

# Nucleo-cytoplasmic shuttling of human Kank protein accompanies intracellular translocation of $\beta$ -catenin

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## Summary

The human Kank protein has a role in controlling the formation of the cytoskeleton by regulating actin polymerization. Besides the cytoplasmic localization as reported before, we observed the nuclear localization of Kank in OS-RC-2 cells. To uncover the mechanism behind this phenomenon, we focused on the nuclear localization signal (NLS) and the nuclear export signal (NES). We found one NLS (NLS1) and two NESs (NES1 and NES2) in the N-terminal region of Kank-L that were absent in Kank-S, and another NLS (NLS2) and NES (NES3) in the common region. These signals were active as mutations introduced into them abolished the nuclear import (for NLS1 and NLS2) or the nuclear export (for NES1 to NES3) of Kank. The localization of Kank in the cells before and

after treatment with leptomycin B suggested that the transportation of Kank from the nucleus to the cytoplasm was mediated by a CRM1-dependent mechanism. TOPFLASH reporter assays revealed a positive relationship between the nuclear import of Kank and the activation of  $\beta$ -catenin-dependent transcription. Kank can bind to  $\beta$ -catenin and regulate the subcellular distribution of  $\beta$ -catenin. Based on the findings shown here, we propose that Kank has multiple functions in the cells and plays different roles in the cytoplasm and the nucleus.

Key words: Kank,  $\beta$ -catenin, Nucleo-cytoplasmic shuttling, Nuclear localization signal, Nuclear export signal

## Introduction

We previously identified a novel gene named *Kank* in the human genome, which encodes a protein with an ankyrin-repeat domain at its C-terminus (Sarkar et al., 2002). Kank protein is predominantly distributed in the cytoplasm in several human kidney cell lines and seems to have a role in controlling the formation of the cytoskeleton through the polymerization of actin (Sarkar et al., 2002; Rodley et al., 2003; Roy et al., 2005). A recent study on the alternative splicing of *Kank* mRNA revealed two isoforms of Kank protein, Kank-L and Kank-S, and Kank-L showed reduced expression in tumor kidney tissues compared with the corresponding normal tissues (Wang et al., 2005). Recently, we observed the nuclear localization of Kank proteins in a tumor kidney cell line, OS-RC-2 cells. This suggests that Kank proteins might be able to enter the nucleus. As the subcellular localization of a protein often controls its function (Gama-Carvalho and Carmo-Fonseca, 2001; Suetsugu and Takenawa, 2003; Kong and Kedes, 2004; Jensik et al., 2004; Kurooka and Yokota, 2005), we investigated the subcellular localization of Kank protein.

In the process of searching for the binding proteins of Kank, we found that  $\beta$ -catenin, an important component in the Wnt signaling pathway, could bind to Kank protein (unpublished results). The Wnt signaling pathway is involved in various differentiation events during embryonic development and leads to the formation of tumors when aberrantly activated (Giles et al., 2003; Lustig and Behrens, 2003). One of the downstream effectors of the pathway is  $\beta$ -catenin, which moves from the cytoplasm to the nucleus, interacts with specific transcription

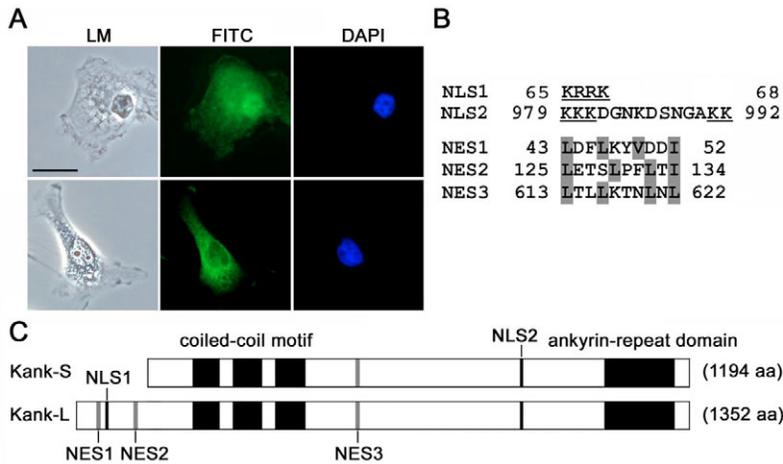
factors, T-cell factor/lymphoid enhancing factor (TCF/LEF), and activates their target genes (Giles et al., 2003; Lustig and Behrens, 2003; Logan and Nusse, 2004). In unstimulated cells, free cytosolic  $\beta$ -catenin is maintained at a low level by serine/threonine phosphorylation of  $\beta$ -catenin, followed by ubiquitination and degradation (Logan and Nusse, 2004). Inhibition of phosphorylation or mutation at the phosphorylation sites of  $\beta$ -catenin increases the chance for survival of  $\beta$ -catenin in the cytoplasm and accumulation in the nucleus (Morin et al., 1997; Logan and Nusse, 2004).

In this study, we demonstrated that Kank protein shuttles between the nucleus and the cytoplasm via a process mediated by nuclear import and export signals, and the nuclear export of Kank depends on the chromosome maintenance region 1 (CRM1) protein. In addition, we detected the binding between Kank and  $\beta$ -catenin in human cells. Our data also suggest that nucleo-cytoplasmic shuttling of Kank accompanies the translocation of  $\beta$ -catenin, which potentiates  $\beta$ -catenin-dependent transcription.

## Results

### Intracellular distribution of Kank protein

The nuclear localization of Kank protein was examined by immunostaining using cultured human kidney cancer cell lines, OS-RC-2 and VMRC-RCW cells (Fig. 1A). Kank protein was present in the nucleus as well as in the cytoplasm in OS-RC-2 cells, although it was predominantly localized in the cytoplasm in VMRC-RCW cells as reported previously (Sarkar et al., 2002). This suggests that there is a mechanism of cell-type



**Fig. 1.** Intracellular distribution of Kank protein and locations of potential NLS and NES motifs.

(A) Intracellular distribution of Kank protein. The localization of endogenous Kank protein in OS-RC-2 (upper) and VMRC-RCW (lower) cells was examined by immunostaining with an FITC-conjugated antibody against Kank protein. The nuclei were stained with DAPI. LM, light microscopy. Bar, 20  $\mu$ m. (B) The sequences of NLS (NLS1 and NLS2) and NES (NES1 to NES3) motifs predicted in Kank-L. The conserved sites are underlined (NLSs) or shadowed (NESs), which were substituted by alanines in the respective mutants. (C) The maps of Kank-L and Kank-S.

specific nuclear localization of Kank protein, which is mediated by nuclear localization signals (NLSs) and nuclear export signals (NESs). We found one NLS motif (NLS1) predicted by the PSORT II software and three NES motifs (NES1 to NES3) in Kank-L predicted by the NetNES 1.1 software (Fig. 1B and Fig. 1C). Although another NLS and two other NESs were also predicted by these software packages, the data are not shown here because they all turned out to be inactive. NLS2 was predicted manually by comparison with typical bipartite NLS motifs because it couldn't be recognized by PSORT II software.

