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Identification of nucleolus localization signal of betanodavirus GGNNV protein α

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Abstract

Betanodavirus greasy grouper (Epinephelus tauvina) nervous necrosis viruses (GGNNV) protein α, a virus capsid protein, was detected in both nucleolus and cytoplasm of infected cells of Asian sea bass (SB) and transfected cells of SB and Cos-7 with pcDNA3.1/RNA2. To study its subcellular localization, ORF of protein α with 338 aa was fused with enhanced green fluorescent protein (EGFP) gene and was detected in transfected cells in the absence of other viral proteins. In both SB and Cos-7 cells, protein α was found to localize EGFP to the nucleolus and cytoplasm. Deletion mutants of protein α indicated that N-terminal 43 amino acid residues were required to import EGFP-α protein into the nucleolus. Further deletions within the 43 amino acid backbone, EGFP/33aa(1–33) and EGFP/30aa(14–43), localized to the nucleolus, suggesting that the 20 amino acids from 14 to 33 of protein α were the domain of nucleolus localization. To further determine the nucleolus targeting sequence, deletion mutations within the 20 amino acids of protein α were constructed. It was found that the deletion of 23RRR25, 29RRR29, or 23RRRANNRRR31 prevented the accumulation of EGFP fusion proteins into the nucleolus, demonstrating that 23RRRANNRRR31 contain the signal required for nucleolus localization. A similar distribution pattern of localization of protein α and its deletion mutants in SB and Cos-7 cells suggested that N-terminal residues of protein α 23RRRANNRRR31 constitute a nucleolus localization signal that functions in both fish and mammalian cells.

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Keywords: Betanodavirus greasy grouper (Epinephelus tauvina) nervous necrosis viruses (GGNNV); Capsid protein α; Enhanced green fluorescent protein (EGFP); Nucleolus localization; Deletion mutations; Nucleolus localization signal; SB and Cos-7 cells

Introduction

Nodaviruses are small, nonenveloped, spherical viruses with bipartite positive-sense RNA genomes, which are capped but not polyadenylated. Two genera have been distinguished in the Nodaviridae family: alphanodaviruses, which are originally isolated from insects and betanodaviruses, which usually infect a wide variety of larval and juvenile marine fish (Ball et al., 1994, 2000). Similar to alphanodaviruses, the genome of betanodaviruses has two coencapsidated genomic RNA segments (RNAs 1 and 2), both of which are required for infectivity. RNA1 encodes protein A, which is the viral component of the viral RNA-dependent RNA polymerase (RdRp). RNA2 encodes protein α, a proteolytic precursor of viral capsid proteins. A subgenomic RNA3 is synthesized during RNA replication from 3’ terminus of RNA1 and encodes one or two small proteins (B1 and B2) with unknown functions. As a member of betanodaviruses, the complete sequence of greasy grouper (Epinephelus tauvina) nervous necrosis viruses (GGNNV) of Singapore strain has been determined in our laboratory. The complete nucleotide sequence of RNA1 is 3103 nucleotides containing an ORF of 982 aa and the RNA2 is 1433 nucleotides containing an ORF of 338 aa (Tan et al., 2001). RNA2 encodes a 37-kDa capsid protein whose recombinant protein α could be used as a sensitive reagent to diagnosis GGNNV infection (Huang et al., 2001). In our previous work, GGNNV was demonstrated to be...
capable of inducing caspase-dependent apoptosis in GGNNV-infected SB cells and protein α was a possible apoptotic inducer in transfected cells (data not shown).

Nucleolus is mainly the site of ribosome biogenesis, a highly complex process leading to the production of preribosomal particles, which are then released to the nucleoplasm and exported to the cytoplasm as mature ribosomal subunits (Carmon-Fonseca et al., 2000). However, more and more exogenous nonribosomal proteins were demonstrated to be transported to the nucleolus under certain conditions. Proteins from porcine reproductive and respiratory syndrome virus (Rowland et al., 1999), Semliki Forest virus (Favre et al., 1994), coronavirus (Hiscox et al., 2001), and Borna disease virus (Pyper et al., 1998) have been reported to localize in the nucleolus and to get involved with various aspects of virus replication. It has been found that a cluster of basic amino acids could lead proteins to the nuclear or nucleolus (Kaffman and O’Shea, 1999).

