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ORIGINAL ARTICLE

Nuclear entry of active caspase-3 is facilitated by its p3-recognition-based specific cleavage activity

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As a critical apoptosis executioner, caspase-3 becomes activated and then enters into the nucleus to exert its function. However, the molecular mechanism of this nuclear entry of active caspase-3 is still unknown. In this study, we revealed that caspase-3 harbors a crm-1-independent nuclear export signal (NES) in its small subunit. Using reverse-caspase-3 as the study model, we found that the function of the NES in caspase-3 was not disturbed by the conformational changes during induced caspase-3 activation. Mutations disrupting the cleavage activity or p3-recognition site resulted in a defect in the nuclear entry of active caspase-3. We provide evidence that the p3-mediated specific cleavage activity of active caspase-3 abrogated the function of the NES. In conclusion, our results demonstrate that during caspase-3 activation, NES is constitutively present. p3-mediated specific cleavage activity abrogates the NES function in caspase-3, thus facilitating the nuclear entry of active caspase-3.

***Keywords*:** nuclear entry, caspase-3, apoptosis, nuclear export signal

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Introduction

Apoptosis is an important physiological event that plays critical roles in the development of multicellular organisms and the maintenance of tissue homeostasis. Apoptosis is also involved in a number of disease processes, such as neuro-degeneration and cancer [1]. Apoptosis-inducing stimuli, such as pro-apoptotic cytokines, UV irradiation and DNA-damaging drugs, induce apoptotic responses characterized by a series of shared morphological changes in the membrane, cytoplasm and nucleus [2].

Many morphological and biochemical apoptotic changes are induced by the activation of the caspase cascade, in which caspase-3 plays a central role [2]. Caspase-3 exists in the form of pro-enzyme, and is located predominantly in the cytoplasm of cells. During apoptosis, caspase-3 is cleaved and activated by upstream caspases [3], and is translocated into the nucleus to cleave its nuclear substrates [4, 5], resulting in characteristic apoptotic nuclear changes such as DNA fragmentation, chromatin condensation and nuclear disruption [3, 6, 7]. It was reported that active caspase-3 is translocated into the nucleus by simple diffusion after disruption of the nuclear-cytoplasmic barrier [8]. Another group found that active caspase-3 may be translocated from the cytoplasm into the nucleus in association with a substrate-like protein(s) in apoptotic cells [4], and suggested that AKAP95 (A-Kinase-Anchoring Protein 95) is a potential carrier [9]. However, there is no direct evidence to demonstrate that deficiency of AKAP5 abrogates the nuclear translocation of active caspase-3. The mechanism of the nuclear translocation of active caspase-3 remains largely unclear.

In this study, we found one crm-1-independent nuclear export signal (NES) but no nuclear localization signal

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Abbreviations: rev-caspase-3 (reverse-caspase-3); NES (nuclear export signal); NLS (nuclear localization signal); LMB (leptomycin B)
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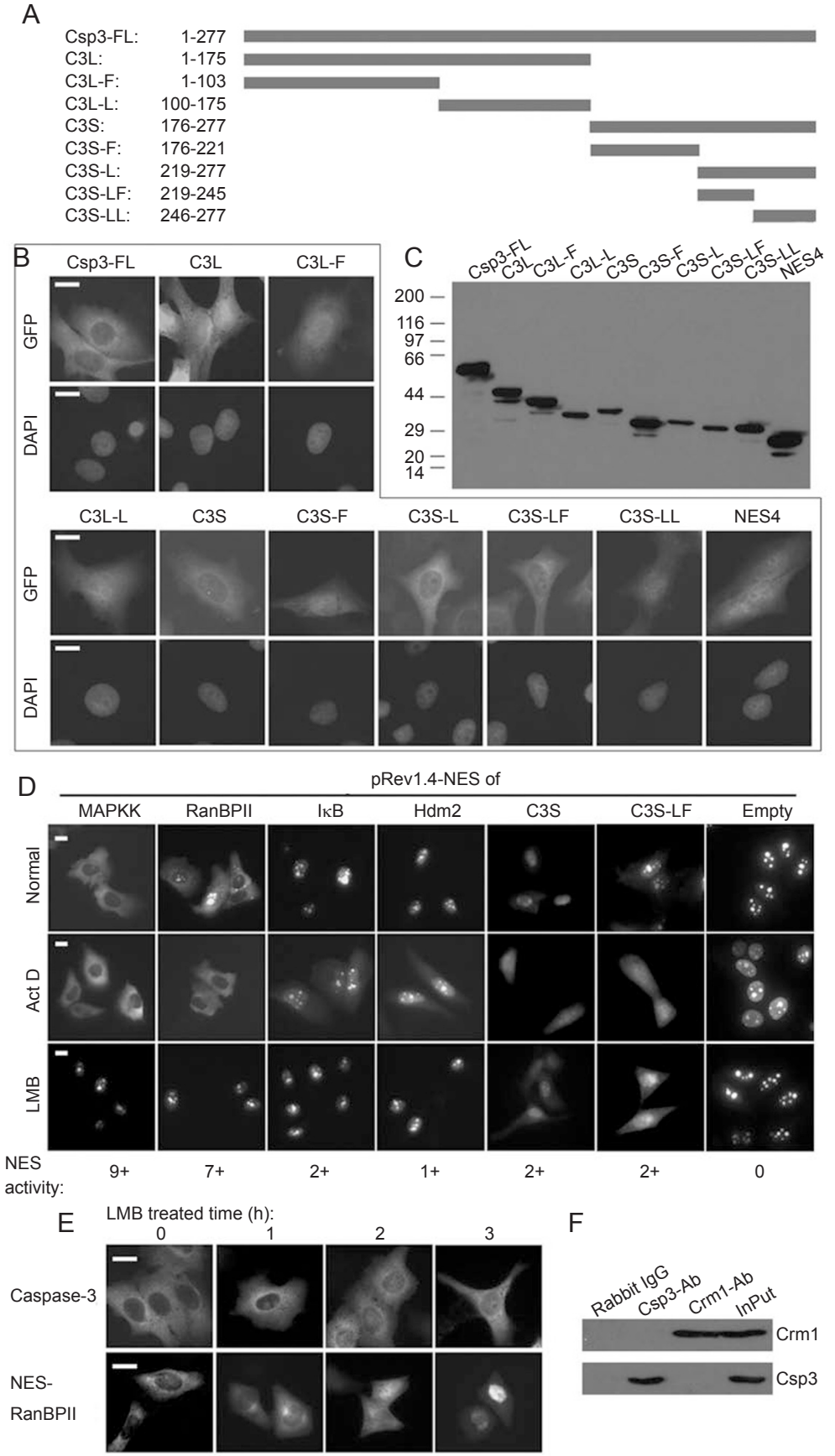
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C3L-F: 1-103
C3L-L: 100-175
C3S: 176-277
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C3S-L: 219-277
C3S-LF: 219-245
C3S-LL: 246-277
B
Csp3-FL
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DAPI
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NES4
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pRev1.4-NES of
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Hdm2
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9+
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LMB treated time (h):
0
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2
3
Caspase-3
NES-RanBPII
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Crm1
Csp3