#### Identification of NLSs in Kank-L

We first prepared GST-fused proteins to examine NLS1 located in the N-terminal region of Kank-L (Fig. 2A). To make a mutation in the NLS1 motif, the amino acids KRRK were substituted with AAAA. The wild-type N-terminal GST-fused form of Kank-L (Nt-WT-GST) was distributed almost equally in the nucleus and cytoplasm in most cells, while the GST-fused construct containing mutant NLS1 (NLS1m-GST) was predominantly detected in the cytoplasm (Fig. 2B and Fig. 2C). This suggested that NLS1 is a functional NLS and the fusion protein cannot be transported into the nucleus without this signal. Since the other NLS motif (NLS2) was located at the region close to the C-terminus (amino acids 979 to 992), we examined its function with a construct (NLS1m-NESnull-GST-NLS2), which was made by fusing NLS2 to the N-terminal fragment of Kank-L with function-less NLS1 and NESs (NLS1m-NESnull-GST) (Fig. 2D). As the motif clearly contributed to the nuclear localization of the Kank fragment (Fig. 2E and Fig. 2F), NLS2 is likely to be another functional NLS.

#### Identification of NESs in Kank-L

We next prepared N-terminal GST-fused versions of Kank-L with mutations in either the NES1 or NES2 motif (Fig. 3A). Note that NES1 and NES2 were located close to NLS1 and these constructs contained NLS1. In the mutant constructs, the region of amino acids LDFLKYVDDI was substituted with ADFAKYADDA (NES1m-GST) or the region LETSLPFLTI was substituted with AETSAPFATA (NES2m-GST). As shown in Fig. 3B (a summary in Fig. 3C), both the NES1 and NES2 mutants were predominantly found in the nucleus although the

wild-type construct was distributed both in the nucleus and in the cytoplasm. These results suggested that NES1 and NES2 were both functional NESs for the transportation of the fusion protein from the nucleus to the cytoplasm.

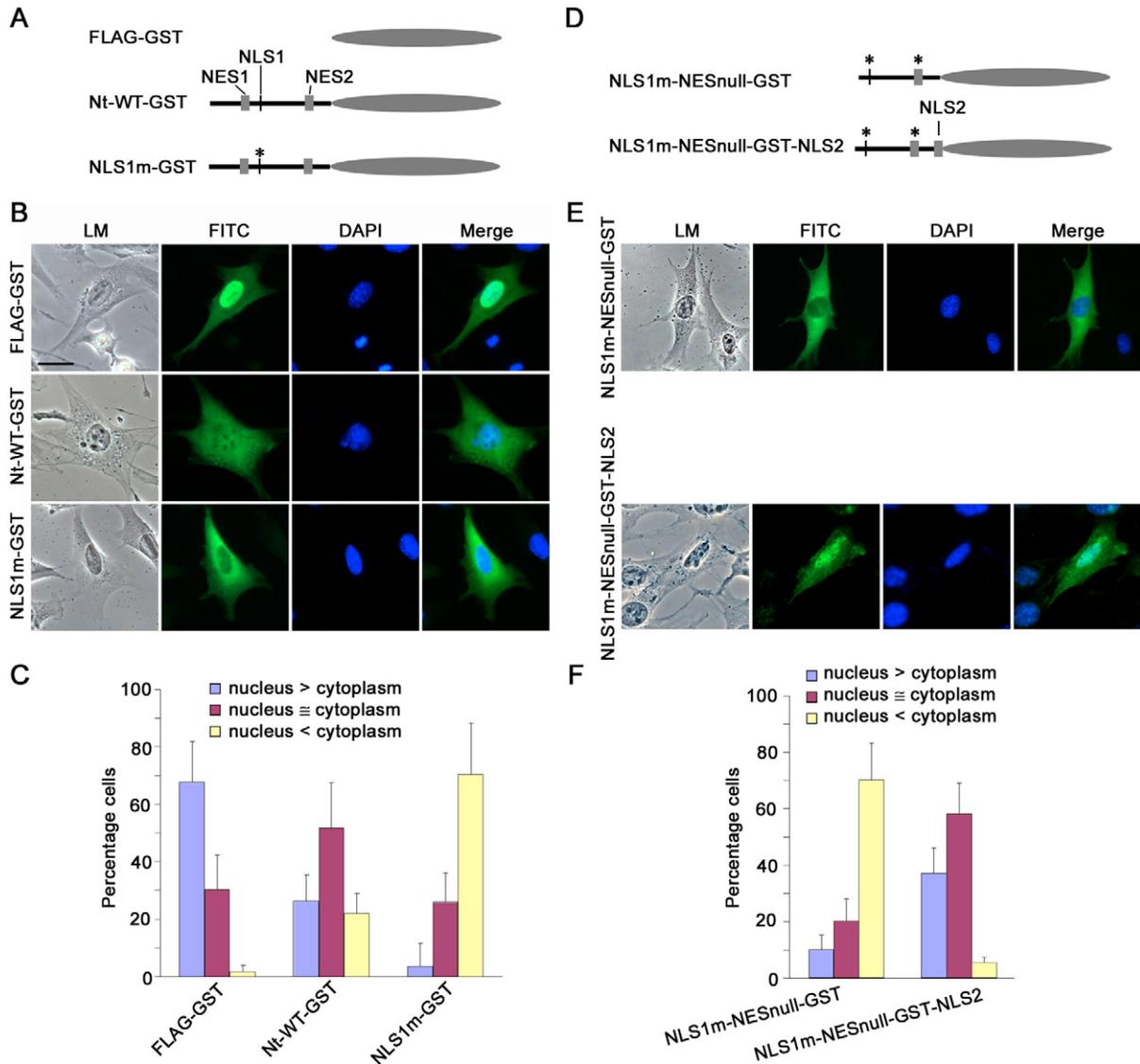
Since NES3 is localized far from NLS1, we used a different strategy to analyze its function. We prepared a GST-fused protein without NES1 but with NLS1 and a mutant NES2 (Fig. 3D). This fusion construct was predominantly distributed to the nucleus because it lacked a functional NES (Fig. 3E; a summary in Fig. 3F). However, after insertion of the region containing the NES3 motif (amino acids LTLKTNLNL) into this construct, the fusion protein was predominantly localized to the cytoplasm (Fig. 3E). This suggested that NES3 was functional for the transportation of the fusion protein from the nucleus to the cytoplasm.

#### Confirmation of the NLSs and NESs in the full-length Kank-L

As both the NLSs and NESs were examined using GST-fused proteins, we examined whether these NLSs and NESs can function in the full-length Kank-L (Fig. 4). For this purpose, we prepared full-length constructs of Kank-L with mutations in NLSs (NLSm) or NESs (NESm) (Fig. 4A). Since mutations in a single NLS or NES did not result in a clear change in the localization (data not shown), we prepared mutant NLS and NES constructs in which all NLSs or NESs were mutated (NLSm and NESm, respectively). NLSm showed a much greater cytoplasmic localization of Kank-L than the wild type of Kank-L (Kank-L-WT), whereas NESm showed a clear nuclear localization (Fig. 4B and Fig. 4C), confirming that these signals function in the full-length Kank-L. To exclude the possibility that the cytoplasmic localization of NLSm resulted from rapid exonuclear transportation by NESs as seen with Kank-L-WT, the cells were treated with leptomycin B (LMB), which blocks the nuclear export (described below). After the treatment with LMB, the subcellular localization of NLSm was still almost the same as untreated cells (Fig. 4C). This suggests that Kank-L protein possesses only two functional NLSs, as reported here.