In the past few years, many studies have shown that the green fluorescent protein (GFP) from jellyfish is a popular tool for localization of a specific protein distribution inside cells when the GFP is fused with that particular protein (Misteli and Spector, 1997; White and Stelzer, 1999; Hiscox et al., 2001; Shih and Lo, 2001). In this study, the protein α of GGNNV could be detected in both nucleolus and cytoplasm of SB cells infected by GGNNV and SB and Cos-7 cells transfected with pcDNA3.1/RNA2. The ORF of protein α was then fused with EGFP and transfected to the SB and Cos-7 cells to study its subcellular localization. It was found that the GGNNV protein α could direct the EGFP to the nucleolus of both SB and Cos-7 cells. Employing a series of deletion mutations, the N-terminal amino acid residues $^{23}$RRRANNRRR$^{31}$ were identified as a nucleolus localization signal which functioned in both fish and mammalian cells, indicating that there is a conserved mechanism for nucleolus localization in both cells.

**Results**

**Localization of protein α in Asian sea bass (SB) cells infected with GGNNV and in SB and Cos-7 cells transfected with pcDNA3.1/RNA2**

To identify the localization of protein α in SB cells infected with GGNNV, SB cells were fixed at 20 h postinfection and analyzed by immunofluorescence assay (IFA). A polyclonal guinea pig antiprotein α sera was used as primary antibody, followed by FITC-conjugated secondary antibody, and the cells were observed under fluorescence microscope. In the case of SB cells, DAPI was used to stain the nuclear boundary to visualize the nucleolus localization of protein α. IFA staining observations of SB cells exhibited two patterns on the localization of protein α, one with nucleolus and cytoplasmic staining (Fig. 1, images a–c) and the other without nucleolus labeling (data not shown). About 10% of GGNNV-infected SB cells showed protein α localization in both the nucleolus and the cytoplasm.

To investigate further the subcellular localization of GGNNV protein α in the absence of other viral proteins, the ORF cDNA of protein α was cloned into the eukaryotic expression vector pcDNA3.1(+), to construct pcDNA3.1/RNA2. SB cells transfected with pcDNA3.1/RNA2 were fixed at 36 h posttransfection and IFA was carried out with polyclonal guinea pig antiprotein α sera. Similarly, Cos-7 cells were transfected with pcDNA3.1/RNA2 to detect the localization of protein α in mammalian cells. In both SB (Fig. 1, images d–f) and Cos-7 cells (Fig. 1, images g–i), protein α was observed in both nucleolus and cytoplasm. The percentage of transfected cells exhibited in both the nucleolus and the cytoplasmic protein α localization is about equal (∼10%) to that of the GGNNV-infected SB cells.

**Localization of enhanced green fluorescent protein (EGFP)-α fusion protein in transfected cells**

To investigate whether the GGNNV protein α could transport an exogen protein to the nucleolus, ORF cDNA of protein α was inserted into pEGFP-C1 to construct pEGFP/
RNA2 (Fig. 2A). In this construct, the expression of the EGFP-α fusion protein was under the control of a cytomegalovirus (CMV) promoter and the localization of protein α could be monitored by green fluorescence.

When the SB cells were transfected with pEGFP/RNA2, at 12 h posttransfection, the expression of EGFP-α fusion protein could be observed by fluorescence microscopy. As shown in Fig. 2B (image d), most green fluorescent signal is in the nucleus, especially enriched in the nucleolus. At 36 h posttransfection, EGFP-α fusion protein could be viewed in both nucleolus and cytoplasm (Fig. 2B, image g). DAPI, which can specifically bind to chromosome, was used to show the nuclear shape in blue color (Fig. 2B, images b, e, and h). The overlapping of green and blue fluorescent signal highlighted the localization of EGFP-α fusion protein in nuclear and nucleolus (Fig. 2B, images f and i). When SB cells were transfected with vector pEGFP-C1 and observed at 36 h posttransfection (Fig. 2B, image a), EGFP diffused throughout whole cell except the nucleolus.

