOG potent NES-mediated export, suggesting that the leucine-rich motif may function in CRM1-mediated nuclear export of hNANOG. Collectively, a NES motif is present in the hNANOG HD and may be functionally involved in CRM1-mediated nuclear export pathway.

1. Introduction

NANOG is a divergent homeodomain (HD)-containing transcription factor whose expression was originally found to be restricted to pluripotent stem cells. NANOG plays an important role in the regulation of self-renewal and pluripotency by repressing and/or activating downstream target genes [1,2]. NANOG is a molecular switch to maintain pluripotency [3] and homo-dimerization is required for interactions with other pluripotent network proteins to promote stem cell pluripotency [4,5]. NANOG is important for stem cell self-renewal, but also plays a novel role in tumor development [6]. Indeed, several studies have reported that NANOG is expressed not only in germ cell tumors but also in other tumors, including breast, cervix, oral, kidney, prostate, and ovarian cancers, as well as in the tumor cell line MCF7 [7–15]. Moreover, ectopic overexpression of NANOG promotes proliferation and transformation in mesenchymal stem cells, as well as NIH3T3 and 293T cells [16–18]. NANOG is differentially expressed in the cytoplasm as well as the nuclei of cancer cells [15,19,20], suggesting a critical role in tumorigenesis, as well as in pluripotent stem cells.

For the regulation of downstream gene expression, nuclear proteins require an active series of nuclear import and nuclear export processes, which are mediated by nuclear localization signals (NLSs) and nuclear export signals (NESs). An NLS is characterized by a short stretch or stretches of highly basic amino acid residues [21,22]. Nuclear proteins containing archetypical NLS sequences are translocated into the nucleus in an energy-dependent process as part of a complex with importin αβ heterodimers. Importin-α contains the NLS binding site and importin-β mediates translocation through the nuclear pore complex [23]. The best-characterized classical nuclear export pathway involves CRM1 (Exportin-1) and a short hydrophobic leucine-rich sequence (LxxLxL) in the nuclear protein [24]. Briefly, proteins harboring leucine-rich NESs are recognized and exported to the cytoplasm by the exportin CRM1, a

Abbreviations: NES, nuclear export signal; LMB, leptomycin B; CRM1, chromosomal region maintenance 1; NLS, nuclear localization signal; HD, homeobox domain.

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member of the karyopherin β superfamily [21,22]. The CRM1-mediated export pathway is effectively inhibited by the actinobacterial toxin leptomycin B (LMB), facilitating experimental identification and verification of this pathway [24].

Human NANOG (hNANOG) is composed of three domains based on the position of the HD sequence: an N-terminal domain (ND) rich in serine, threonine, and acidic residues; an HD containing the DNA binding motif; and a C-terminal domain (CD) containing two potent transactivation subdomains [25,26]. Even though hNANOG shares only 58% amino acid homology with mouse NANOG, the HD is well conserved in mouse, rat, dog and chimpanzee orthologs, implying a conservation of function. The homeobox proteins are important regulators of cell differentiation and proliferation [27,28]. A recent study revealed that a short sequence in the well conserved HD of NANOG orthologs was sufficient to induce pluripotency in NANOG-deficient somatic cells [29]. Two NLS motifs are present in the HD and are required for complete nuclear localization of hNANOG [30]. In addition, similar to OCT6, which acts as a transcriptional regulator during neurogenesis, an NES, as well as an NLS, is localized in the POU HD [31,32]. Based on these observations, we examined the nucleocytoplasmic distribution of NANOG using a variety of EGFP-fusion proteins and identified a functional hNANOG NES motif that mediates export, potentially through a CRM1-dependent pathway.

2. Materials and methods

2.1. Plasmid constructs

All plasmid constructs were produced as described in our previous report [30]. Briefly, hNANOG HD deletions and site-directed mutants were produced using the QuickChange Site-Directed Mutagenesis method (Stratagene, La Jolla, CA) and ligated into pEGFP-C1 (Clontech, Palo Alto, CA) to produce EGFP-fusion proteins. All constructs were confirmed by sequencing.