#### Effect of leptomycin B on the subcellular localization of Kank protein

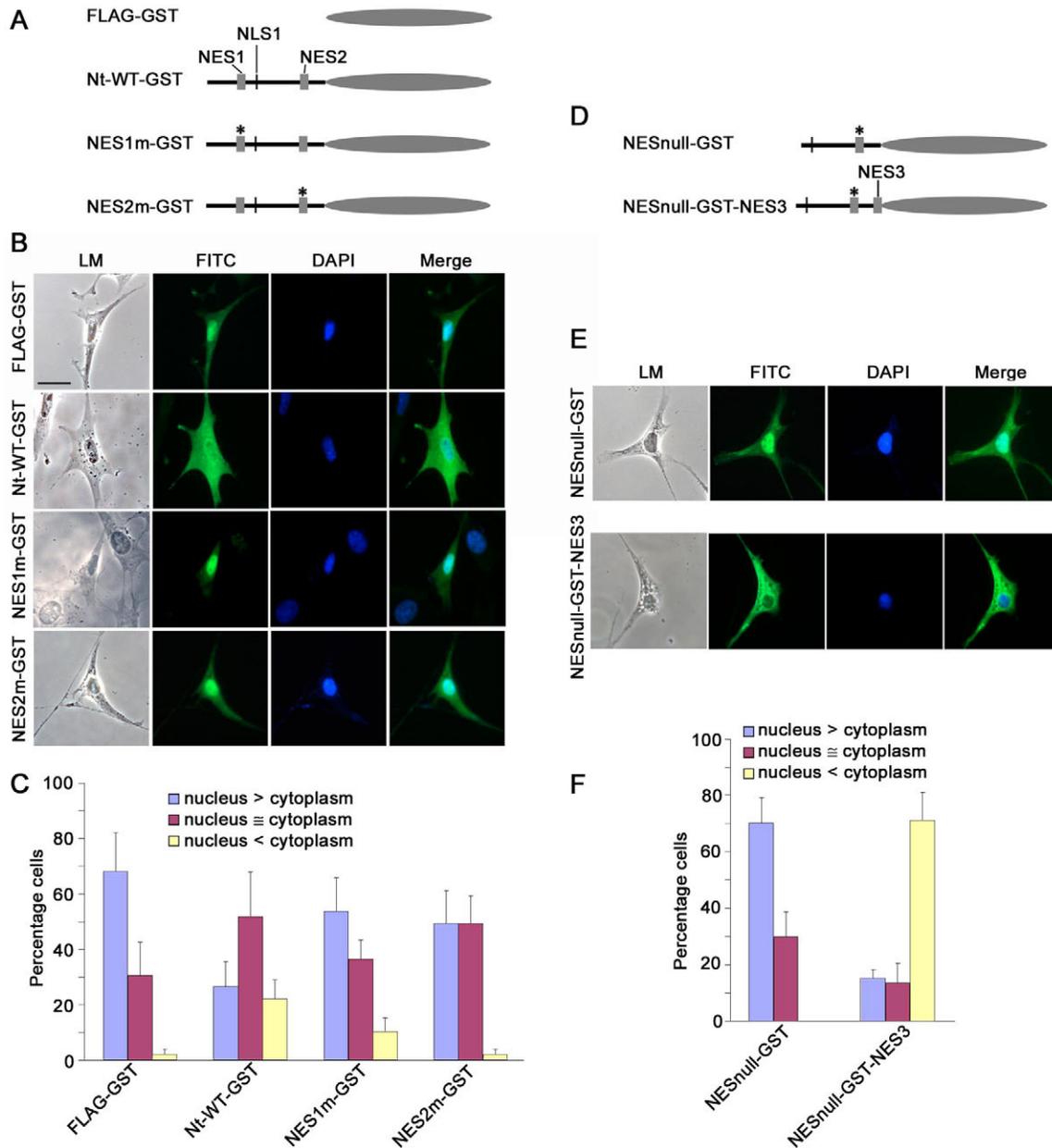
It is known that a protein containing an NES can be transported



**Fig. 2.** Identification of NLSs. (A) Schematic structures of FLAG-tagged GST (FLAG-GST) or GST constructs of FLAG-tagged N-terminal Kank with wild-type (Nt-WT-GST) or mutant NLS1 (NLSm-GST). (B) Representative images of NIH3T3 cells transiently transfected with the constructs shown in (A). The localization of FLAG-tagged GST-fused proteins was monitored by immunostaining with an FITC-conjugated antibody and the nucleus was stained with DAPI. LM, light microscopy. (C) Quantification of the cells according to the localization of GST-fused proteins. The cells with FITC signals predominantly in the nucleus or in the cytoplasm, or located equally in both, were classified and scored respectively. (D) Schematic structures of GST constructs of FLAG-tagged N-terminal Kank with mutant NLS1 and NESs (NLS1m-NESnull-GST), or mutant NLS1 and NESs, and intact NLS2 (NLS1m-NESnull-GST-NLS2). (E) Representative images of NIH3T3 cells transiently transfected with the fusion constructs shown in (D). The localization of FLAG-tagged GST-fused protein was monitored by immunostaining with an FITC-conjugated antibody and the nucleus was stained with DAPI. (F) Quantification of the cells according to the localization of GST-fused proteins. Bar, 20  $\mu$ m.

from the nucleus to the cytoplasm mediated by CRM1 (Fornerod et al., 1997; Stade et al., 1997; Gama-Carvalho and Carmo-Fonseca, 2001; Harel and Forbes, 2004), and this process can be inhibited specifically by an antibiotic, leptomycin B (LMB) (Wolff et al., 1997; Kudo et al., 1998; Kudo et al., 1999; Fasken et al., 2000). To examine whether the export of Kank depends on CRM1, we examined the effects of LMB. While Kank protein in human OS-RC-2 cells showed a cytoplasmic localization without LMB treatment, Kank was detected in the nucleus in most cells after treatment with LMB

for 8 hours (Fig. 5A). Since the anti-Kank antibody cannot discriminate Kank-L from Kank-S, we transfected vectors expressing FLAG-tagged Kank-L and Kank-S into mouse NIH3T3 cells, respectively. While both Kank-L and Kank-S showed a cytoplasmic localization without LMB treatment, the localization of Kank-L and Kank-S gradually shifted to the nucleus after LMB treatment (Fig. 5B-D). When the shift of Kank-L was compared with that of Kank-S, it seemed that Kank-L was accumulated in the nucleus more quickly than Kank-S (Fig. 5C,D).