To detect the subcellular localization of the fusion protein in mammalian cells, Cos-7 cells were also transfected with pEGFP-RNA2. At 12 h posttransfection (Fig. 2B, image D), EGFP-α fusion protein was seen concentrating in the nucleoli. At 36 h posttransfection, EGFP-α protein could be observed in the cytoplasm and nucleoli (Fig. 2B, image G). By contrast, EGFP alone was distributed diffusely throughout the cytoplasm and nucleoplasm with no specific pattern (Fig. 2B, image A). The localization of
The nucleolus localization signal resides in the N-terminus of protein α

As protein α showed similar distribution in both cell types tested, experiments for the identification of the nucleolus localization signal were performed in Cos-7 cells only. Four N-terminal and C-terminal truncated protein α were constructed and fused with EGFP (Fig. 3A): pEGFP-RNA2/N90 (deleted N-terminal amino acids 1–90), pEGFP-RNA2/N78 (deleted N-terminal amino acids 1–78), pEGFP-RNA2/N43 (deleted N-terminal amino acids 1–43), and pEGFP-RNA2/C39 (deleted C-terminal amino acids 1–39). These recombinant plasmids were transfected to Cos-7 cells and viewed by confocal microscopy at 36 h posttransfection. EGFP/RNA2/N90 (data not shown), EGFP/RNA2/N78 (data not shown), and EGFP/RNA2/N43 (Fig. 3B, image a) were detected by antiprotein sera (images e and h) using indirect IFA at 36 h posttransfection. The merged image represents a digital superimposition of green and red signals, where areas of fluorescence colocalization are yellow-orange, indicating expression of fusion proteins (images f and i).

From the above result, it could be deduced that there are nucleolus localization signals in the protein α which can lead exogenous protein EGFP to the nucleolus of both fish and mammalian cells.
showed the same distribution in Cos-7 cells; that is, at 36 h posttransfection, most protein was distributed in the cytoplasm and only background signal was observed in the nucleoplasm, but was completely excluded from nucleoli. However, the expression image of EGFP/RNA2/C39 (Fig. 3B, image g) was the same as that of wild-type EGFP/H9251 fusion protein. The indirect IFA with antiprotein/H9251 sera further demonstrated the localization of EGFP/RNA2/N43 and EGFP/RNA2/C39 in transfected Cos-7 cells. The merged image (Fig. 3B, image f) of EGFP/RNA2/N43 fusion protein (Fig. 3B, image d, green) and red image of antiprotein α sera (Fig. 3B, image e) showed empty nucleoli. However, in the Cos-7 cell transfected with pEGFP/RNA2/C39 (Fig. 3B, image g), the indirect IFA showed the expression of EGFP/RNA2/C39 in nucleolus and cytoplasm (Fig. 3D, images h and i).

The expression of EGFP/RNA2/N90, EGFP/RNA2/N78, EGFP/RNA2/N43, and EGFP/RNA2/C39 was confirmed by Western blot analysis with anti-GFP antibody which detected the expected proteins (Fig. 3C). Taken together, these results indicated that the nucleolar localization signal of protein α is located in its N-terminal 43 amino acids.

**Defining the nucleolus localization domain of protein α**

To identify the functional domain of protein α responsible for nucleolus localization, the N-terminal 43 amino acids of protein α were fused to EGFP (Fig. 4A) and transiently expressed in Cos-7 cells. The N-terminal 43 amino acids of protein α were able to lead EGFP into the nucleoli of Cos-7 cells (Fig. 4B). To further confirm the nucleolar localization of EGFP/43aa observed by confocal microscopy, Cos-7 cells expressing EGFP/43aa was fractionated into cytoplasmic and nuclear component and mAb
against GFP was used in Western blot assay. EGFP-43aa fusion protein was not detected in the cytoplasmic fraction (Fig. 5, lane 3), whereas in the nuclear fraction, the EGFP-43aa fusion protein was in high concentration (Fig. 5, lane 1). However, EGFP as a control was found in both cytoplasmic (Fig. 5, lane 4) and nuclear fraction (Fig. 5, lane 2). There was more EGFP in the cytoplasmic extract than in the nuclear fraction. Antinucleolin mAb was used as a control to test the fidelity of the subcellular fractionations from cytoplasm (lane 5) and nuclear (lane 6).

