Identification and characterization of the nuclear import and export signals of the mammalian Ste20-like protein kinase 3

Wan-Shu Lee[a], Chiung-Yueh Hsu[a], Pei-Ling Wang[a], Chi-Ying Fred Huang[b], Chia-Hua Chang[a], Chiun-Jye Yuan[a,*]

[a] *Department of Biological Science and Technology, National Chiao Tung University, 75 Po-Ai Street, Hsinchu 300, Taiwan, ROC*
[b] *Division of Molecular and Genomic Medicine, National Health Research Institutes, Taipei 115, Taiwan, ROC*

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Abstract Mst3, a human Ste20-like protein kinase, has been recently demonstrated to undergo a caspase-mediated cleavage during apoptosis. The proteolytic cleavage of the C-terminus of Mst3 caused nuclear translocation of its kinase domain. This work provides evidence that Mst3 may contain a bipartite-like nuclear localization sequence (NLS) at the C-terminus of its kinase domain (residues 278–292). The removal of NLS from the kinase domain of Mst3 led to the cytoplasmic accumulation of EGFP-Mst3$^{\Delta 277}$. The presence of nuclear exporting signals in the Mst3 was also demonstrated by leptomycin B-treatment and serial deletion of the C-terminal regulatory domain of Mst3. A nuclear export signal was also postulated to be in the regions of amino acids 335–386. In conclusion, Mst3 contains both NLS and NES signals, which may cooperate to control the subcellular distribution of Mst3.

Keywords: Mst3; Ste20; Nuclear localization sequence; Nuclear exporting signal; Nuclear trafficking

1. Introduction

Ste20, a sensory protein kinase upstream in the MAP kinase pathway, plays an important role in regulating the pheromone-response pathway of budding yeast [1]. Twenty eight mammalian counterparts of Ste20 have been identified so far and can be categorized into two main subfamilies, based on their amino acid sequences and the physical location of their kinase domains in the polypeptide chains. Each p21-Rac/Cdc42 activated kinase (PAK) family member contains a kinase domain at its C-terminus, while its N-terminal regulatory domain has a binding site for a small GTPase [2]. The germinal center kinase (GCK) family, however, has an N-terminal kinase domain and a C-terminal regulatory domain [3]. The Mst (mammalian SPS1/Ste20-like) protein kinases, Mst1, -2, -3, and -4, are members of this latter subfamily [3–8]. Based on the similarity between the sequences inside and outside their kinase domains, and additional information obtained from *Drosophila* and/or *C. elegant* orthologs, the Mst family kinases can be further divided into the GCK-II subgroup (Mst1 and Mst2) and the GCK-III subgroup (Mst3 and Mst4) [9].

Mst3 was recently shown to undergo caspase-mediated proteolytic cleavage during apoptosis [10]. Cleavage of Mst3 by caspases yields two fragments, Mst3/N and Mst3/C, with sizes of around 35 and 15 kDa, respectively. The Asp313 was identified later to be the cutting site for caspases. Interestingly, the removal of the C-terminus by mutagenesis results in nuclear translocation of the Mst3$^{WT\Delta 314}$. This observation supports the hypothesis that the Mst3$^{WT\Delta 314}$ may contain a nuclear localization sequence (NLS), which is exposed upon caspase cleavage and may be a prerequisite for its role in apoptosis. The presence of a putative nuclear export signal (NES) in the C-terminal regulatory domain of Mst3 has also been postulated [10].

This study shows that Mst3 contained both NLS and NES signals. The NLS is present at the C-terminus of the Mst3 kinase domain, whereas the C-terminal regulatory domain contains at least one NES signal. The evidence presented in this work further reveals that the dynamic subcellular distribution of Mst3 can be mediated by coordination between these two signals.

2. Materials and methods

2.1. Materials

Restriction enzymes were purchased from New England Bio-Labs. Fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM), penicillin G, streptomycin, and Lipofectamine were obtained from Life Technologies. Leptomycin B (LMB) was obtained from Roche. The oligonucleotides were synthesized by BioBasic Inc., UK. All other reagents used were of reagent grade.

2.2. Cell lines and cell culture

The human cervical carcinoma cell line (HeLa) was maintained in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated in a humidified 37 °C incubation chamber that contained 5% CO_2. The cells were transiently transfected using Lipofectamine^{TM} as directed by the manufacturer (GIBCO Life Technologies).