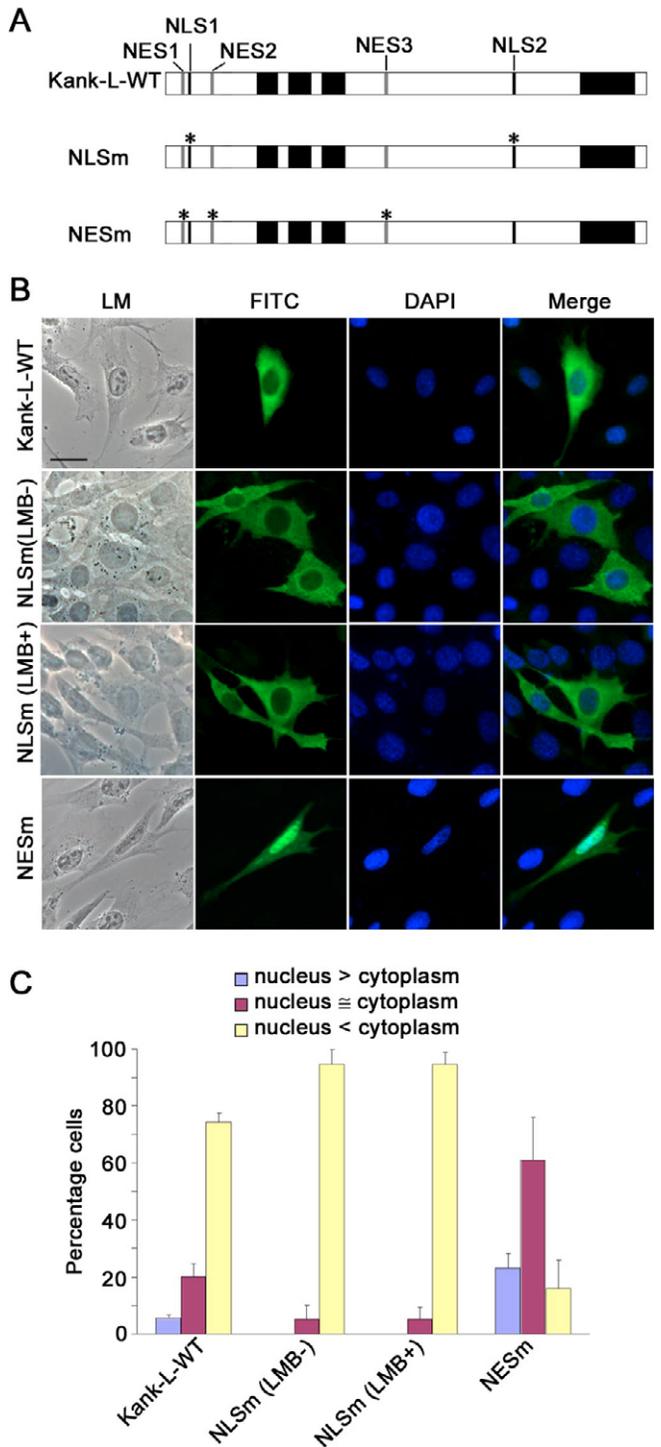


**Fig. 3.** Identification of NESs. (A) Schematic structures of FLAG-tagged GST (FLAG-GST) or GST constructs of FLAG-tagged N-terminal Kank with wild-type (Nt-WT-GST), or mutant NES1 (NES1m-GST) or mutant NES2 (NES2m-GST). (B) Representative images of NIH3T3 cells transiently transfected with the fusion constructs shown in (A). The localization of FLAG-tagged GST fused protein was monitored by immunostaining with an FITC-conjugated antibody and the nucleus was stained with DAPI. LM, light microscopy. (C) Quantification of the cells according to the localization of GST-fused proteins. (D) Schematic structures of GST constructs of FLAG-tagged partial Kank without NESs (NESnull-GST) or with NES3 (NESnull-GST-NES3). In NESnull-GST, the first NES was deleted and the second was mutated. (E) Representative images of NIH3T3 cells transiently transfected with the fusion constructs shown in (D). The localization of FLAG-tagged GST fused protein was monitored by immunostaining with an FITC-conjugated antibody and the nucleus was stained with DAPI. (F) Quantification of the cells according to the localization of GST-fused proteins. Bar, 20  $\mu$ m.

**Effect of nucleo-cytoplasmic shuttling of Kank on  $\beta$ -catenin-dependent transcription and nuclear localization**

In an attempt to explore the function of Kank protein, we screened for the proteins binding to Kank. We found that endogenous Kank can bind to endogenous  $\beta$ -catenin, which was revealed by immunoprecipitation (Fig. 6). We then examined the effect of nucleo-cytoplasmic shuttling of Kank

on  $\beta$ -catenin-dependent transcription (Fig. 7A). After being transported into the nucleus,  $\beta$ -catenin associates with the transcription factor LEF/TCF, which results in the transcription of target genes (Logan and Nusse, 2004). We wondered whether Kank protein and its mutants have any effect on this process and examined the transactivation of  $\beta$ -catenin-LEF using the TOPFLASH/ FOPFLASH reporter



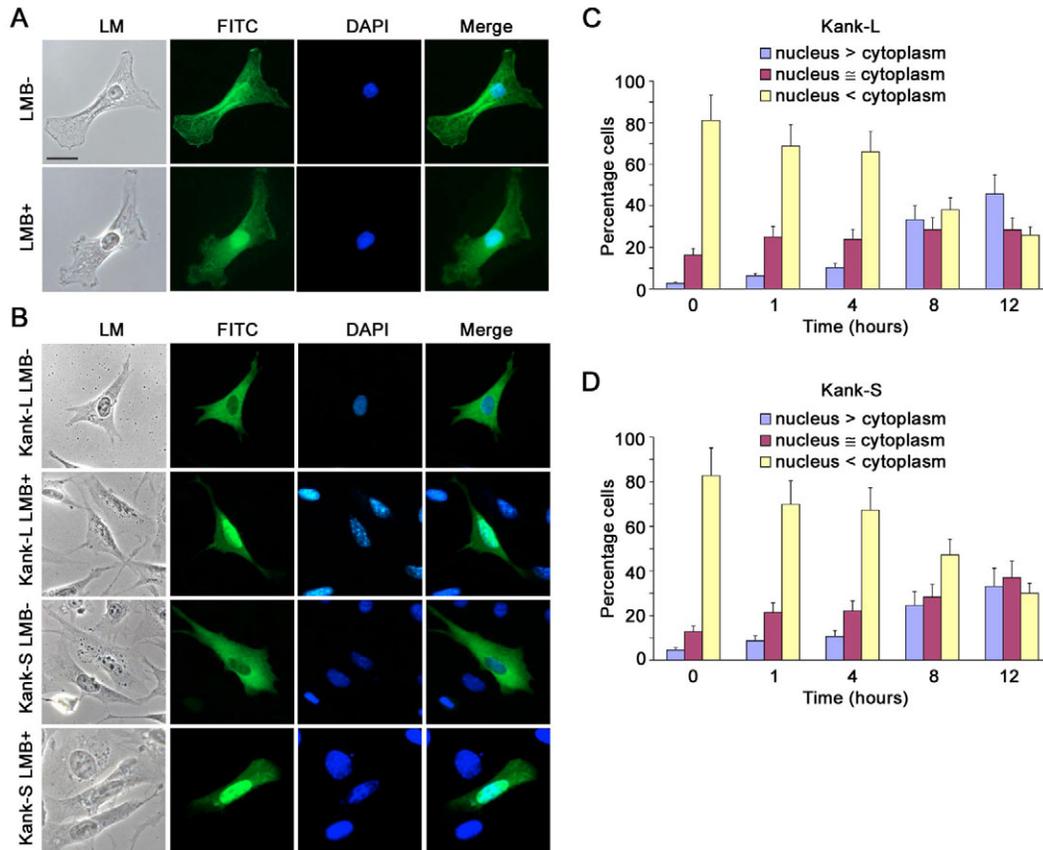
**Fig. 4.** Localization of full length Kank-L and its mutants. (A) Schematic structures of FLAG-tagged full-length Kank-L constructs with wild-type NLS and NES motifs (Kank-L-WT), or with mutant NLS (NLSm) or NES (NESm) motifs. (B) Representative images of NIH3T3 cells transiently transfected with the fusion constructs shown in (A). The cells transfected with NLSm were treated with or without leptomycin B. The localization of FLAG-tagged GST fused protein was monitored by immunostaining with an FITC-conjugated antibody and the nucleus was stained with DAPI. LM, light microscopy. (C) Quantification of the cells according to the localization of GST-fused proteins. Bar, 20  $\mu$ m.