2.2. Cell culture and preparation for transient transfection

COS-7 and HEK293T cells (American type cell collection) were grown in Dulbecco's Modified Eagle's Medium (Welgene, Daegu, South Korea) supplemented with 10% Fetal Bovine Serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin. To trace localization patterns, COS-7 cells were seeded onto glass coverslips in 6-well plates, grown overnight and transfected with 2 μg EGFP-fusion protein vector expressing hNANOG HD and cognate mutants using the Exgen 500 *in vitro* transfection reagent (Fermentas, Hanover, MD). After 48 h, the cells were fixed for 20 min in 4% paraformaldehyde, permeabilized in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) for 10 min, blocked in 3% BSA-supplemented PBS for 1 h, and finally stained with 4',6-diamidino-2-phenylindol (DAPI) to stain the nuclear DNA. After the coverslips were mounted in Faramount aqueous mounting solution (DakoCytomation, Carpinteria, CA), cells were photographed using a Leica confocal microscope and processed using the LAS AF Lite 2.4.1 (Leica Microsystems GmbH, Wetzlar, Germany) and Adobe Photoshop software packages, as described previously [30]. A minimum of three independent experiments were conducted for all transfection experiments.

2.3. Western blot analysis

For Western blotting, HEK293T cells were co-transfected with EGFP-hNANOG HD or EGFP-hNANOG HD-ΔNLSs and the myc-tagged LacZ expression vector as a cytoplasmic marker, using the Exgen 500 *in vitro* transfection reagent (Fermentas). Cells were harvested two days post-transfection. Cytoplasmic and nuclear extracts were then prepared using a modified nuclear extract miniprep method [33]. The extracts were separated by SDS–PAGE, transferred onto a polyvinylidene fluoride membrane (PVDF) (GE healthcare, Waukesha, WI, USA), and probed with mouse-anti-GFP (Santacruz, CA) and mouse-anti-myc (Abfrontier, Seoul, KR) antibodies.

2.4. LMB binding assay

COS-7 cells were transfected with the hNANOG HD-ΔNLSs mutant and treated with leptomycin B (LMB, 5 ng/ml; Sigma) for different times (0, 2, 5, and 8 h). EGFP localization was observed using a confocal scanning microscope.

3. Results and discussion

3.1. Identification of a potent NES motif within the hNANOG homeodomain

hNANOG has two potent NLS motifs within the HD. A partial HD lacking either or both NLS motif(s) results in a predominantly cytoplasmic distribution, indicating that an intact HD is required for complete nuclear localization [30]. In addition, OCT6 has a leucine-rich NES within its POU HD that is implicated in nuclear shuttling [31]. Based on these observations, an amino acid sequence analysis of the hNANOG HD was conducted. A leucine-rich motif consisting of ^{125}MQE**L**SNI**L**N**L**^{134} was found in the middle of the hNANOG HD (Fig. 1A), which is similar to the OCT6 POU HD NES [^{367}ITG**L**ADS**L**Q**L**^{376}; 31]. Sequences analysis using NetNES software (http://www.cbs.dtu.dk/services/NetNES/) and NES Finder (http://research.nki.nl/fornerodlab/) also revealed that ^{125}MQE**L**SNI**L**N**L**^{134} within HD is a NES classical motif (Fig. 1B). Such "classical sequences" are relatively hydrophobic short linear peptide enriched in leucine residues, usually consisting of the three subtypes of spacing such as 3-2-1, 2-2-1 and 2-3-1 [X-R(2-3)X-R(2-3)-X-R-X, where X is leucine, isoleucine, methionine or valine and R is any amino acid] [34,35].