To narrow down the exact location of the localization signal, two additional deletion fragments from the N-terminal of proteins α were fused to EGFP (Fig. 4A) and transfected to Cos-7 cells. At 36 h posttransfection, transfected Cos-7 cells were fixed and antinucleolin mAb was used to define the location of nucleoli. Both EGFP/33aa (1–33) (Fig. 4B, image d) and EGFP/30aa (14–43) (Fig. 4B, image g) were located in the nucleoli of Cos-7 cells. The EGFP fusion proteins could be recognized by anti-GFP polyclonal antibody with expected sizes that were 32.7 kDa EGFP/33aa, 32.8 kDa EGFP/33aa, and 33.5 kDa EGFP/43aa in lanes i, ii, and iii, respectively (Fig. 4C). Taken together, a putative domain of nucleolar localization of protein α was identified to be between amino acid residues 14 and 33 of protein α.

Identification of $^{23}$RRRANNRRR$^{31}$ as the nucleolus localization signal of protein α

We next examined the amino acids from 14 to 33 to find which amino acids were more important in the nucleolar localization. Amino acid sequence analyses revealed that the Arg-rich region between amino acids 23 and 31 of protein α resembles nucleolar localization signals (NoLS) described in several other viral proteins and cellular proteins, such as human immunodeficiency virus type 1 (HIV-1) Rev and Tat (Kubota et al., 1996; Siomi et al., 1990), human T lymphotropic virus type I (HTLV-I) Rex (Siomi et al., 1988), African swine fever virus (ASFV) I14L (Goatley et al., 1999), the MEQ protein of Marek’s disease virus (MDV) (Liu et al., 1997), parathyroid hormone-related protein (PTHrP) (Henderson et al., 1995), death effector domain-containing DNA-binding protein (DEDD) (Stegh et al., 1998), and angiogenin (Lixin et al., 2001). Table 1 shows a comparison between the NoLS of these proteins and the Arg-rich domain at N terminal of protein α.

To determine whether the Arg-rich domain between amino acids 23 and 31 of protein α is required for nucleolar localization, three constructs encoding fusion proteins containing different deletion mutations were transfected to Cos-7 cells (Fig. 6A). It was found that at 12 h posttransfection, for all these constructs, most signal was diffused in nucleoplasm, but was completely excluded from the nucleoli (Fig. 6B, images a, d, and g). After 36 h transfection, the fluorescent signal was viewed in cytoplasm, but not in the nucleolus (Fig. 6B, images ai, di, and gi). These phenomena were consistent with our conjecture that the amino acid residues $^{23}$RRRANNRRR$^{31}$ of protein α played a vital role in the nucleolus targeting of protein α in transfected cells. When the amino acids $^{23}$RRR$^{25}$ or $^{29}$RRR$^{31}$ were truncated from protein α, the specific nucleolar localization of the EGFP-α fusion protein was abolished, indicating that both $^{23}$RRR$^{25}$ and $^{29}$RRR$^{31}$ were responsible for directing nucleolar localization, but individually incapable of localizing EGFP-α fusion proteins to the nucleoli.

### Table 1

| Proteins Sequences |
|---------------------|
| Protein α          |
| MEQ                |
| Rev                |
| Rex                |
| I14L               |
| PTHrP              |
| DEDD               |
| Angiogenin         |
| Note: Sequences from MEQ (from MDV), Tat and Rev (from HIV), Rex (from HTLV-I), I14L (from ASFV), parathyroid hormone-related peptide (PTHrP), death effector domain-containing DNA-binding protein (DEDD), and angiogenin, which are required for nucleolar localization, are compared with N-terminal domain of protein α. The basic residues are shown in bold. |
Subcellular localization of deletion mutations of protein α in SB cells

As the data of identifying the nucleolus localization signal were based on the Cos-7 cells, it was necessary to investigate whether this nucleolus localization signal worked in betanodavirus GGNNV host fish cells. We transiently expressed those different deletion mutants which functioned in Cos-7 cells into SB cells. The distribution of EGFP fusion proteins showed a similar pattern, as we predicted. At 36 h posttransfection, EGFP-RNA2/N43 was distributed in the nucleoplasm and cytoplasm except the nucleolus (Fig. 7, image a) and EGFP/43aa was enriched in the nucleolus (Fig. 7, image d). Following the deletion amino acids of 23RRR (Fig. 7, image g), 29RRR (Fig. 7, image j), or 23RRRANNRRR (Fig. 7, image m) from protein α, the localization of EGFP-α fusion protein disappeared from the nucleoli. In addition, DAPI staining was carried out to show the nuclear in blue (Fig. 7, images b, e, h, k, and n). These observations demonstrated that the nucleolus localization signal 23RRRANNRRR (Fig. 7, image i) identified in Cos-7 cells played the same important role in betanodavirus GGNNV host SB cells.