genes which were co-expressed with the plasmids used in Fig. 4 (Kank-L-WT, NESm and NLSm), or plasmids either expressing Kank-S or Kank-L with mutated NLS1 (NLS1m). In the FOPFLASH samples where there was a mutation at the LEF/TCF binding site, there was no difference among the samples (data not shown), suggesting that Kank and its mutants had no effect on this process without binding between the  $\beta$ -catenin-LEF complex and LEF/TCF binding sites on the reporter gene. In the TOPFLASH samples, conversely, the wild-type Kank-L (Kank-L-WT) up-regulated the expression and an NES-null mutant (NESm) had an effect stronger than the wild type, while an NLS-null mutant (NLSm) had a weaker effect than the wild type, NLS1m and Kank-S. Kank-S showed an intermediate effect, which was similar to that of NLS1m (Fig. 7A). This suggests that the nuclear accumulation of Kank protein could enhance  $\beta$ -catenin-dependent transcription and Kank-L is more efficient than Kank-S because of NLS1 in its extra N-terminal region.

Since the expression of Kank shown in Fig. 7A was directed by ectopic vectors, we wonder whether the endogenous expression of Kank regulates  $\beta$ -catenin-dependent transcription. We performed RNA interference using the esiRNA technique to suppress the expression of endogenous Kank in HEK293T cells. The efficiency of RNAi was examined by western blot (Fig. 7B, upper panel), in which a *Xenopus* elongation factor was used as a negative control. As shown in Fig. 7B (lower panel), esiRNA reduced the level of endogenous Kank protein to a fourth compared with the negative control. After transfecting a plasmid expressing  $\beta$ -catenin-S33Y, a mutant  $\beta$ -catenin resistant to degradation, luciferase activity was increased more than 2.5-fold in TOPFLASH samples (Fig. 7C). However, in the Kank knockdown sample, the activity was decreased to about two-thirds compared with the sample without the knockdown (Fig. 7C). This suggests that Kank affected  $\beta$ -catenin-dependent transcription in mammalian cells.

To understand how Kank regulates  $\beta$ -catenin-dependent transcription, we examined whether Kank affects the subcellular distribution of  $\beta$ -catenin (Fig. 7D,E). While  $\beta$ -catenin-S33Y was distributed equally in the nucleus and the cytoplasm, the percentage of nuclear  $\beta$ -catenin was increased by the co-expression of wild-type Kank-L (Fig. 7D,E). In the cells expressing  $\beta$ -catenin with the NLS-null mutant (NLSm), the percentage of cytoplasmic  $\beta$ -catenin increased compared with wild-type Kank-L. By contrast, the expression of the NES-null mutant (NESm) resulted in an increased percentage of nuclear  $\beta$ -catenin. These results suggested that Kank can promote the nuclear localization of  $\beta$ -catenin and potentiate  $\beta$ -catenin-dependent transcription.

To examine whether the mutations in the NLS and NESs of Kank affect the binding between Kank and  $\beta$ -catenin, we co-transfected the vectors expressing wild-type or mutant Kank and  $\beta$ -catenin-S33Y into HEK293T cells. Immunoprecipitation of wild-type and mutant Kank proteins with the anti-FLAG antibody all resulted in co-precipitation of  $\beta$ -catenin (Fig. 7F), suggesting that the mutations in the NLS and NESs of Kank did not affect the binding between Kank and  $\beta$ -catenin. Binding between Kank and  $\beta$ -catenin was detected using wild-type  $\beta$ -catenin (data not shown).

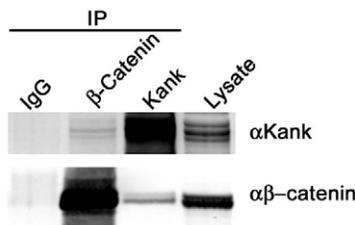


**Fig. 5.** The effect of leptomycin B on the localization of Kank protein. (A) Representative images of OS-RC-2 cells showing endogenous Kank protein detected with anti-Kank antibody before or after treatment with leptomycin B (LMB). LMB<sup>-</sup>, the cells without LMB treatment; LMB<sup>+</sup>, the cells treated with LMB for 8 hours. (B) Representative images of NIH3T3 cells transiently transfected with the constructs of Kank-L or Kank-S and the nucleus was stained with DAPI. (C) Quantification of the cells according to the localization of FLAG-tagged Kank-L protein after treatment with LMB for the periods indicated. (D) Quantification of the cells according to the localization of FLAG-tagged Kank-S protein after treatment with LMB for the periods indicated. The results in (C) and (D) were scored from triplicate transfections. Bar, 20 μm.

**Discussion**

Nucleo-cytoplasmic transportation of proteins plays an important role in the regulation of many cellular processes, including signal transduction, cell cycle progression and cell proliferation (Gama-Carvalho and Carmo-Fonseca, 2001). In general, these shuttling proteins contain both a nuclear localization signal (NLS) and a nuclear export signal (NES),

which are required for the import to the nucleus and the export to the cytoplasm, respectively (Gorlich and Kutay, 1999; Moroianu, 1999; Harel and Forbes, 2004). A well-documented example is p53 protein, which was identified as a nucleo-cytoplasmic shuttling protein. According to a current model, the activity of p53 is regulated both by restraining the import to the nucleus and by enhancing the exit to the cytoplasm under the normal condition (Boyd et al., 2000; Geyer et al., 2000; Liang and Clarke, 2001).



**Fig. 6.** In vivo binding assay between Kank and β-catenin. Immunoprecipitation (IP) of the lysate from VMRC-RCW cells with an anti-Kank antibody, an anti-β-catenin antibody or with control IgG was examined by western blotting using antibodies against Kank or β-catenin.