First, to determine whether the leucine-rich motif is an NES in the hNANOG HD, an EGFP-fused deletion mutant lacking the two NLS motifs was constructed and the subcellular localization pattern of the mutant was examined in COS-7 cells (Fig. 2). A deletion mutant lacking the two NLS motifs (hNANOG HD-ΔNLSs) was amplified by PCR, ligated into a pEGFP-C1 fusion protein expression vector and confirmed by sequencing analysis (Fig. 2A). To trace the nucleocytoplasmic localization pattern, recombinant EGFP expression vectors were transfected into COS-7 cells and the localization was examined by confocal microscopy two days post-transfection (Fig. 2B). As negative and positive controls, EGFP alone was evenly distributed in the cytoplasm as well as the nucleus, and EGFP-fused hNANOG HD was strictly localized to the nucleus. However, the EGFP-fused hNANOG HD-ΔNLSs mutant was predominantly localized in the cytoplasm. The localization patterns were further examined by preparing nuclear and cytoplasmic fractions and analyzing the fractions by Western blot (Fig. 2C). Lysates were obtained from HEK293T cells co-transfected with EGFP, EGFP-fused wild-type hNANOG HD, or EGFP-fused hNANOG HD-ΔNLSs and a myc-tagged LacZ construct as a cytoplasmic marker. Subsequently, nuclear and cytoplasmic fractions were separated by SDS–PAGE and probed with anti-EGFP and anti-c-myc antibodies. As expected, hNANOG HD-ΔNLSs was prominently detected in the cytoplasmic extract, while intact hNANOG HD expression was detected in the nuclear extract (Fig. 2C). LacZ expression (116 kDa) was only detected in the cytoplasmic fraction of all the samples tested. These results confirmed that the hNANOG HD-ΔNLSs, which possesses only the NES motif, was

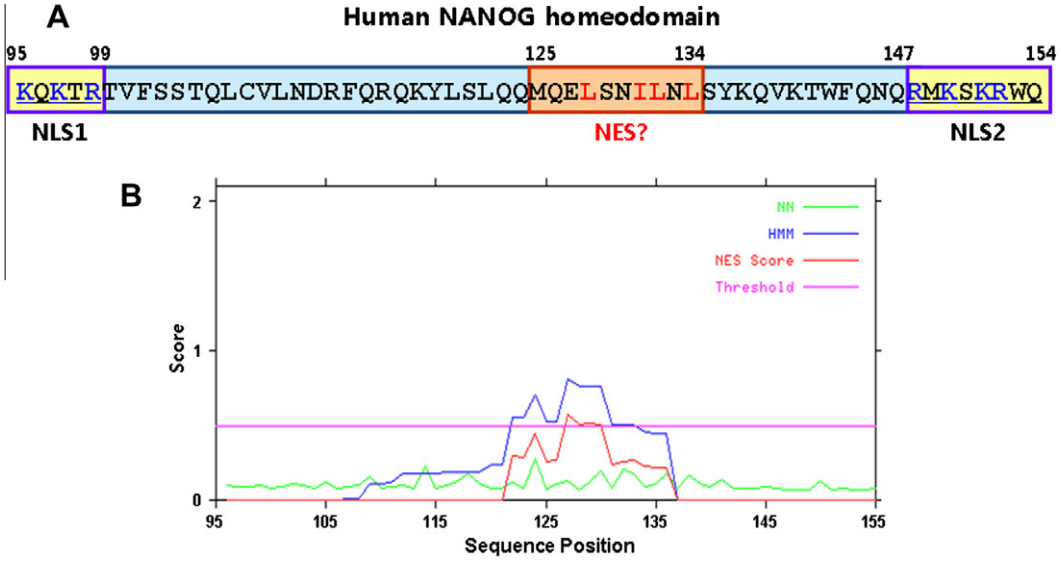

Fig. 1. Sequence analysis of the hNANOG HD containing a putative NES motif. (A) Nuclear localization signal (NLS) 1 and 2 are indicated at the N- or C-terminal ends of the hNANOG HD. A putative leucine-rich NES sequence (^{125}MQE**L**SNI**L**N**L**^{134}) is located in the middle of the HD, which is similar to the hOCT6 POU HD NES (^{367}ITGLADSLQL^{376}; [31]). (B) Using NetNES software, ^{125}MQE**L**SNI**L**N**L**^{134} is shown as a NES classical motif present in hNANOG HD. Graphical plot of the values (NES score) was calculated by the prediction server from the Markov Model (HMM), and Artificial Neural Network (NN) scores. If the NES score exceeds the threshold, the residue concerned is predicted to be involved in a nuclear export signal [34].