2.3. Plasmid construction and site-directed mutagenesis

As shown in Fig. 1, Mst3 deletion mutants, including Mst3$^{WT\Delta 417}$ (residues 1–416), Mst3$^{WT\Delta 386}$ (residues 1–385), Mst3$^{WT\Delta 337}$ (residues 1–336), Mst3$^{WT\Delta 295}$ (residues 1–294), and Mst3$^{WT\Delta 278}$ (residues 1–277), as well as Mst3 fragments, such as Mst3$^{312-431}$ (residues 312–431), Mst3$^{335-431}$ (residues 335–431), and Mst3$^{385-416}$ (residues 385–416),

* Corresponding author. Fax: +886-3-572-9288.
E-mail address: cjyuan@mail.nctu.edu.tw (C.-J. Yuan).

Abbreviations: GFP, green fluorescence protein; Mst, mammalian Ste20-like protein kinase; NLS, nuclear localization sequence; NES, nuclear exporting signal; LMB, leptomycin B

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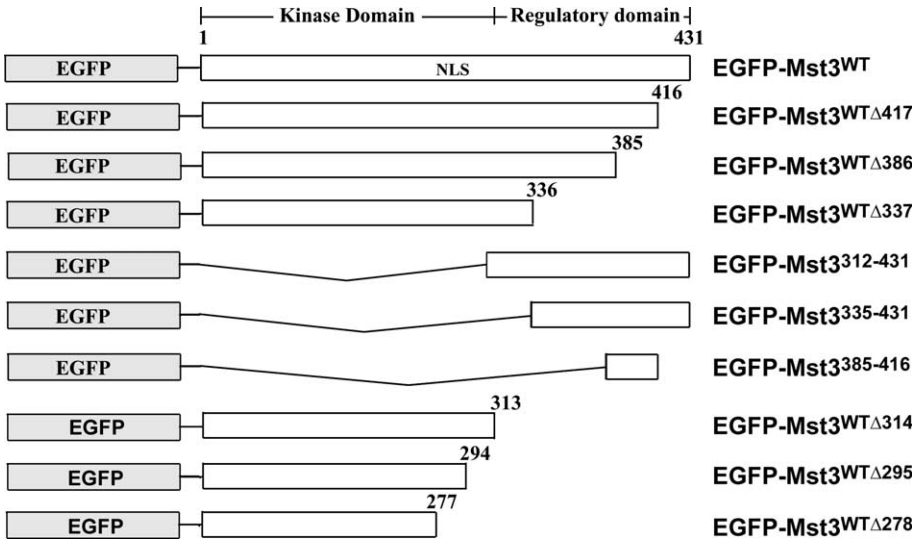

Fig. 1. EGFP-tagged wild-type Mst3 and its mutants. NLS is the proposed nuclear localization sequence of Mst3.

were generated by polymerase chain reaction. These cDNA fragments, with a stop-codon added to their 3′-end, were inserted into the pEGFP-C2 vector (CLONTECH) between *Hin*dIII and *Eco*RI sites.

The NLS-deficient mutants of $Mst3^{WT\Delta314}$, $Mst3^{\Delta314(278A/279A)}$ (with K278 and K279 replaced by Ala), $Mst3^{\Delta314(291A/292A)}$ (with K291 and R292 replaced by Ala), and $Mst3^{\Delta314(4A)}$ (with K278, K279, K291, and R292 replaced by Ala), were generated according to the QuikChange™ Site-Directed Mutagenesis protocol (Stratagene). The primers 5′-CGACAGGTACGCGGCATGGAAG-3′ and 5′-CGGCCTTCC-ATGCCGCGTACCTGTCG-3′ were used to yield $Mst3^{\Delta314(291A/292A)}$. The primers 5′-CTACGCAATGCAGCGGCAACTTCCTACTTGA-CCG-3′ and 5′-CGGTCA-AGTAGGAAGTTGCCGCTGCATTG-CGTAG-3′ were used to obtain $Mst3^{\Delta314(278A/279A)}$. The DNA sequences of subcloned cDNAs were confirmed using an automatic DNA sequencer ABI-377 (Perkin–Elmer).