To determine the presence of NLSs and NESs in shuttling proteins, a series of deletion mutants are needed for examining their subcellular localization. Recently, the prediction of NLS and NES motifs by bioinformatics became available (Nair et al., 2003; la Cour et al., 2004), which enable us to save a time for predicting these motifs and for preparing point mutants. Since the human Kank protein is very large (more than 1000 amino acids for both Kank-L and Kank-S isoforms), it is rather difficult to identify NLSs or NESs in the full-length protein directly. Our strategy adopted here is: first to predict NLS and NES motifs in Kank protein by bioinformatic methods, then to prepare small fragments containing both NLSs and NESs, which are fused to GST protein for quick screening, and finally, to examine point mutations in the full-length Kank protein for

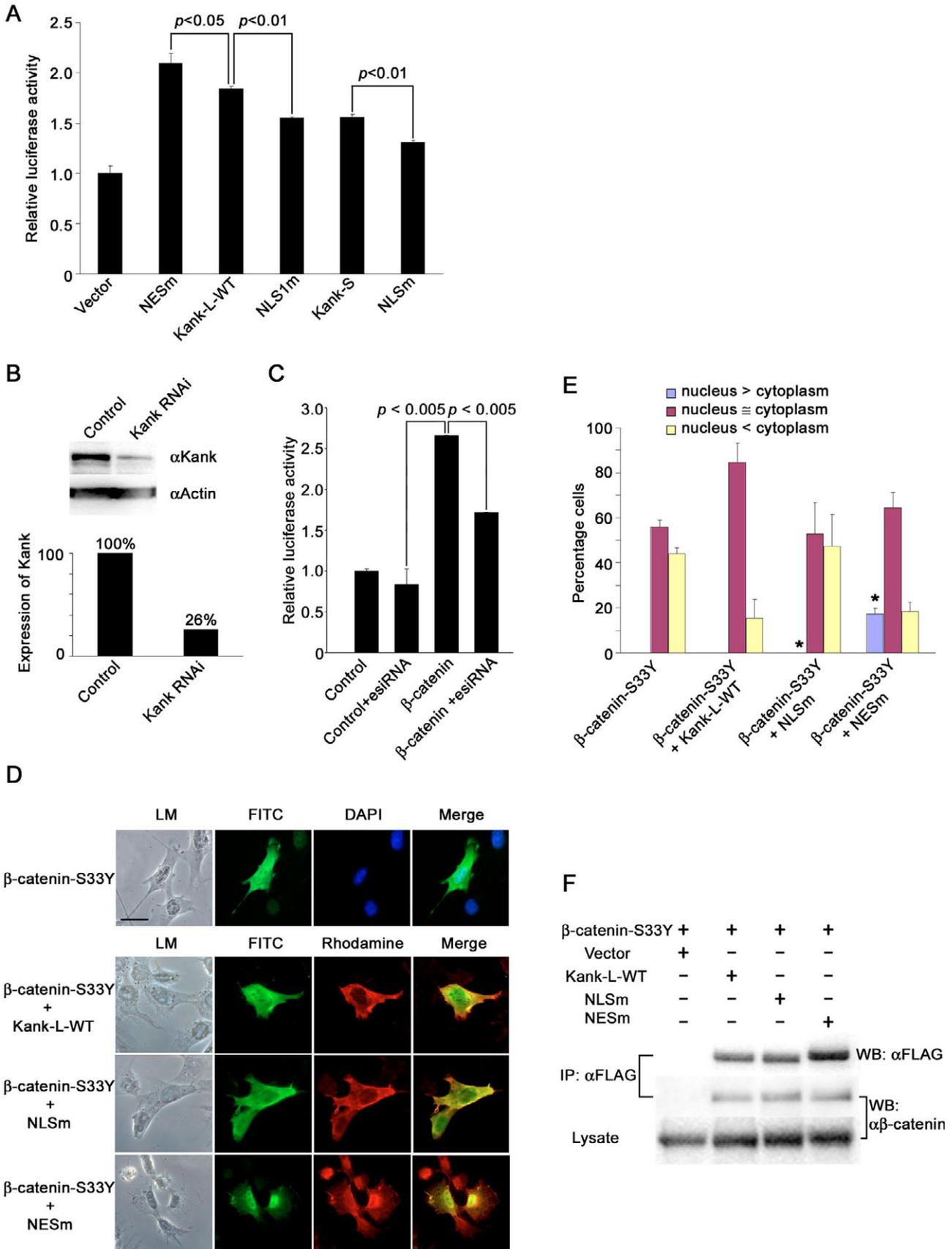


Fig. 7. See next page for legend.

**Fig. 7.** The effect of Kank protein on  $\beta$ -catenin-dependent transcription and nuclear localization. (A) The effect of Kank protein on  $\beta$ -catenin-dependent transcription examined by TOPFLASH reporter gene assay. SW480 cells were transfected with the reporter gene plasmid (TOPFLASH) together with vectors expressing wild-type and mutant Kank proteins. The pCMV Tag-2B cloning vector (Vector) was used to show the background level of  $\beta$ -catenin-dependent transcription. The constructs for Kank-L-WT, NESm and NLSm are the same as shown in Fig. 4. The constructs for Kank-L with a function-less NLS1 (NLS1m) and Kank-S were also included. Luciferase activity was normalized against *Renilla* luciferase activity. The results were obtained from triplicate transfections. (B) The efficiency of Kank RNAi. The level of endogenous Kank protein was examined by western blotting (top) and quantified (bottom). (C) The requirement of endogenous Kank protein for  $\beta$ -catenin-dependent transcription examined by TOPFLASH reporter gene assay. The reporter gene plasmid (TOPFLASH) with or without  $\beta$ -catenin-S33Y or esiRNA against Kank were transfected into HEK293T cells. Luciferase activity was normalized against *Renilla* luciferase activity. The results were obtained from triplicate transfections. (D) Representative images of NIH3T3 cells transiently transfected with the constructs expressing  $\beta$ -catenin-S33Y alone or  $\beta$ -catenin-S33Y with FLAG-tagged wild-type Kank-L or FLAG-tagged Kank-L with mutant NLS (NLSm) or NES (NESm) motifs.  $\beta$ -catenin was visualized with an FITC-conjugated antibody and Kank was visualized with a rhodamine-conjugated antibody. The nucleus was stained with DAPI. (E) Quantification of the Kank-positive cells according to the localization of  $\beta$ -catenin protein. The asterisks indicate  $P < 0.05$  by *t* test. (F) Binding between FLAG-tagged Kank and  $\beta$ -catenin. All constructs used were the same as those used in (D). IP, immunoprecipitation. Bar, 20  $\mu$ m.

confirmation. Using this strategy, we demonstrated that Kank is a nucleo-cytoplasmic shuttling protein. The nuclear import or export of Kank-L is likely mediated respectively by two NLS motifs or by three NES motifs through the CRM1/exportin1 pathway. Among a total of five potential NES motifs in Kank-L (data not shown), only three were verified by the experiments reported here. Similarly, among three NLS motifs predicted, two NLSs were experimentally confirmed, one in the N-terminal region of Kank-L (KRRK from positions 65 to 68; NLS1) and the other, a bipartite NLS, in the common region of Kank-L and Kank-S (KKKDGNGKDSNGAKK from positions 979 to 992; NLS2).

Kank was detected in the cytoplasm by immunofluorescence microscopy (Sarkar et al., 2002; Rodley et al., 2003; Roy et al., 2005). After the treatment with LMB for a long time (more than 8 hours, Fig. 5B-D), nuclear accumulation of Kank protein was observed. However, the cytoplasmic localization of Kank protein was still detected at a higher level compared with some  $\beta$ -catenin binding proteins (Henderson, 2000; Wiechens et al., 2004), which indicates that Kank might bind to some proteins in the cytoplasm and keep a part of Kank protein in the cytoplasm. Therefore, our findings demonstrated that the cytoplasmic distribution of Kank was achieved, if not all, by the NES-mediated nuclear export. Since Kank has a role in controlling the formation of the cytoskeleton through the polymerization of actin (Sarkar et al., 2002; Rodley et al., 2003), it is possible that Kank protein has multiple functions in the cell and play different roles in different subcellular localizations. The Kank protein molecules localized in the cytoplasm might be involved in the polymerization of actin, while the molecules transported into

the nucleus might be involved in some other molecular events as discussed below.