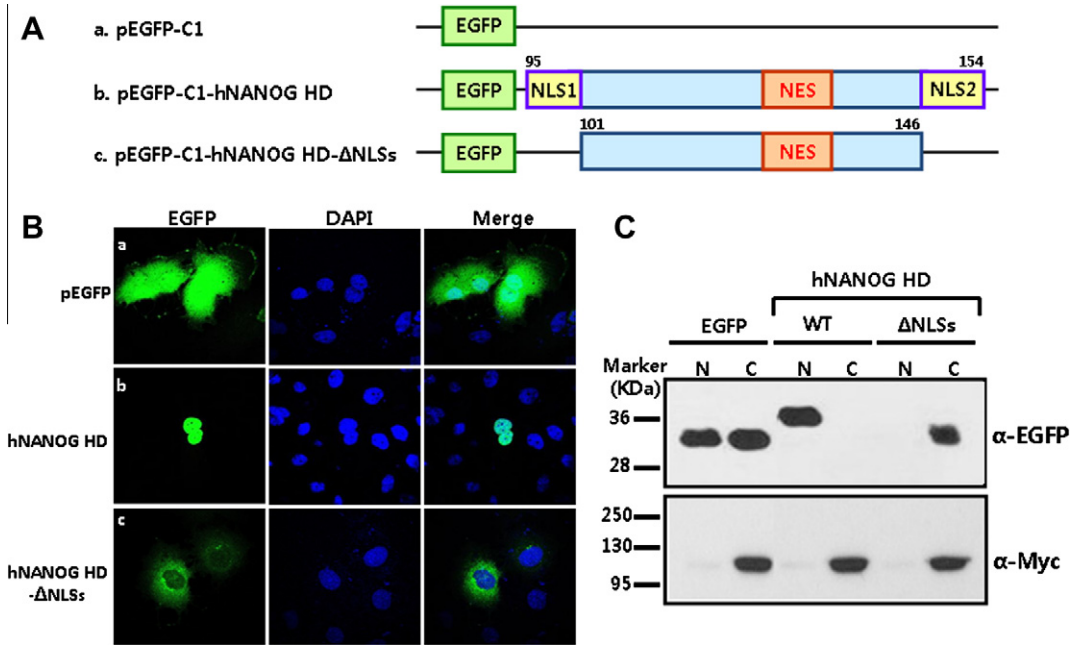

Fig. 2. Subcellular localization of hNANOG HD and hNANOG HD-ΔNLSs. (A) Schematic representation of hNANOG HD and hNANOG HD-ΔNLSs, each fused to EGFP. (B) Confocal laser scanning microscopy of COS-7 cells transfected with EGFP-hNANOG HD or EGFP-hNANOG HD-ΔNLSs and counter-stained DAPI to show the location of the nuclear DNA. (C) Cytoplasmic and nuclear fractionation of HEK293T cells transfected with EGFP-hNANOG HD or EGFP-hNANOG HD-ΔNLSs. Cytoplasmic and nuclear extracts were subjected to Western blot analysis with anti-EGFP and anti-c-myc antibodies.

predominantly cytoplasmic. These data demonstrated the requirement for NLS motifs for complete nuclear localization and also suggested that additional motif(s) might be present to block nuclear transport since EGFP-fused hNANOG HD-ΔNLSs are small enough to be passively transported into the nucleus.

3.2. Leucine-rich residues may be important for hNANOG HD nuclear export

To identify the precise amino acid sequences of a putative NES motif and examine the effects on nucleocytoplasmic distribution, we performed site-directed mutagenesis of leucine and isoleucine residues to alanines within the putative NES motif ^{125}MQE**L**S-NI**L**N**L**^{134} in an hNANOG HD fragment containing either the N-terminal or C-terminal NLS motif (NLS1 and 2; Fig. 3A). Cytoplasmic and nuclear EGFP expression was observed in both hNANOG HD (92–146) lacking the C-terminal NLS2 (hNANOG HD-ΔNLS2) and hNANOG HD (101–154) lacking the N-terminal NLS1 (hNANOG HD-ΔNLS1) (Fig. 3B). The hNANOG HD-ΔNLS1 had more cytoplasmic localization, and the hNANOG HD-ΔNLS2 had more nuclear localization. These data further validate our previous report that both NLS motifs are required for complete nuclear localization