2.4. Fluorescence microscopy

The location of the EGFP in living cells was monitored using an inverted fluorescence microscope (Leica DELM model). The HeLa cells were cultured in a 35 mm cultural dish with a sterilized cover glass. After transfection and recovery for 18 h, cells were fixed with 4% paraformaldehyde in pH 7.4 phosphate buffer saline at 4 °C for 20 min. To counterstain the nucleus, the paraformaldehyde-fixed cells were treated with 0.2% Triton X-100 at 4 °C for 20 min and then stained with 200 ng/ml DAPI (4′,6′-diamidino-2-phenylindole) at 4 °C for 20 min. The raw photographic images were acquired using a Kodak digital camera and processed with Free PLUS software (Media Cybernetics, Silver Spring, MD, USA). Each experiment was conducted under the same conditions at least twice.

3. Results

3.1. Identification of NLS in Mst3

An earlier work has shown that the C-terminus-truncated Mst3 mutant, $Mst3^{WT\Delta314}$, exhibits nuclear translocation [10]. Upon sequence alignment, a short basic residue-rich region, ^{278}KKTSYLTELIDRYKRWK^{294}, in Mst3 was found to resemble the bipartite-type NLS [11,12]. To verify that this sequence is indeed the NLS, EGFP-tagged $Mst3^{WT}$, $Mst3^{WT\Delta314}$, $Mst3^{WT\Delta295}$, and $Mst3^{WT\Delta278}$ (Fig. 1) were transiently expressed in 293T cells. As expected, EGFP-$Mst3^{WT\Delta295}$, like EGFP-$Mst3^{WT\Delta314}$, was present in the nucleus, whereas EGFP-$Mst3^{WT}$ and EGFP-$Mst3^{WT\Delta278}$ were mostly distributed in the cytoplasm (Fig. 2A). The EGFP alone was found to be distributed in both the cytoplasm and the nucleus (Fig. 2A), probably because a small protein such as EGFP (about 26 kDa) can slip through the nuclear pores by passive diffusion. These results reveal that the NLS of Mst3 may be present in the region between amino acids 278 and 294.

Bipartite NLS is characterized by two basic-residue clusters in the sequence. When the key residues in proposed NLS, Lys278, Lys279, Lys291, and Arg292, were changed to Ala by site-directed mutagenesis (Table 1), most EGFP-$Mst3^{\Delta314(4A)}$ was found to be present in the cytoplasm (Fig. 2B), whereas partial mutation of NLS caused impaired nuclear localization of $Mst3^{\Delta314(278A/279A)}$ and $Mst3^{\Delta314(291A/292A)}$ (Fig. 2B and Table 1). These results reveal that the Mst3 contains a NLS that is most likely located between amino acids 278 and 292. Although the involvement of K294 is unknown, it may make little or no contribution to the NLS, according to the results in Fig. 2B and Table 1.

3.2. Leptomycin B inhibits nuclear export of Mst3

Several lines of evidence demonstrate that nucleocytoplasmic translocation of proteins can be mediated by both NLS and NES signals [13–16]. Although NLS has been shown to be a prerequisite for the nuclear localization of Mst3, the presence of NES that mediates the cytoplasmic distribution of full-length Mst3 cannot be ruled out. To examine this possibility, the EGFP-$Mst3^{WT}$ transiently expressed-HeLa cells were treated with 10 ng/ml LMB, an inhibitor of the Crm1-mediated export. As shown in Fig. 3, the originally cytoplasmic-localized EGFP-$Mst3^{WT}$ began to accumulate in the nucleus after LMB treatment. This result suggests that Mst3 may contain a NES-like sequence, which is functionally active and that the Crm1/exprotin-dependent nuclear exporting mechanism may be responsible for the nuclear export of the full-length Mst3.

3.3. NES controls the subcellular localization of Mst3

The amino acid sequence alignment revealed that the C-terminal regulatory domain of Mst3 may contain two Leu/Ile-rich sequences that are characteristic of NES signals (Fig. 4A). Therefore, the C-terminal regulatory domain of EGFP-tagged