When we searched for the binding proteins of Kank, we found that  $\beta$ -catenin could bind to Kank in mammalian cells (Fig. 6). However, this binding is likely to be indirect from the results of in vitro pull-down assay (data not shown). Then, we wondered what was the effect of Kank on the function of  $\beta$ -catenin. Here, we have demonstrated that the nuclear import of Kank promoted the nuclear localization of  $\beta$ -catenin, which resulted in the activation of  $\beta$ -catenin-mediated transcription (Fig. 7A-E). Although we detected binding between endogenous Kank and endogenous  $\beta$ -catenin (Fig. 6), the subcellular distributions of Kank and  $\beta$ -catenin do not overlap completely (Fig. 7D). Our explanation for this phenomenon is that the nuclear export of Kank protein is greater than its nuclear import because of preferential localization of Kank in the cytoplasm, and thus, Kank protein might be transported to the cytoplasm soon after its import into the nucleus. By contrast,  $\beta$ -catenin is kept in the nucleus to exert its function. However, we cannot exclude the possibility that Kank affects  $\beta$ -catenin-dependent transcription through other signaling pathways because a mutant completely depleting of the functional nuclear import signal (NLSm) still showed a low but distinct level of enhancement in  $\beta$ -catenin-dependent transcription (Fig. 7A).

In conclusion, we show that Kank is a nucleo-cytoplasmic shuttling protein and the nuclear import of Kank protein accompanies with the nuclear import of  $\beta$ -catenin, which up-regulates  $\beta$ -catenin-dependent transcription. Our data indicate that one should not consider Kank protein as a static molecule that merely localizes in the cytoplasm. On the contrary, Kank shuttles in and out of the nucleus so as to exert multiple functions in the cell and play different roles in the cytoplasm and the nucleus.

## Materials and Methods

### Bioinformatic analysis

NLS and NES motifs were predicted using the software PSORT II (<http://psort.nibb.ac.jp/form2.html>) and NetNES 1.1 (<http://www.cbs.dtu.dk/services/NetNES/>), respectively.

### Cell culture

Mouse NIH3T3 and human HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco). Human OS-RC-2, VMRC-RCW and SW480 cells were grown in RPMI1640 medium (Gibco), Minimum Essential Medium (Gibco) and Leibovitz's L-15 medium (Gibco), respectively. All culture media were supplemented with 10% (v/v) fetal bovine serum (Gibco). Cells were incubated at 37°C in a humidified atmosphere containing 5% (v/v) CO<sub>2</sub>.

### Plasmid construction

For the construction of FLAG-tagged Kank N-terminus-GST plasmids, a *Bam*HI-*Eco*RI cDNA fragment encoding the N-terminal 158 amino acids in Kank-L was inserted into the *Bam*HI/*Xho*I site of the vector pCMV-Tag 2B (Stratagene) together with a PCR-amplified *Eco*RI-*Xho*I fragment from the GST gene. Deletion and mutant constructs of FLAG-tagged Kank-L N-terminus-GST were generated by PCR amplification using the wild-type construct as a template and appropriate primer pairs. To generate NLS1m-NESnull-GST-NLS2, a pair of oligonucleotides encoding NLS2 (KKKDGNGKDSNGAKK), 5'-AATCAAGAAGAAAGATG-GTAACAAAGATTCAAATGGCGCAAAAAAGG-3' (forward) and 5'-AATTC-CTTTTTGCGCCATTGAATCTTGTGTTACCATCTTCTTCTTG-3' (reverse), were annealed and inserted into the *Eco*RI site, which was located between the Kank N-terminal fragment and GST. To generate NESnull-GST-NES3, a pair of primers encoding NES3 and its neighboring amino acids (DLTLKTNLNLK), 5'-AATTC-GACCTCACACTCCTCAAGACAAACTTGAATCTCAAAG-3' (forward) and 5'-AATTCCTTGGAGATTCAAGTTTGTCTTGGAGAGTGTGAGGTCG-3' (reverse), were annealed and inserted into the *Eco*RI site, which was located between the Kank N-terminal fragment and GST. The mutant constructs of full-length Kank-L were generated by PCR amplification using the FLAG-tagged wild-type Kank-L

construct as a template and appropriate primers. All of the constructs were verified by DNA sequencing.

### Immunofluorescence staining and microscopy

Plasmid DNA (1.5 µg) was transiently transfected into NIH3T3 cells grown on fibronectin-coated glass coverslips with PolyFect (QIAGEN). Twenty-four hours after transfection, the cells were fixed with 3.7% formaldehyde in PBS for 15 minutes. The cells were then treated with 0.5% Triton X-100 for 5 minutes and with fresh 0.1% sodium borohydride in PBS for another 5 minutes (sodium borohydride treatment was omitted in the case of examination of β-catenin localization). Next, after the cells had been treated with 5% goat serum for 30 minutes, they were incubated with anti-FLAG M2 (Sigma) or anti-β-catenin (BD Biosciences) antibodies for 1 hour, and visualized using FITC- or Rhodamine-labeled antibodies (Molecular Probe) under a fluorescence microscope (Axioskop, Zeiss). DAPI staining was performed for 10 minutes after incubation with a secondary antibody when necessary. In all experiments presented here, more than one hundred FITC-positive cells were counted and classified according to their subcellular localization.

### Western blotting and immunoprecipitation

Western blotting was performed as described previously (Wang et al., 2005). For the assay in vivo of binding between Kank and β-catenin, Protein-G Sepharose (Amersham Biosciences) was incubated with the cell lysate and either anti-Kank antibody or anti-β-catenin antibody (or rabbit IgG for a negative control), and then washed with TBS (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl) five times. The proteins were dissociated from Protein-G Sepharose by heating the resin at 100°C for 3 minutes after addition of 20 µl of 2× SDS-PAGE sample buffer [125 mM Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 0.004% bromophenol blue and 100 mM DTT]. For in vitro binding assay, after the FLAG-M2-Agarose was incubated with cell lysate for one hour at 4°C and subsequently washed with TBS five times, the binding proteins were eluted with 50 µl of 1 mg/ml FLAG peptide (Sigma).

### Luciferase assay

Kank construct DNA (4.4 µg/well) mixed with 0.5 µg/well of TOPFLASH, a positive control plasmid containing TCF/LEF-1-binding sites, or FOPFLASH, a negative control plasmid containing a mutant binding site, was transfected into human colon cancer SW480 cells in 6-well plates using Lipofectamine 2000 reagent (Invitrogen). The luciferase activity in the cell extracts was determined using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activity was normalized to the *Renilla* luciferase activity. Each transfection was carried out in triplicate and measurements were repeated at least twice. To quantify the effect of esiRNA on β-catenin-dependent transcription, the luciferase assay was performed in HEK293T cells using 0.5 µg of TOPFLASH or FOPFLASH, and 0.01 µg of pRLTK in the presence or absence of 0.5 µg of β-catenin-S33Y or 4 µg of esiRNA.