(NLS) residing at the small subunit of caspase-3. This NES was not disturbed and was still functional when caspase-3 was in the active form. Mutations disrupting the cleavage activity or the p3 recognition site caused a defect in the nuclear entry of active caspase-3, indicating that the function of the NES in caspase-3 would need to be abrogated by the p3-recognition-based specific cleavage for nuclear entry. Our results suggest that the nuclear entry of active caspase-3 is not mediated by passive diffusion, but by its p3-recognition-based specific cleavage activity.

Results

Caspase-3 harbors a crm-1-independent NES in its small subunit

Identification and characterization of the subcellular localization signals in caspase-3 would be important to illustrate the molecular mechanism of the nuclear entry of active caspase-3. To identify the subcellular localization signals in caspase-3, we constructed a series of GFP-fused truncated forms based on the two subunits of caspase-3 (Figure 1A). The results showed that the small subunit (C3S) as well as the full-length caspase-3 displayed evident cytoplasm localization, while the large subunit (C3L) and its truncated forms were distributed throughout the whole cell (Figure 1B and 1C). We then divided the small subunit into several parts to narrow down the cytoplasm localization region, and found it resided in C3S-LF, which was from AA219 to AA245 (Figure 1B and 1C). These results indicate that there is a cytoplasm distribution signal residing in the small subunit of caspase-3, and that there is no obvious NLS in caspase-3.

To determine whether the cytoplasm distribution signal in caspase-3 is a NES, we performed a NES-evaluating assay described by Henderson *et al.* [11]. Briefly, the HIV rev-GFP fusion protein would shift from the nucleus to the cytoplasm after a short exposure of cells to actinomycin D (Act D), whereas the NES-mutated form of rev-GFP (Rev1.4) would remain in the nucleus. Insertion of a NES sequence between the Rev1.4 and the GFP would restore the export activity like the one observed in the wild-type rev-GFP. Therefore, the export activity of the candidate sequence could be determined by a shift in localization from the nucleus to the cytoplasm following Act D treatment for 1 to 3 h.

We then examined the NES activity of C3S and C3S-LF using the NES-evaluating assay, and the NESs of MAPKK, RanBPII, IκB and Hdm2 were used as the control. Results showed that the NES activity of MAPKK, RanBPII, IκB and Hdm2 was 9+, 7+, 2+ and 1+, respectively, as reported (Table 1, Figure 1D) [11]. pEGFP-Rev1.4-C3S and C3S-LF did not exhibit any absolute cytoplasmic localization. After Act D treatment, nuclear-cytoplasmic distributions increased from 30% and 20.3% to 65.4% and 50.8% respectively, indicating that C3S and C3S-LF harbored a nuclear export activity that had a 2+ rank (Table 1, Figure 1D). These results showed that caspase-3 harbors a weak NES in its small subunit.

Since many NESs are regulated by crm-1, we then examined whether the NES of caspase-3 is crm-1 dependent. We treated the above pEGFP-Rev1.4-NES transfected cells with the crm-1 inhibitor leptomycin B (LMB). The results showed that cells transfected with pEGFP-Rev1.4-NES of MAPKK, RanBPII, IκB and Hdm2 all exhibited nearly 100% nuclear localization, while the nuclear localization percentage of C3S and C3S-LF only increased slightly (Table 1, Figure 1D), indicating that the cytoplasmic distribution of C3S and C3S-LF was not sensitive to LMB. We also transfected pEGFP-caspase-3 into Hela cells and treated them with LMB. The results showed that only a small portion of caspase-3 shifted from the cytoplasm to the nucleus, and no absolute nu-

Figure 1 Caspase-3 harbors a crm-1-independent nuclear export signal (NES) in its small subunit. **(A)** Construction of caspase-3 truncation mutants that contain NES candidates. C3L, the large subunit of caspase-3; C3S, the small subunit of caspase-3; C3L-F, the former part of the large subunit; C3L-L, the latter part of the large subunit; C3S-F, the former part of the small subunit; C3S-LF, the latter part of the small subunit; C3S-LF, the former part of C3S-L; C3S-LL, the latter part of C3S-L. **(B)** GFP-fused caspase-3 truncation mutants were transfected into HeLa cells for 24 h, then the cells were fixed and stained with DAPI for observation. Bar = 20 μM. **(C)** GFP-fused caspase-3 truncation mutants were transfected into HeLa cells for 24 h, then the cells were harvested for western blot using anti-GFP antibody. **(D)** Caspase-3-truncated forms that contain the cytoplasmic distribution activity, C3S and C3S-LF, and other known NESs of MAPKK, RanBPII, IκB and Hdm2, were constructed into pEGFP-Rev1.4 separately. The constructions were transfected into three parallel wells of Hela cells. At 24 h post-transfection, cells were treated with Act D or LMB separately for 3 h, then observed immediately under a fluorescence microscope. Bar = 20 μM. **(E)** HeLa cells were transfected with pEGFP-C2-caspase-3 or pEGFP-NES of RanBPII for 24 h, then treated with LMB and observed under a fluorescence microscope at the indicated time. Bar = 20 μM. **(F)** Extracts of HeLa cells were immunoprecipitated with anti-crm-1 or anti-caspase-3 antibodies, and then the precipitated complex was analyzed by western blot. Rabbit IgG was included as a control.