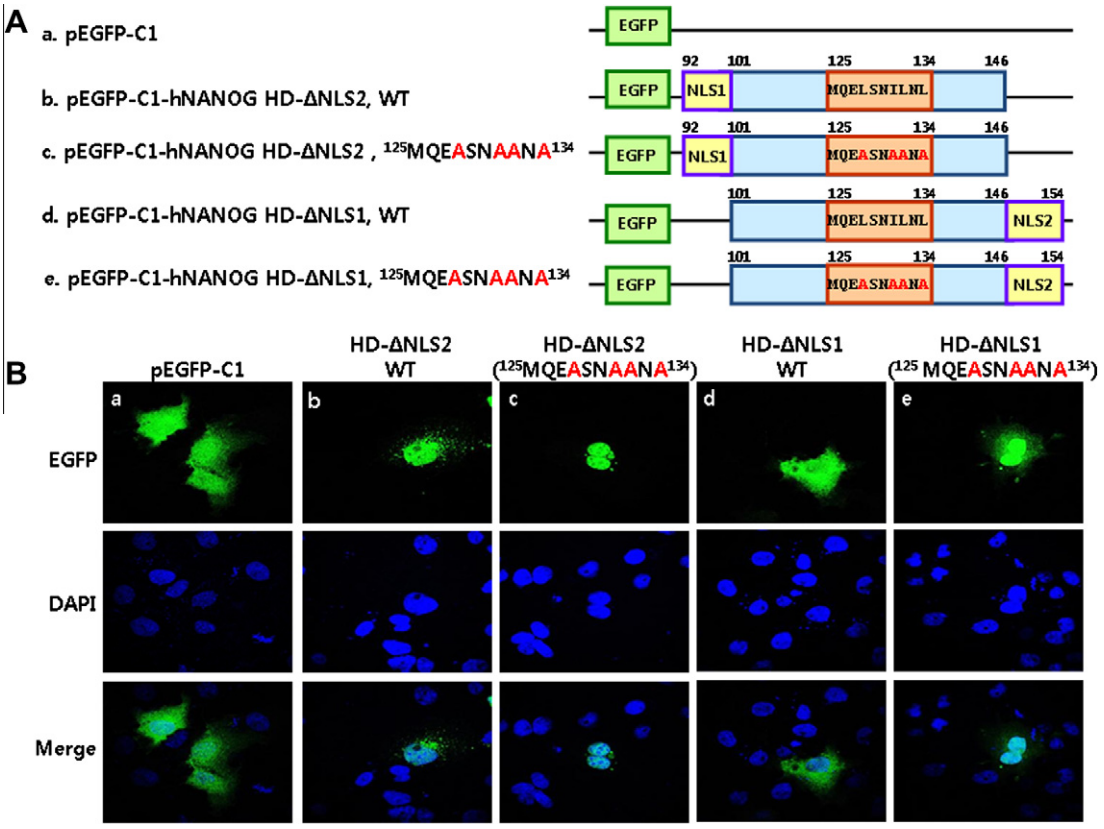

Fig. 3. Site-directed mutagenesis of leucine and isoleucine residues within the hNANOG HD containing either NLS1 or NLS2. (A) Schematic representation of hNANOG HD deletion and site-directed mutants fused to EGFP. (B) Confocal laser scanning microscopy of COS-7 cells transfected with EGFP-tagged fusion proteins and counter-stained with DAPI.

[30]. Nevertheless, in both hNANOG HD-ΔNLS1 and hNANOG HD-ΔNLS2, mutagenesis of leucine and isoleucine residues within the putative NES motif clearly led to a predominant localization in the nucleus. These data indicated that the ^{125}MQE**L**SNI**L**N**L**^{134} sequence functions as an NES in hNANOG HD.

Several studies have reported that a leucine-rich sequence is crucial for nuclear export [31,36–38]. SOX10, which belongs to a family of transcription factors that contain a high mobility group (HMG) DNA binding domain and plays fundamental roles in neural development, has an active leucine-rich NES as well as two NLSs in