### Endoribonuclease-prepared siRNA (esiRNA)

Suppressing the Kank function with esiRNA was performed as described elsewhere (Yang et al., 2002; Kittler et al., 2004; Kittler et al., 2005) with some modifications. Briefly, the human *Kank* gene was cloned into pEGFP-C1 vector. For T7-based RNA transcription, the templates were prepared by PCR using primers 5'-CGT-AATACGACTCACTATCGGGCATGGTCTGCTGGAGTTCGTG-3' (forward) and 5'-CGTAATACGACTCACTATAGGGCCTCTACAAATGTGGTATGGCTG-3' (reverse). PCR products were purified by QIAquick PCR purification kit (QIAGEN). Double strand RNA synthesized with MEGAscript T7 kit (Ambion) was digested with ShortCut RNase III (New England Biolabs). A *Xenopus* elongation factor was used as a negative control.

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### References

Boyd, S. D., Tsai, K. Y. and Jacks, T. (2000). An intact HDM2 RING-finger domain is required for nuclear exclusion of p53. *Nat. Cell Biol.* **2**, 563-568.  
 Fasken, M. B., Saunders, R., Rosenberg, M. and Brighty, D. W. (2000). A leptomycin B-sensitive homologue of human CRM1 promotes nuclear export of nuclear export sequence-containing proteins in *Drosophila* cells. *J. Biol. Chem.* **275**, 1878-1886.  
 Fornerod, M., Ohno, M., Yoshida, M. and Mattaj, J. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051-1060.

Gama-Carvalho, M. and Carmo-Fonseca, M. (2001). The rules and roles of nucleocytoplasmic shuttling proteins. *FEBS Lett.* **498**, 157-163.  
 Geyer, R. K., Yu, Z. K. and Maki, C. G. (2000). The MDM2 RING-finger domain is required to promote p53 nuclear export. *Nat. Cell Biol.* **2**, 569-573.  
 Giles, R. H., van Es, J. H. and Clevers, H. (2003). Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim. Biophys. Acta* **1653**, 1-24.  
 Gorlich, D. and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell Dev. Biol.* **15**, 607-660.  
 Harel, A. and Forbes, D. J. (2004). Importin beta: conducting a much larger cellular symphony. *Mol. Cell* **16**, 319-330.  
 Henderson, B. R. (2000). Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. *Nat. Cell Biol.* **2**, 653-660.  
 Jensch, P. J., Huggenvik, J. I. and Collard, M. W. (2004). Identification of a nuclear export signal and protein interaction domains in deformed epidermal autoregulatory factor-1 (DEAF-1). *J. Biol. Chem.* **279**, 32692-32699.  
 Kittler, R., Putz, G., Pelletier, L., Poser, I., Heninger, A. K., Drechsel, D., Fischer, S., Konstantinova, L., Habermann, B., Grabner, H. et al. (2004). An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. *Nature* **432**, 1036-1040.  
 Kittler, R., Heninger, A. K., Franke, K., Habermann, B. and Buchholz, F. (2005). Production of endoribonuclease-prepared short interfering RNAs for gene silencing in mammalian cells. *Nat. Methods* **2**, 779-784.  
 Kong, K. Y. and Kedes, L. (2004). Cytoplasmic nuclear transfer of the actin-capping protein tropomodulin. *J. Biol. Chem.* **279**, 30856-30864.  
 Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E. P., Yoneda, Y., Yanagida, M., Horinouchi, S. and Yoshida, M. (1998). Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. *Exp. Cell Res.* **242**, 540-547.  
 Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E. P., Wolff, B., Yoshida, M. and Horinouchi, S. (1999). Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc. Natl. Acad. Sci. USA* **96**, 9112-9117.  
 Kurooka, H. and Yokota, Y. (2005). Nucleo-cytoplasmic shuttling of Id2, a negative regulator of basic helix-loop-helix transcription factors. *J. Biol. Chem.* **280**, 4313-4320.  
 la Cour, T., Kiemer, L., Molgaard, A., Gupta, R., Skriver, K. and Brunak, S. (2004). Analysis and prediction of leucine-rich nuclear export signals. *Protein Eng. Des. Sel.* **17**, 527-536.  
 Liang, S. H. and Clarke, M. F. (2001). Regulation of p53 localization. *Eur. J. Biochem.* **268**, 2779-2783.  
 Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781-810.  
 Lustig, B. and Behrens, J. (2003). The Wnt signaling pathway and its role in tumor development. *J. Cancer Res. Clin. Oncol.* **129**, 199-221.  
 Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997). Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science* **275**, 1787-1790.  
 Moroianu, J. (1999). Nuclear import and export pathways. *J. Cell Biochem. Suppl.* **32-33**, 76-83.  
 Nair, R., Carter, P. and Rost, B. (2003). NLSdb: database of nuclear localization signals. *Nucleic Acids Res.* **31**, 397-399.  
 Rodley, P., Hatano, N., Nishikawa, N. S., Roy, B. C., Sarkar, S. and Kiyama, R. (2003). A differential genomic cloning for cancer study: an outline and applications. *Recent Res. Dev. Mol. Biol.* **1**, 13-27.  
 Roy, B. C., Aoyagi, T., Sarkar, S., Nomura, K., Kanda, H., Iwaya, K., Tachibana, M. and Kiyama, R. (2005). Pathological characterization of Kank in renal cell carcinoma. *Exp. Mol. Pathol.* **78**, 41-48.  
 Sarkar, S., Roy, B. C., Hatano, N., Aoyagi, T., Gohji, K. and Kiyama, R. (2002). A novel ankyrin repeat-containing gene (*Kank*) located at 9p24 is a growth suppressor of renal cell carcinoma. *J. Biol. Chem.* **277**, 36585-36591.  
 Stade, K., Ford, C. S., Guthrie, C. and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell* **90**, 1041-1050.  
 Suetsugu, S. and Takenawa, T. (2003). Translocation of N-WASP by nuclear localization and export signals into the nucleus modulates expression of HSP90. *J. Biol. Chem.* **278**, 42515-42523.  
 Wang, Y., Onishi, Y., Kakinuma, N., Roy, B. C., Aoyagi, T. and Kiyama, R. (2005). Alternative splicing of the human *Kank* gene produces two types of Kank protein. *Biochem. Biophys. Res. Commun.* **330**, 1247-1253.  
 Wiechens, N., Heinle, K., Englmeier, L., Schohl, A. and Fagotto, F. (2004). Nucleo-cytoplasmic shuttling of Axin, a negative regulator of the Wnt-beta-catenin pathway. *J. Biol. Chem.* **279**, 5263-5267.  
 Wolff, B., Sanglier, J. J. and Wang, Y. (1997). Leptomycin B is an inhibitor of nuclear export: inhibition of nucleocytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. *Chem. Biol.* **4**, 139-147.  
 Yang, D., Buchholz, F., Huang, Z., Goga, A., Chen, C. Y., Brodsky, F. M. and Bishop, J. M. (2002). Short RNA duplexes produced by hydrolysis with *Escherichia coli* RNase III mediate effective RNA interference in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**, 9942-9947.