Table 1 Evaluating NES activity of caspase-3 using cell scoring system

Construct	Drug	Cellular localization of GFP (%)			*n*	Activity
		Nu	Nu+Cyto	Cyto		
NES of MAPKK	None	0	8.7	91.3	275	9+
	Act D	0	0	100	254	
	LMB	95	5	0	239	
NES of RanBPII	None	0	76.5	23.5	255	7+
	Act D	0	14.7	85.3	272	
	LMB	100	0	0	233	
NES of IκB	None	76.1	23.9	0	230	2+
	Act D	20.6	79.4	0	306	
	LMB	100	0	0	241	
NES of Hdm2	None	92.2	7.8	0	280	1+
	Act D	55.1	44.9	0	245	
	LMB	100	0	0	213	
C3S	None	70	30	0	250	2+
	Act D	34.6	65.4	0	286	
	LMB	76.1	23.9	0	339	
C3S-LF	None	79.7	20.3	0	246	2+
	Act D	49.2	50.8	0	315	
	LMB	88.3	11.7	0	282	

Caspase-3 truncated forms that contained the cytoplasmic distribution activity, C3S and C3S-LF, and other known NESs of MAPKK, RanBPII, IκB and Hdm2, were constructed into pEGFP-Rev1.4 separately. The NES-containing constructs were transfected into Hela cells. At 24 h post-transfection, cells were treated with Act D or LMB for 3 h, then observed immediately under a fluorescence microscope. The NES activities were evaluated according to the methods described by Henderson *et al.* [11]. A minimum of 200 cells were counted per slide, and labels were masked to ensure unbiased counting. This type of analysis was performed at least twice for each sample. Cyto, cytoplasmic; Nu, nuclear.

clear accumulation was observed (Figure 1E). For further confirmation, we examined whether crm-1 could bind with caspase-3 by immunoprecipitation, and the result showed that there was no interaction between caspase-3 and crm-1 (Figure 1F). These results indicate that the NES of caspase-3 is not dependent on crm-1.

Mutation analysis of the NES region of caspase-3

To precisely identify the NES sequence in caspase-3, we performed NetNES analysis (www.cbs.dtu.dk/services/NetNES/) and found five NES candidates, NES1, NES2, NES3, NES4 and NES5 (Figure 2A). Our truncation analysis showed that the NES of caspase-3 resided at C3S-LF (from AA219 to AA245), which covered the region of NES4 (from AA219 to AA236), implying that NES4 might be the most likely candidate. Unlike C3S-LF, putative NES4 alone could not result in GFP localization in the cytoplasm, indicating that the other nine amino acids were also needed (Figure 1B and 1C).

We mutated caspase-3 in the region of NES4 for further examination (Figure 2A). The results showed that the I235A mutation disrupted the cytoplasm localization of caspase-3, whereas the L219A, L223A and L236A mutations had little effect on caspase-3 distribution. The double mutants L219/223A and I235A/L236A also showed disrupted cytoplasm localization (Figure 2B, Table 2). Furthermore, we found that all the mutations that affected caspase-3 distribution led to increased percentages of dead cells at various levels (Table 2). These results indicate that NES4 is important for caspase-3 to localize in the cytoplasm.

NES in caspase-3 is not disturbed by conformational changes accompanying the activation of caspase-3, but is abrogated by its p3-mediated cleavage activity

Caspase-3 displays an obvious conformational change after its activation and harbors a specific cleavage activity. To investigate whether the NES in caspase-3 is disturbed after activation, and whether and how the properties of active caspase-3 contribute to its nuclear entry, we used reverse-caspase-3 (rev-caspase-3) as the study model, which mimics the conformation of active caspase-3 and harbors the same specific cleavage activity as the active caspase-3 (Figure 3) [10, 12].

Rev-caspase-3 is composed of a reverse arrangement of the large subunit and the small subunit of caspase-3 to

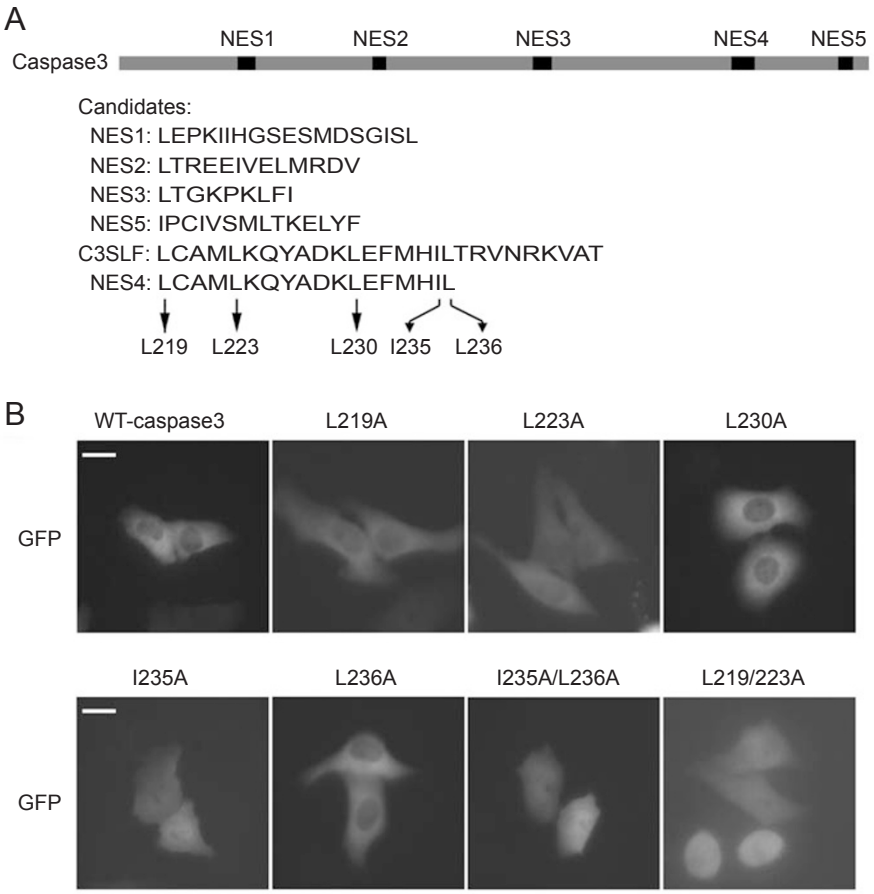

Figure 2 Identification of important amino acids in the NES of caspase-3. **(A)** Sequence of NES candidates in caspase-3 and the potentially important amino acids in the NES4 of caspase-3. **(B)** Caspase-3 was mutated at the potentially important amino acids in the NES4, and constructed into pEGFP-C2. The mutant constructions were transfected into HeLa cells for 24 h for direct fluorescence observation without fixation. Bar = 20 μM.