Discussion

Localization to the nucleolus has been a part of the strategy for viruses to control both host cell and virus subgenomic RNA translation. The coronavirus N protein which is located in the nucleolus disrupted host cell division (Wurm et al., 2001); the HIV regulating proteins Tat and Rev made use of nucleolus for the trafficking of HIV-1 RNA (Michenzi et al., 2000; Siomi et al., 1990). In addition, some cellular proteins, including Werner syndrome (WRN) protein (Marciniak et al., 1998), DEDD (Stegh et al., 1998), and PTHR (Henderson et al., 1995), which localize in nucleoli, have been found to be involved in the apoptosis events and the regulation of proliferation and differentiation. In this study, we have demonstrated nucleolar localization of GGNNV protein α in infected SB cells by IFA using monospecific antibody against protein α. Subsequently, transient expression of protein α showed its nucleolar distribution in fish and mammalian cells. However, the role of nucleoli in GGNNV infection needs further study.

The stages of the cell cycle may modulate protein targeting of specific subcellular compartment (Hiscox et al., 2001). Nucleoli are present when cells are in interphase...
In the present study, at various time points, about 10% of infected or transfected cells exhibited nucleolus and cytoplasmic localization of GGNNV protein. A possible reason for this observation could be that the fewer infected or transfected cells are at interphase, which showed localization of GGNNV protein to the nucleolus, whereas other cells are in other phases of mitosis. Hiscox et al., (2001) also reported fewer cells with nucleolus localization of IBV N protein. However, mitosis alone cannot account for the absence of protein α from the nucleoli in the majority of infected or transfected cells, which suggests that localization of GGNNV protein α to the nucleoli might be dependent on particular events of the cycle that the infected or transfected cell is undergoing.

Protein import into the cell nucleus occurs through nuclear pore complexes either by diffusion or by signal-mediated transport. Proteins less than 40 kDa are able to enter the nucleus by passive diffusion; others generally require a specific nuclear localization signal (NLS), which is ATP dependent. NLSs have been identified for a number of viral and cellular proteins which have been classified as either monopartite or bipartite, depending on whether or not stretches of basic residues are interrupted by a 10 amino acid space region (Garcia-Bustos et al., 1991). Recently, several sequences have been defined as nucleolus localization signals. They consisted of clustered basic Arg/Lys residues and also functioned as nuclear transporting signals (Hiscox et al., 1996; Siomi et al., 1988; Goatley et al., 1999; Zhu et al., 1999).

In this study, EGFP was used to study the subcellular localization of protein α and search for its NLS. EGFP-α fusion protein with a mass of 67 kDa could not be expected to diffuse into the nucleoplasm unless it contained an NLS/NoLS. The results of subcellular fraction analysis indicate that EGFP protein can diffuse to the nucleus, but its concentration in the nuclear extract is low. However, when EGFP was fused with N-terminal 43 aa of protein α, the fusion protein was mostly detected in the nuclear extract. So, we predicted that there must be a nucleolus localization signal in the 43 aa which contributes to the nucleolar localization of EGFP/43aa. There are two groups of basic amino acids in the N-terminal amino acids of protein α: 2RKGEKKLAK10 and 23RRRANNRRR31, which resembled nucleolar localization signals that have been described in other viral and cellular proteins. The distribution of the deletion mutants of protein α in transfected cells demonstrated that 23RRRANNRRR31 is a nucleolus localization signal. In this study, nucleolus localization signal of protein α was first identified in betanodaviruses, which may yield new insights into the mechanism of betanodaviruses infection. When 23RRR25 or 29RRR31 or 23RRRANNRRR31 was deleted from protein α, EGFP fusion protein, which is too large (>40 kDa) to pass through the nuclear pore complexes by simple diffusion, was still observed in the nucleus. However, when the N-terminal 43 aa was deleted from protein α, EGFP/RNA2/N43 disappeared from the nucleus. These observations led to the possible suggestion that the protein α may contain other signals within 43 aa, such as 2RKGEKKLAK10, to be involved in its nuclear localization in the transfected and infected SB cells.