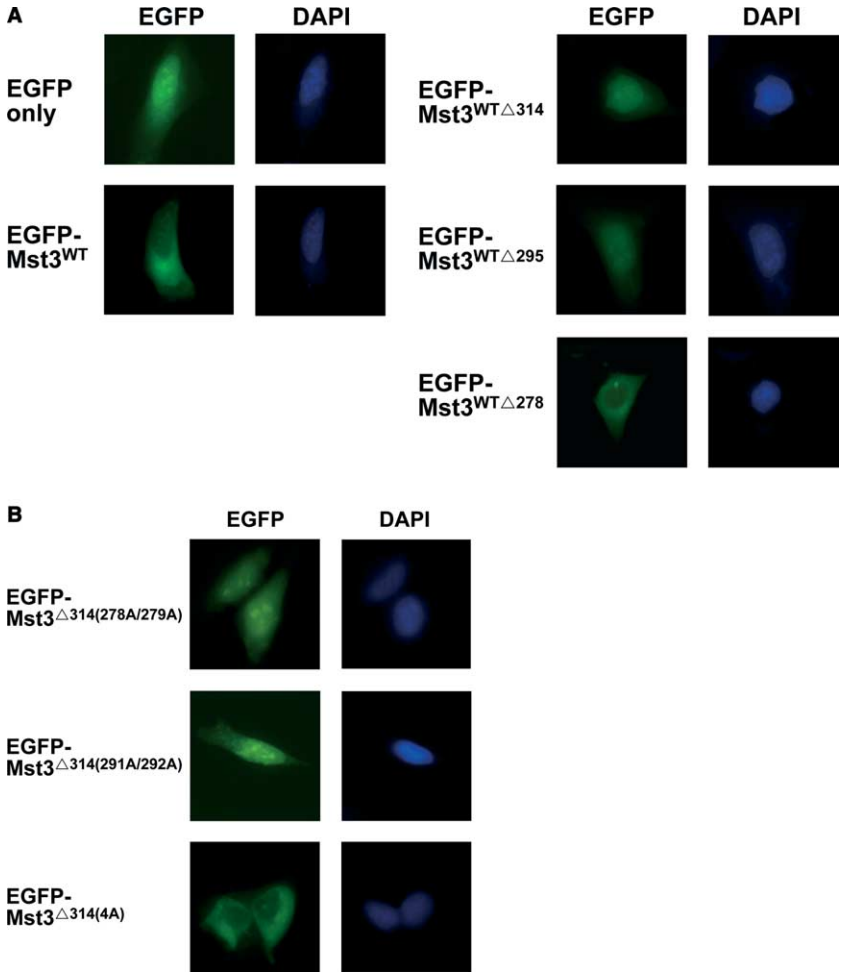

Fig. 2. NLS in Mst3 was revealed by deletion analysis. (A) HeLa cells transiently transfected with EGFP-Mst3^{WT}, EGFP-Mst3$^{WT\Delta 314}$, EGFP-Mst3$^{WT\Delta 295}$, or EGFP-Mst3$^{WT\Delta 278}$, were monitored under a fluorescent microscope. (B) HeLa cells transiently transfected with Mst3$^{\Delta 314(278A/279A)}$, Mst3$^{\Delta 314(291A/292A)}$, or Mst3$^{\Delta 314(4A)}$ were monitored under a fluorescent microscope. Nuclei were stained with DAPI following transfection, as described in Section 2.

Table 1
The subcellular distribution of Mst3 mutants

EGFP-Mst3 mutants	Amino acid sequence[a]	Nucleus (N) or Cytoplasm (C)
Mst3-NLS	^{278}KKTSYLTELIDRYKRWK^{294}	N
Mst3$^{\Delta 314(278A/279A)}$	AA	C = N
Mst3$^{\Delta 314(291A/292A)}$	AA	N>C
Mst3$^{\Delta 314(4A)}$	AA AA	C

[a] The amino acid sequence of human Mst3 between residues 278 and 294 is shown. The basic residue clusters in NLS are underlined. N and C represent the cellular location of Mst3 mutants in nucleus (N) and cytoplasm (C), respectively.

Mst3 was serially deleted (Fig. 1) and transiently expressed in HeLa cells. As shown in Fig. 4B, green fluorescence began to accumulate in the nucleus when the regions between amino acids 386–416 and 337–385 were lost. This result demonstrates that the C-terminal regulatory domain of Mst3 contains the NES. To further identify the NES in Mst3, various EGFP-tagged C-terminal fragments of Mst3 (Fig. 1) were constructed and transiently expressed in the HeLa cells. Interestingly, a significant amount of the Mst3$^{312–431}$- or Mst3$^{335–431}$-tagged EFGP was found to accumulate in the cytoplasm, while Mst3$^{385–416}$-tagged EGFP remained in the nucleus. These results suggest that Mst3 may contain a NES, which is in the region between amino acids 335 and 385.