Table 2 Analysis of the caspase-3 mutants in NES region

Caspase-3	Percentage of the cells (%)			*n*	Fluorescence intensity
	Normal cell		Dead cell		
	Cyto	Nu+Cyto			
WT	76.7	9.7	13.6	331	Strong
L219A	63.5	24.2	12.3	364	Weak
L223A	42.9	17.8	39.3	448	Weak
L230A	75.0	10.0	15.0	320	Strong
I235A	0	30.7	69.3	316	Strong
L236A	44.8	9.7	45.5	495	Strong
I235A/L236A	0	74.1	25.9	351	Weak
L219/223A	0	78.1	21.9	292	Weak

Caspase-3 was mutated at the potentially important amino acids in the NES4, and constructed into pEGFP-C2 for transfection into HeLa cells. At 24 h post-transfection, cells were stained with PI and observed under a fluorescence microscope. Mutant distributions were counted by GFP localization, and dead cells were counted by positive staining of PI. Fluorescence intensity indicated the GFP fluorescence intensity of the GFP-fused mutant protein. A minimum of 200 cells were counted per slide, and labels were masked to ensure unbiased counting. This type of analysis was performed at least twice for each mutant. Cyto, cytoplasmic; Nu, nuclear.

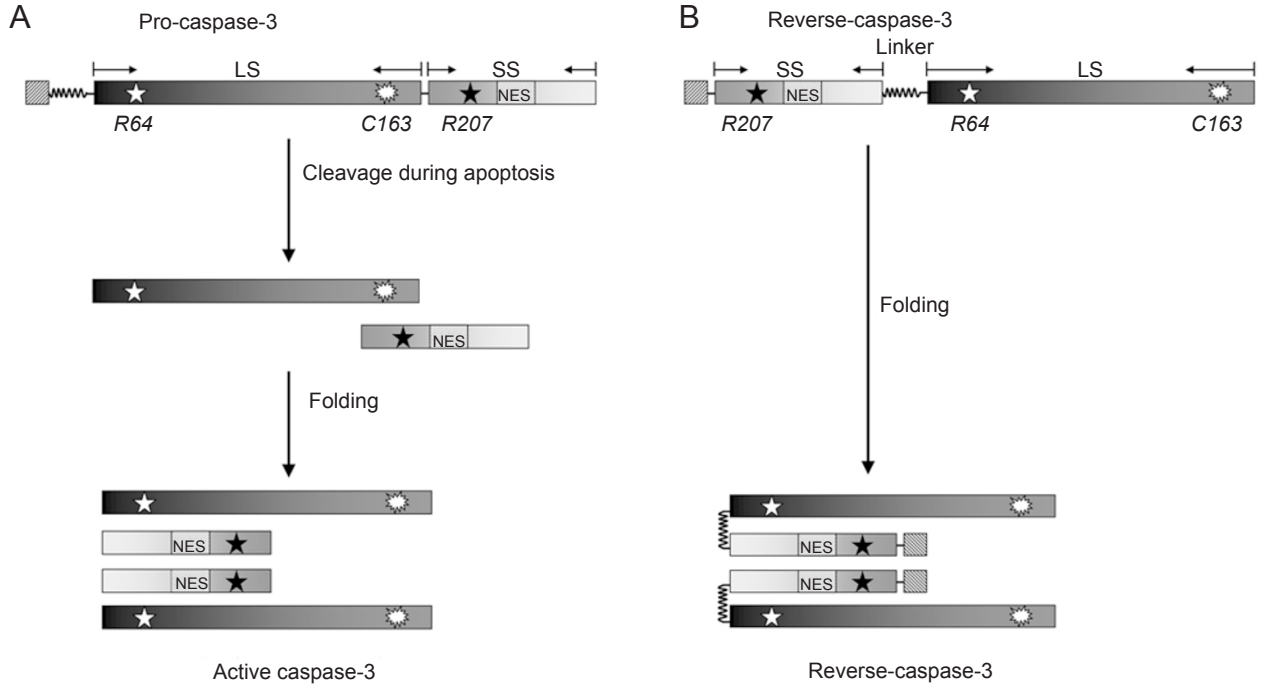

Figure 3 Schematic representation of rev-caspase-3. **(A)** Processing and folding of pro-caspase-3 into mature active caspase-3 during apoptosis. **(B)** Spontaneous folding of rev-caspase-3 into mature zymogen. LS and SS indicate the large subunit and small subunit of caspase-3, respectively. In rev-caspase-3, one part of the caspase-3 pro-domain was inserted between SS and LS as the linker. C163 was the cleavage center of active caspase-3; R64 and R207 were the essential amino acids for substrate recognition at the p1 and p3 recognition sites, respectively [13, 14].

mimic the conformation of active caspase-3, and harbors the constitutive specific cleavage activity, which is the same as that of the active caspase-3 (Figure 3). A previous investigation had examined the relationship between the properties of active caspase-3 and its nuclear entry by transfecting the related mutant caspase-3, inducing apoptosis, and then separating the nucleus from the apoptotic cells to examine caspase-3 by western blot [4]. In this process, the purified nuclear fraction could be contaminated by the cytoplasm components, as the nuclear envelope (NE) was harshly destroyed during the purification of nuclei from apoptotic cells. Moreover, mutant caspase-3 could be cleaved by endogenous active caspase-3 during induced apoptosis, and might subsequently enter into the nucleus by forming a hetero-tetramer with the endogenous active caspase-3 [3]. By using tagged mutant rev-caspase-3, which overcomes the disadvantages of the methods employed by the previous investigation, the results would be more reliable for examining the relationship between the properties of active caspase-3 and its nuclear entry.

The wild-type rev-caspase-3 caused rapid apoptosis when overexpressed due to its high cleavage activity (Supplementary information, Figure S1). Almost all the transfected cells were round, and GFP fluorescence intensity was very weak. Nuclear substrates of caspase-3, PARP and Lamin B1, were degraded when the wild-type rev-caspase-3 was overexpressed (Supplementary information, Figure S2), indicating that the wild-type rev-caspase-3 had the same nuclear entry ability as the activated caspase-3.

To explore whether the NES in caspase-3 is disrupted by the conformational changes that occur during caspase-3 activation, we mutated cysteine 163 of rev-caspase-3, the cleavage center of active caspase-3, to serine in order to disrupt its cleavage activity but maintain the active caspase-3 conformation [13, 14]. The results from both the GFP-fused C163S and the FLAG-tagged C163S experiments showed that the C163S mutant resided in the cytoplasm (Figure 4A and 4B, and Supplementary information, Table S1), and the results from western blotting confirmed the expression of these two mutants (Figure 4D and 4E). These results showed that the NES in caspase-3 was intact and still functional when caspase-3 was in the active conformation. Our findings also demonstrated that cleavage activity was essential for active caspase-3 to enter into the nucleus.