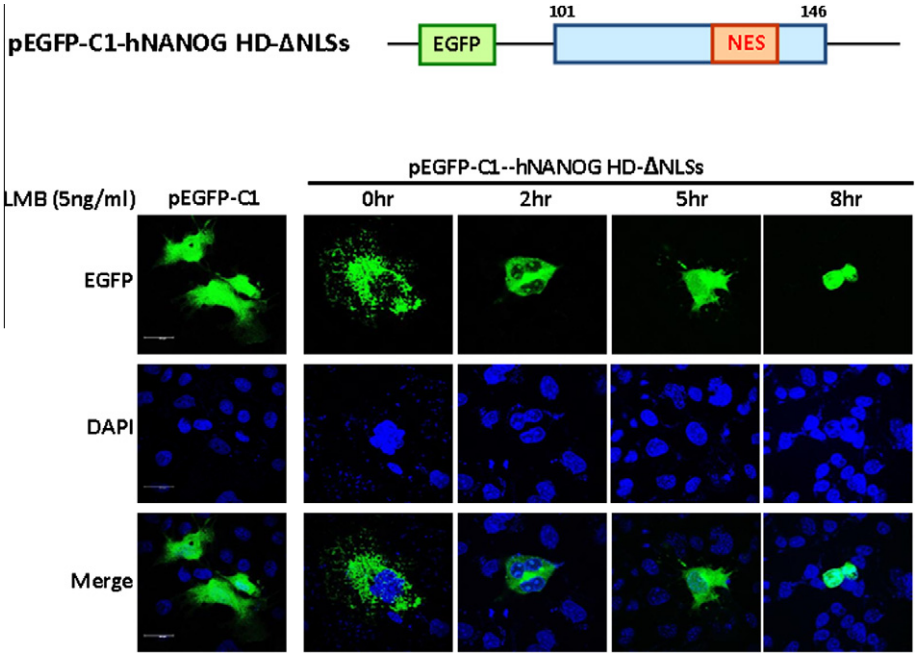

Fig. 4. LMB binding assay. Confocal laser scanning microscopy of COS-7 cells transfected with EGFP-hNANOG HD-ΔNLSs and subsequently treated with LMB (5 ng/ml) for various times (0, 2, 5, and 8 h).

the HMG domain. Mutation of leucines to alanines or treatment of cells with the CRM1-specific nuclear export inhibitor LMB inhibited nuclear export and consequently nucleocytoplasmic shuttling of SOX10 [36]. OCT6 also contains a leucine-rich NES motif as well as an N-terminal NLS within the POU HD, which shares a high degree of similarity with the hNANOG NES motif identified in this study. OCT6 is able to shuttle between nucleus and cytoplasm, and mutation of the OCT6 NES sequence reduced DNA binding and transcriptional activity [31].

3.3. LMB treatment inhibits the hNANOG potent NES motif-mediated nuclear export

The best-characterized nuclear export pathway employs the karyopherin receptor CRM1 (Exportin-1), which interacts with a short, hydrophobic, leucine-rich segment (LxxLxL) within the protein to be translocated [24]. CRM1-mediated export is effectively inhibited by the actinobacterial toxin LMB. To investigate the correlation between the putative hNANOG NES motif and CRM1, COS-7 cells overexpressing hNANOG HD-ΔNLSs containing only the NES motif were treated with LMB (5 ng/ml) for various times (0, 2, 5, or 8 h). In the absence of LMB, the mutant was predominantly cytoplasmic. In the presence of LMB, the mutant accumulated in the nucleus over time, with maximal redistribution by 8 h of LMB treatment (Fig. 4). These data suggest that LMB successfully inhibited the hNANOG potent NES-mediated nuclear export, suggesting that the leucine-rich motif may function in CRM1-mediated nuclear export of hNANOG.

Homeodomain proteins are important transcription factors and play a critical role in cell proliferation and differentiation [39]. A recent report suggested that pluripotency could be recreated by direct reprogramming, which is dependent on the HD-containing transcription factor NANOG. Moreover, this reprogramming capacity exists in a unique DNA binding domain that is conserved in a variety of species [29], suggesting that this highly conserved HD is a critical and fundamental domain that enables NANOG to exert a variety of functions during vertebrate development. hNANOG has two NLS motifs within the HD and both are required for complete nuclear localization [30]. In this study, hNANOG was shown to also contain a potent NES motif that plays an important role in nuclear export. In addition, the NES motif may be functionally involved in CRM1-dependent nuclear export of hNANOG, although further studies will be required to understand the mechanisms regulating nuclear shuttling.

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