4. Discussion

Nucleocytoplasmic transport is an important process that provides an opportunity for protein kinases to specifically regulate genes and cellular functions by gaining controlled access to their downstream targets in the nucleus [17,18]. The calcium/CaM-dependent protein kinase IV (CaMK-IV), for

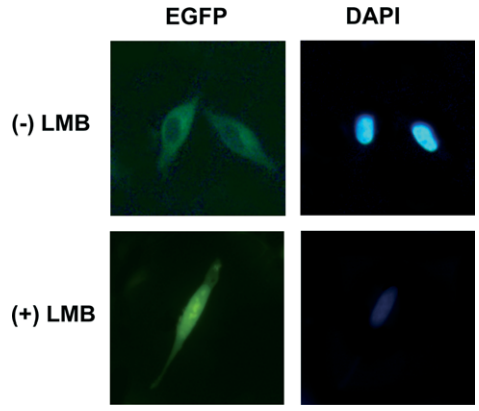

Fig. 3. Effect of LMB on the subcellular distribution of EGFP-Mst3WT. HeLa cells, expressing EGFP-Mst3^{WT}, were treated with (+LMB) or without (−LMB) 10 ng/ml leptomycin B at 37 °C for 1 h. Cells were then fixed and stained with DAPI. Observations were made under a fluorescence microscope. LMB treatment resulted in the nuclear localization of EGFP-Mst3^{WT}, as identified by EGFP fluorescence.

example, translocates to the nucleus and phosphorylates the cyclic AMP response element-binding protein (CREB) [19,20]. The nuclear translocation of p42/p44 MAPK is essential for growth factor-induced DNA replication [21,22] and cell transformation [23].

The bi-directional nuclear trafficking of proteins is usually initiated by the recognition and association of nuclear transport receptors, such as importins and Crm1, to short stretches of amino acids known as the NLS [11–14] and the NES [13–16,24,25] in the proteins ready to be transported. As shown previously, removing the C-terminal regulatory domain of Mst3 can change the subcellular distribution of Mst3 [10]. Although the molecular mechanism for the nuclear translocation of Mst3 was unclear, the presence of the NLS was postulated. The results of this work reveal that an NLS is present at the C-terminus of Mst3 kinase domain. The proteolytic cleavage of Mst3 during apoptosis may expose the NLS signal and subsequently mediate the translocation of truncated Mst3 to the nucleus. Although no general consensus sequence is present, NLS seems to be characterized by a cluster of basic amino acids [12,26,27]. Upon amino acid alignment, a bipartite type NLS that contains two basic-residue clusters [11,12] in Mst3 is postulated. The mutation of the basic residues in the NLS markedly affected the subcellular distribution of Mst3 in cells (Fig. 2B and Table 1).

Interestingly, the full-length Mst3 moved from the cytoplasm to the nucleus, when the cells were treated with LMB, an inhibitor of the Crm1-mediated export process. This finding suggests that Mst3 may contain a NES signal. This hypothesis was demonstrated by studying the subcellular localization of the C-terminal deletion mutants of Mst3 (Fig. 4). Upon amino acid sequence alignment, the presence of two possible NESs in the regions of amino acids 335–385 and 385–416 was postulated (Fig. 4A). The region between residues 335 and 385 is probably the one that contains NES, based on the C-terminal deletion analysis of Mst3 (Fig. 4B). Although EGFP-Mst4$^{\Delta 386}$ also accumulated in the nucleus, indicating that the residues 385–416 may contain an NES, this region did not exhibit NES activity when fused with EGFP, perhaps because the EGFP-Mst3$^{\Delta 386}$ or the EGFP-Mst3$^{385–416}$ does not fold properly.