To investigate the effects of substrate recognition

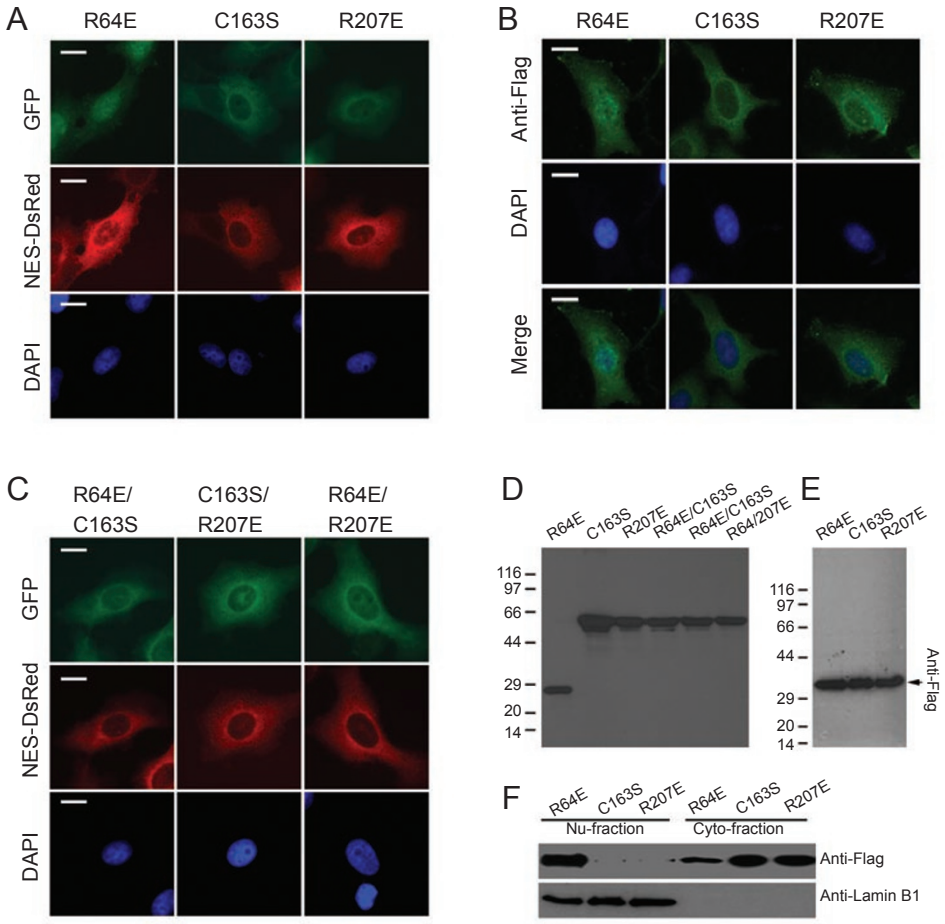

Figure 4 NES in the caspase-3 is not disturbed by the activation of caspase-3, and p3-mediated cleavage activity is essential for the active caspase-3 to enter the nucleus. **(A)** GFP-fused rev-caspase-3 mutants, R64E, C163S or R207E, were co-transfected with MAPKK-NES-DsRed into HeLa cells separately; cells were fixed 24 h post-transfection and stained with DAPI for fluorescence observation. Bar = 20 μM. **(B)** Flag-tagged rev-caspase-3 mutants, R64E, C163S or R207E, were transfected into HeLa cells separately; cells were fixed 24 h post-transfection and probed with anti-Flag antibody for immune-fluorescence observation. Bar = 20 μM. **(C)** GFP-fused rev-caspase-3 double mutants, R64E/C163S, C163S/R207E or R64E/R207E, were co-transfected with MAPKK-NES-DsRed into HeLa cells separately; cells were fixed 24 h post-transfection and stained with DAPI for fluorescence observation. Bar = 20 μM. **(D)** Cells transfected with GFP-fused rev-caspase-3 mutants were harvested for western blot using the anti-GFP antibody. **(E)** Cells transfected with Flag-tagged rev-caspase-3 mutants were harvested for western blot using the anti-Flag antibody. **(F)** Cells transfected with Flag-tagged rev-caspase-3 mutants were fractionized into nuclear and cytoplasmic fractions for western blot using the anti-Flag antibody. Anti-Lamin B1 antibody was used to detect nuclear-cytoplasmic contamination.

activity on the nuclear entry of active caspase-3, we mutated arginine 64 or arginine 207 of rev-caspase-3 to glutamic acid to disrupt the substrate recognition activities at the p1 and p3 recognition sites, respectively [13, 14]. The results showed that both the GFP-fused R207E and the FLAG-tagged R207E resided in the cytoplasm (Figure 4A and 4B, and Supplementary information, Table S1). R64E-GFP was distributed in the whole cell, but the R64E-GFP fusion protein was cleaved between GFP and R64E (Figure 4A and 4D). A caspase-3-recognized IETD motif resided between GFP and R64E (Supplementary information, Data S1), indicating that the R64E mutant retained the cleavage ability. Lamin B1 and PARP were not degraded by the R64E mutant (Supplementary information, Figure S2), indicating that the R64E mutant harbored a limited activity for substrate recognition and cleavage. This was also consistent with the phenomenon that R64E induced a much lower level of apoptosis than the wild-type rev-caspase-3 (Supplementary information, Figure S1). To verify the location of R64E, we replaced the IETD motif with the FLAG epitope for indirect immunofluorescence observation. Such replacement did not

influence the distribution of C163S or R207E (data not shown). The results showed no cleavage in the fusion protein (Figure 4E), and R64E was distributed throughout the cell (Figure 4B and Supplementary information, Table S1). We also prepared purified nuclear and cytoplasmic fractions for further investigation, and found that among the three mutants examined only the R64E mutant existed in the nuclear fraction (Figure 4F).

We constructed double mutants in order to rule out the possibility that the R64E mutation might cause the change of the subcellular localization signal in the rev-caspase-3. We observed that all the double mutants, namely R64E/R207E, R64E/C163S and R207E/C163S, resided in the cytoplasm (Figure 4C and 4D, and Supplementary information, Table S1). These results indicate that the subcellular localization signal in the rev-caspase-3 is not disrupted by the R64E mutation.

These results confirm that the NES in caspase-3 is not disturbed by the conformational changes accompanying caspase-3 activation. It also showed that the p3-mediated cleavage activity abrogated the function of the NES in caspase-3, which was essential for the active caspase-3 to enter into the nucleus.

p3-mediated cleavage activity abrogates the functions of crm-1-dependent NESs with low NES activity

The above results showed that p3-mediated specific cleavage activity could abrogate the function of the NES

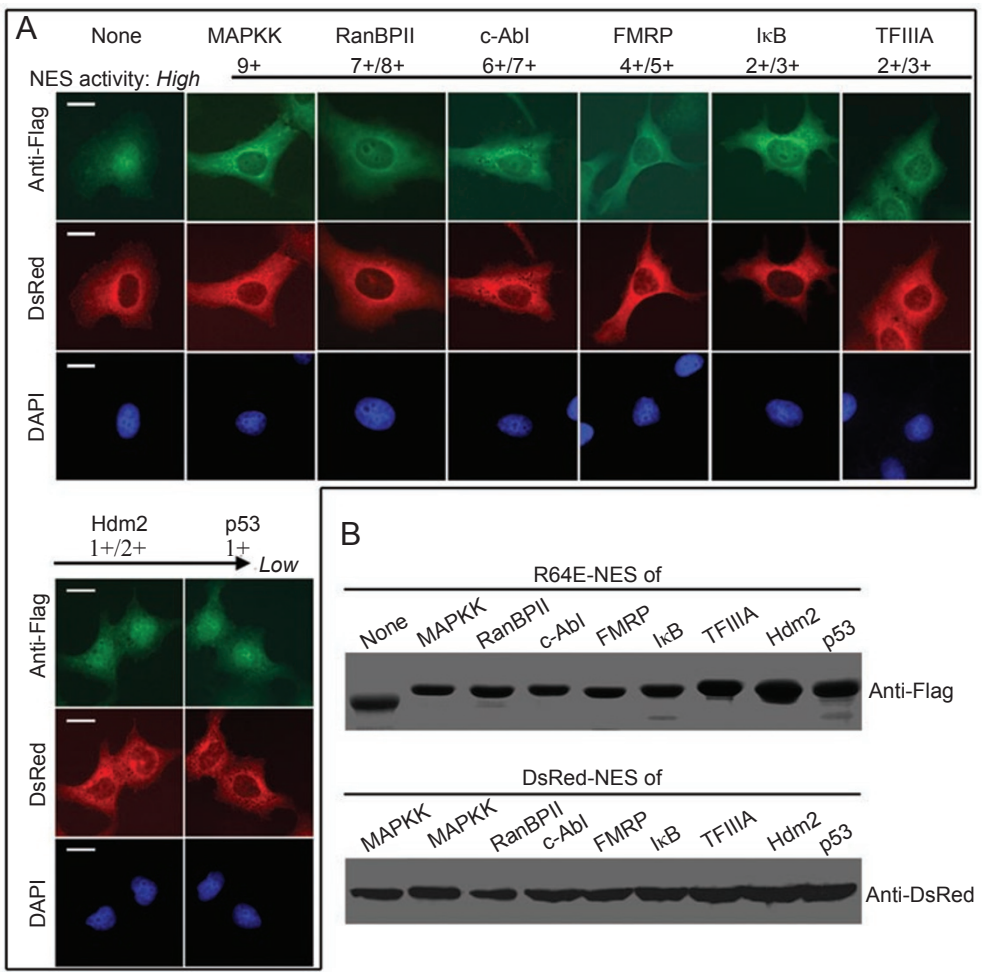

Figure 5 p3-mediated cleavage activity abrogates the functions of other crm-1-dependent NESs with low NES activity. **(A)** Different active NESs of different activities, from high to low activity, including NESs of MAPKK, cAbl, RanBPII, FMRP, IκB, TFIIIA, Hdm2 and p53, were added to the C terminus of R64E-rev-caspase-3, or fused with DsRed separately. NES-R64E-rev-caspase-3 was co-transfected with the same NES-DsRed into HeLa cells. For R64E-rev-caspase-3, MAPKK-NES-DsRed was co-transfected. At 24 h post-transfection, cells were fixed and probed with anti-Flag antibody for immune-fluorescence observation. The values of the NES activities were reported by Henderson *et al.* [11], and confirmed by our experiments. Bar = 20 μM. **(B)** The co-transfected cells were harvested for western blot using anti-Flag and anti-DsRed antibodies.

in caspase-3. Since the NES in caspase-3 is not dependent on crm-1, we further examined whether R64E-rev-caspase-3 could counteract the crm-1-dependent NES activity. We fused the R64E mutant with different crm-1-dependent NESs from high activity to low activity [11], and found that only R64E-rev-caspase-3 conjugated to the NES of Hdm2 or p53 (with low NES activity) resided in the nucleus (Figure 5A). NES-conjugated DsRed was used as the control to detect whether the crm-1-dependent pathway was disrupted, and the results showed that all the NES-conjugated DsRed resided in the cytoplasm (Figure 5A), indicating that the nuclear entry of R64E-rev-caspase-3-NES of Hdm2 or p53 was not a result of the disruption of the crm-1-dependent pathway, but that it was due to the nuclear entry ability of the R64E mutant. Western blot results confirmed the expression of these fusion proteins (Figure 5B). Our findings indicate that the p3-mediated cleavage activity of active caspase-3 not only abrogates its own NES function, but could also neutralize the crm-1-dependent NES with low NES activity when it is fused in cis.

Discussion

Many pro-apoptotic proteins display cyto-nuclear translocation during apoptosis, and there are several mechanisms reportedly involved. First, the pro-apoptotic protein harbors a masked NLS sequence in normal cells, which is exposed to phosphorylation, ubiquitination or other mechanisms during apoptosis, leading to protein nuclear localization, such as WOX1 [15], DEDD [16] and DIO-1 [17]. Second, the pro-apoptotic protein harbors both NLS and NESs, and exhibits whole cell or putative cytoplasmic distribution. During apoptosis, the NES is masked by protein modifications such as phosphorylation or ubiquitination, leading to protein nuclear localization, such as TRADD [18], GAPDH [19, 20], MST1 [21] and Daxx [22]. Third, there are some proteins that undergo cyto-nuclear translocation by protein interaction or other as yet poorly understood mechanisms, such as AIF [23] and EndoG [24].