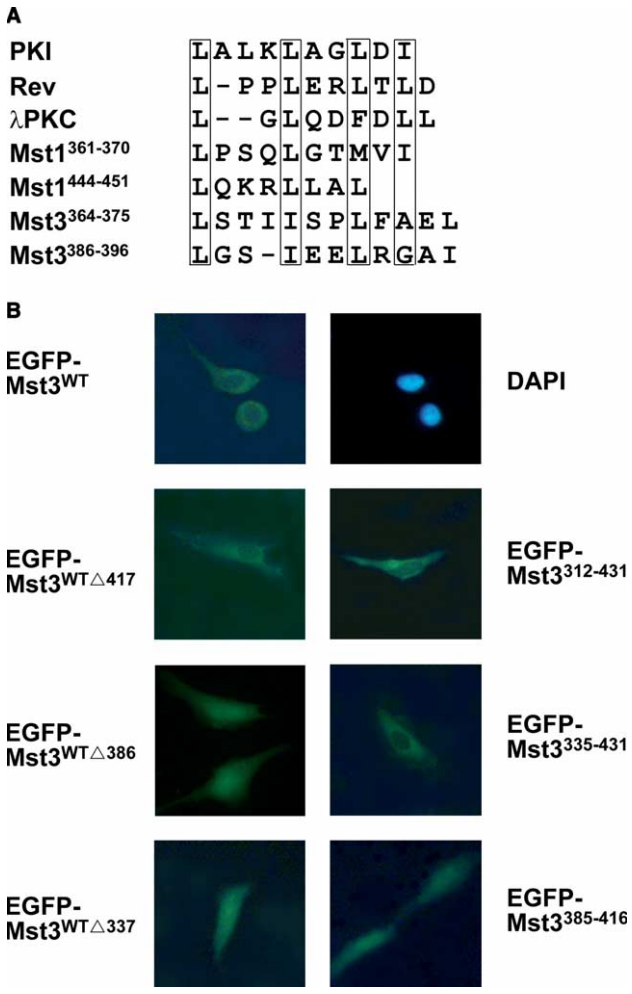

Fig. 4. Mst3 contains NES signals that promote its nuclear translocation. (A) Comparison of proposed NES sequences with those in protein kinase A inhibitor (PKI) [16], human immunodeficiency virus-1 Rev protein (Rev) [15], λPKC [24], and Mst1 [25]. (B) HeLa cells were transiently transfected with EGFP-Mst3^{WT}, EGFP-Mst3$^{WT\Delta 417}$, EGFP-Mst3$^{WT\Delta 386}$, EGFP-Mst3$^{WT\Delta 337}$, EGFP-Mst3$^{312–431}$, EGFP-Mst3$^{335–431}$, or EGFP-Mst3$^{380–416}$. Eighteen hours after transfection, cells were fixed and stained with DAPI as described in Section 2. The subcellualr distribution of Mst3 mutants was monitored by EGFP fluorescence.

Therefore, the presence of an NES in the region 385–416 was not established. Short stretch ^{364}LSTIISPLFAEL^{375} was found to be highly homologous to the known NES sequences (Fig. 4A), so further investigations using site-directed mutagenesis may help to determine the presence of NES in this region.

In summary, Mst3 may contain both NLS and NES, which may cooperate in mediating the dynamic subcellular distribution of Mst3 in cells. In the resting state, the NLS of wild-type Mst3 may be shielded by the C-terminal regulatory domain. Therefore, the NES dominates and keeps Mst3 out of the nucleus. Only when caspase removes the C-terminal regulatory domain of Mst3, the NLS exposed and the nuclear translocation of Mst3 induced. Alternatively, the predominant cytoplasmic steady-state localization of inactive Mst3 may result from the fact that the rate of nuclear export exceeds that of import. Removing the regulatory domain that contains the

NESs with caspase cleavage simply shifts the equilibrium between the import and export of the kinase domain toward the nuclear side. This assertion is demonstrated by the result that the full-length Mst3 appears to shuttle continuously between the nucleus and the cytoplasm, since LMB treatment promotes nuclear accumulation (Fig. 3).

Mst3 has been shown to play an important role in triggering apoptotic cell death. The nuclear localization of Mst3 may promote cell apoptosis, as suggested by a previous work, in which $Mst3^{WT\Delta 314}$ induced more cell death than did $Mst3^{WT}$ [10]. Crm1-mediated export not only provides a mechanism for the cytoplasmic confinement of full-length Mst3, but also provides a means of spatially separating the active kinase domain from the regulatory domain during apoptosis. The identification and characterization of the NLS, and the elucidation of potential NESs are important steps toward understanding the mechanism of Mst3 action.

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