Our finding demonstrates that pro-caspase-3 harbors a NES but not NLS (Figures 1 and 2). The NES of caspase-3 is not dependent on the crm-1 pathway, although it is similar to other known crm-1-dependent NESs with leucin-rich properties [11]. Our co-immunoprecipation data indicate that caspase-3 does not bind to crm-1 (Figure 1F). Furthermore, localization of caspase-3 was not very sensitive to the crm-1 inhibitor LMB. After LMB treatment, only a small portion of caspase-3 shifted from the cytoplasm to the nucleus, and no absolute nuclear accumulation was observed (Figure 1E). This slight shift might be due to the apoptosis-inducing ability of LMB [25, 26], which would result in the activation and subsequent nuclear entry of caspase-3. Other possibilities cannot be ruled out. Nevertheless, the main pathway that regulates the nuclear export of caspase-3 is not dependent on crm-1. Since the NES resides in the small subunit of pro-caspase-3, it is unlikely that the amino acid sequence of the NES would be disturbed by the activation of caspase-3. Rev-caspase-3 mutants, which are deficient of p3-recognition activity or cleavage activity, or both, and yet harbor the same conformation as the active caspase-3, displayed an absolute cytoplasmic distribution (Figure 4). This indicates that the NES in the active caspase-3 is still functional, and there is no NLS reassembled in the active caspase-3.

Cleavage activity and specific substrate-recognition activity are the two main features of the active caspase-3 [2]. We found that both p3 recognition activity and cleavage activity were necessary for the active caspase-3 to enter into the nucleus (Figure 4). However, a previous study showed that only the p3-recognition activity was important to this process [4], which could be due to the different experimental methods employed. In their study, the cells that contained C163S mutant caspase-3 were induced to undergo apoptosis, and the nuclei were then separated for western blot analysis [4]. Apart from the easy cytoplasmic contamination due to the harshly destroyed NE, endogenous caspase-3 was also activated during the induced apoptosis, which could cleave the C163S mutant caspase-3, allowing the formation of a hetero-tetramer with endogenous caspase-3 for entry into the nuclei [3]. This also explains their result, showing that mutation at the cleavage site between the p17 and p12 subunits inhibits nuclear translocation of caspase-3 [4], as this mutant could not be cleaved to form a hetero-tetramer with the endogenous caspase-3. In our study, rev-caspase-3 was used as the study model. It is composed of a reverse arrangement of the large subunit and the small subunit of caspase-3 to mimic the conformation of active caspase-3, and had been reported to harbor the same constitutive specific cleavage activity as the active caspase-3 [10, 12]. Using tagged rev-caspase-3 and its mutants, it was feasible to reliably detect the effects of these mutations on the nuclear entry of active caspase-3 *in vivo*. Our results showed that p3-mediated cleavage activity of caspase-3 leads to the dysfunction of its NES in the absence of any apoptotic stimuli (Figures 4 and 5), suggesting that the apoptotic surroundings are not necessary for the active caspase-3 to enter into the nucleus. As long as caspase-3 is activated, the p3-mediated cleavage activity will cause a direct entry of active caspase-3 into the nucleus even if the cells are non-apoptotic.

The nuclear entry of active caspase-3 can be divided into two steps. The first is to cross the NE. The active caspase-3 enters into the nucleus at the early stage of apoptosis, when the NE is still intact and functional [27]. As an ~ 60 kDa complex, it is difficult for the active caspase-3 to cross NE by passive diffusion. Furthermore, there is no NLS residing in the pro-caspase-3, or reassembled during the activation of caspase-3 (Figures 1 and 4). Therefore, this process has to be accomplished with the assistance of a carrier protein. Once the active caspase-3 enters into the nucleus, it will be exported back to the cytoplasm, since its NES is still functional. Therefore, the second step is to prevent the export of the active caspase-3. This could be achieved by associating with the carrier protein or by disrupting the export pathway of caspase-3. As p3-mediated cleavage activity is essential for the nuclear entry of active caspase-3, one or more proteins recognized by a p3-recognition site have to be cleaved to facilitate the entry of active caspase-3 into the nucleus. The effectors (carrier and export preventer) responsible for the above two sequential steps could be same or different; nevertheless, at least the export preventer should be a caspase-3 substrate.

Previous investigators have hypothesized that the active caspase-3 enters into the nucleus by a substrate-like carrier protein [4] and have suggested that AKAP95 might be the potential carrier [9]. AKAP95 is a nuclear-localized protein, and contains a pseudo caspase-3 recognition motif that can be recognized and bound but not cleaved by the active caspase-3. It was suggested that active caspase-3 was carried into the nucleus by interacting with the pseudo caspase-3 recognition motif of AKAP95 [9]. In our study, we found that C163S-rev-caspase-3, deficient in cleavage activity but retaining the substrate recognition activity, still resided in the cytoplasm (Figure 4), indicating that AKAP95 alone is unable to carry the active caspase-3 into the nucleus. Moreover, even if the active caspase-3 is carried by AKAP95 to enter into the nucleus, AKAP95 has to be dissociated from the active caspase-3, otherwise the active caspase-3 would not be able to recognize other substrates via its substrate-recognition site, which has already been occupied by AKAP95; in this case, the released active caspase-3 will be exported back into the cytoplasm since its NES is still functional (Figure 4). Therefore, prevention of the export of active caspase-3 seems critical for the active caspase-3 to enter into the nucleus. Export prevention could be achieved by two ways. The first is by associating with the carrier protein or other nuclear proteins, although in this way it seems difficult for the active caspase-3 to exert its function. We prefer another possibility, which is to disrupt the export pathway of caspase-3 by degrading the components of the pathway, such as the related exportin and nucleoporins. We analyzed the nucleoporins that are substrates of caspase-3, including RanBPII, Tpr, Nup153, Nup98 and Nup93 [27], but did not obtain any positive results (data not shown). Further studies, especially the identification of the caspase-3 exportin, would be necessary.

In this study, we demonstrate that NES resides at the small subunit of caspase-3, which is not regulated by crm-1. This NES is constitutively present and functional even when caspase-3 is activated. p3-mediated cleavage activity of active caspase-3 abrogates the function of the NES in the active casapse-3, facilitating its entry into the nucleus.

Materials and Methods

Plasmids construction

Caspase-3 was amplified from the cDNA of Hela cells, and then constructed into pEGFP-C2 (Clontech) by *Eco*RI/*Sal*I. Truncated forms of caspase-3 were amplified by the corresponding primers and constructed into pEGFP-C2 by *Eco*RI/*Sal*I. Rev-caspase-3 was constructed according to the methods described by Srinivasula *et al.* [10], and ligated into pEGFP-C2 by *Eco*RI/*Sal*I. Mutants R64E, C163S, R207E and the double mutants of rev-caspase-3 were created by Geneedit (Promega) and constructed into pEGFP-C2 and pcDNA3.1-Flag (Invitrogen, Grand Island, NY, USA) by *Eco*RI/*Sal*I or *Eco*RI/*Xho*I.

The pEGFP-Rev1.4 plasmid was constructed as described by Henderson *et al.* [11]. Briefly, NES mutant HIV Rev (Rev1.4) was generated by PCR from the template pRSV-Rev, and constructed into pEGFP-N1 by *Nhe*I/*Bgl*II. A multiple cloning site (MCS) in the translation frame of EGFP was inserted between *Bgl*II and *Bam*HI, and the plasmid was designated as pEGFP-Rev1.4. C3S and C3S-F, the candidate NES4 of caspase-3, or the NES of MAPKK (NLVDLQKKLEELELDEQQ), cAbl (AINKLENNLRELQICPAT), RanBPII (HAEKVAEKLEALSVKEET), FMRP (KEVDQLRLERLQIDEQL), IκB (PATRIQQQLGQLTLENLQ), TFIIIA (SKADPLPVLENLTLKSSN), Hdm2 (SDSISLSFDESLALCVIR) and p53 (RFEMFRELNEALELKDA) [11] were constructed into pEGFP-Rev1.4 or DsRed-N2 (Clontech) between *Bgl*II/*Eco*RI sites.

Cell culture and cell transfection

HeLa cells were cultured in DMEM (Invitrogen) with 10% fetal bovine serum (HyClone Laboratories Inc.) and 100 U/ml penicillin/streptomycin at 37 °C, 5% CO_2. For transfection, cells were plated in 35-mm-diameter culture dishes at about 80% confluency and transfected with the plasmids using Lipofectamine2000 (Invitrogen) according to the protocol provided by the manufacturer.

Immuno-fluorescence

Cells were cultured onto glass coverslips in a 24-well culture plate (Nunc). After transfection with DNA plasmids for 24 h, cells were fixed with 4% paraformaldehyde in PBS for 15 min, followed by extraction with 0.5% Triton X100 in PBS for 3 min, immunostained with anti-FLAG antibody (Sigma), and then with

FITC goat anti-mouse IgG (Santa Cruz). After washing three times with PBS, the cells were kept on mounting solutions for observation under a fluorescence microscope (Zeiss M200).

Immunoprecipitation

Cells from a 100-mm dish were lysed in 1 ml of lysis buffer (15 mM Tris, pH 7.5, 120 mM NaCl, 0.5% Triton X-100, 25 mM KCl, 2 mM EDTA, 0.1 mM DTT) plus a protease inhibitor cocktail (Sigma). For each immunoprecipitation, a 0.4-ml aliquot of lysate was incubated with 0.5 μg of the indicated antibody or control IgG, and 25 μl of 1:1 slurry of Protein A sepharose beads (Pharmacia) at 4 °C for at least 2 h. The precipitated immune-complex was washed with lysis buffer five times, and then fractionated on SDS-PAGE for western blot analysis.

Subcellular fractionation

Cells were washed with PBS and lysed in hypotonic solution (10 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 42 mM KCl, 10 mM lactacystin) using a Dounce homogenizer at 4 °C, and centrifuged at 600× *g* for 10 min. The supernatant and pellet were designated as the crude cytoplasmic and nuclear fractions, respectively. The crude cytoplasmic fraction was centrifuged at 100 000× *g* for 90 min, and the supernatant was collected as the purified cytoplasmic fraction. The crude nuclear fraction was extensively washed with hypotonic solution, and centrifuged through a 2 M sucrose cushion at 150 000× *g* for 60 min. The pellet was then collected as the purified nuclear fraction.

Western blotting

After being resolved on 6%-15% SDS-PAGE gels, the protein samples were transferred onto a nitrocellulose (NC) membrane in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 100 V for 1 h. The NC membrane was then blocked in TTBS (20 mM Tris-HCl (pH 7.4), 500 mM NaCl and 0.3% Tween-20) containing 5% non-fat milk for 1 h at room temperature and probed with anti-GFP antibody (Santa Cruz, sc-9996), anti-FLAG antibody (Sigma, F3040), anti-crm-1 antibody (Santa Cruz, sc-7825), anti-caspase-3 antibody (Santa Cruz, sc-7148, sc-1224), anti-PARP antibody (Santa Cruz, sc-25780) or anti-Lamin B1 antibody (Santa Cruz, sc-20682). The NC membrane was washed three times with TTBS, blocked for 30 min in TTBS containing 5% non-fat milk at room temperature, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit or anti-goat secondary antibodies (Sigma) at room temperature. The membrane was washed three times with TTBS, and then developed with enhanced chemiluminescence (PIERCE) for visualization.

NES scoring assay

The NES scoring assay was described by Henderson *et al.* [11]. Briefly, the HIV rev-GFP fusion protein would shift from the nucleus to the cytoplasm after a short exposure of cells to Act D (Sigma), while the NES-mutated form of rev-GFP (Rev1.4) would remain in the nucleus. Insertion of an NES sequence between the Rev1.4 and the GFP would restore the export activity observed in the wild-type rev-GFP. Therefore, the export activity of the candidate NES could be determined by a shift in localization from the nucleus to the cytoplasm following Act D treatment of 1 to 3 h.

The candidate NES sequence was cloned into pEGFP-Rev1.4 and transfected into Hela cells. At 24 h post-transfection, cells were treated with 5 μg/ml Act D for 3 h; cycloheximide (Sigma) at a final concentration of 15 μg/ml was added to all samples to ensure that cytoplasmic GFP arose from nuclear export but not from the newly translated protein. The subcellular localization of the GFP-fusion proteins was determined with 200 randomly selected cells at least per sample, and the activity of the functional NESs was rated according to the scoring system described by Henderson *et al.* [11]. The crm-1 dependence of the functional NESs was further confirmed by transfected cells using 6 ng/ml LMB (Sigma) treatment for 3 h.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)