The mechanism of nucleolus localization of exogenous proteins depends on their nucleolar localization signals to bind either to an acid region in the nucleolus such as B23, or to rRNA, or to ribosome proteins (Li, 1997; Kubota et al., 1995).
cells. Although protein α failed to localize to the nucleolus when amino acids 23RRRANNRRR31 were deleted, the apoptosis could not be prevented in transfected SB and Cos-7 cells (data not shown), indicating there is no relationship between nucleolus localization of protein α and its ability to induce apoptosis. Since protein α is located in both cytoplasm and nucleolus in transfected and infected cells, its cytoplasm localization might be involved in its apoptosis.

Materials and methods

Cells and viruses

Betanodaviruses (GGNNV) and SB cell line (Chong et al., 1987, Chong et al., 1990) were obtained from Agri-food and Veterinary Authority of Singapore (AVA). The SB cells were grown in modified Eagle’s medium (MEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 0.34% NaCl, 0.12% HEPES, and 2 mM glutamine at 23°C. GGNNV was originally isolated in 1992 from brain, head, kidney, and liver of greasy grouper E. tauvina, in Singapore. The SB cells were infected with the betanodaviruses GGNNV for propagation and inoculated cultures were harvested when 90% of cells in the monolayer showed specific CPE.

Cos-7 cells (a monkey kidney cell line) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% FBS and antibiotic (GIBCO) and grown at 37°C in 5% CO₂.

Antibodies

Polyclonal antiprotein α sera were produced by immunizing guinea pig with recombinant protein α expressed in Escherichia coli. The sera were then bled after boosting two times and purified by Column–protein A. The antibody was confirmed by Western blot analysis using GGNNV-infected SB cells and a specific 37-kDa protein could be detected (data not shown). Polyclonal anti-GFP sera was purchased from Molecular Probes (USA) and mAb anti-nucleolin was purchased from MBL (Japan).

Plasmids construction and cell transfection

Protein α encoding sequence and all deletion fragments were cloned into pcDNA3.1(+) vector (Clontech, USA) or into pEGFP-C1 vector (Clontech) in-frame with the EGFP and sequenced to verify reading frame. The primers and restriction enzymes site used to construct different recombinant plasmids were shown in Table 2.

The deletion mutants of pEGFP/RNA2/d1RRR, pEGFP/43AA/d2RRR, and pEGFP/43AA/d9aa acquired by using QuickChange Site-Directed Mutagenesis Kit (Stratagene) and primers are shown in Table 2. Briefly, recombinant
plasmid pEGFP/RNA2 was amplified by pfuTurbo DNA polymerase with designed primers and then digested with DpnI restriction enzyme at 37°C for 1 h, followed by transformation with 1 μl reaction. The mutated plasmids were screened by sequencing with primer pEGFP: ACTACCT-GAGCACCAGTGCC.

Confluent monolayers (60–80%) of SB or Cos-7 cells grown on four-well chamber slides (IWAKI, Japan) were transfected with 0.5 μg (per chamber) of plasmid DNA purified by Qiagen plasmid Midi kits (USA) mixed with Lipofectamine plus reagent, according to the instructions of the manufacturer (Life Technologies, USA).

Indirect immunofluorescence assay and confocal microscopy

Transfected Cos-7 cells were cultured on four-well chamber slides (IWAKI). At 36 h posttransfection, cells were rinsed with phosphate-buffered saline and subjected to fixation with 4% paraformaldehyde for 25 min at room temperature and then permeabilized for 5 min in 0.2% Triton X-100 in PBS at room temperature for IFA. IFA was performed by incubating cells with primary antibodies (mAb antinucleolin or polyclonal antiprotein α sera) for 1 h at room temperature and then washed with phosphate-buffered saline with 0.05% Tween20 (PBST), followed by secondary antibodies conjugated with tetramethyl rhodamine isocyanate for 40 min at room temperature. Images were viewed and collected with a Zeiss confocal microscope LSM510.

DAPI staining

SB cells transfected with plasmids were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 in PBS as described above. Then cells were stained with DAPI (Molecular Probes) according to the manufacturer’s instructions, viewed using a Leica 550 IW fluorescence microscope, and digital photographs were taken. Subcellular fractionation

Cos-7 cells were cultured in 25 × 25 cm flasks and transfected with plasmid pEGFP-C1 or pEGFP/43AA. At 48 h posttransfection, cells were rinsed twice with ice-cold PBS and harvested by centrifuging at 2000 rpm for 5 min. The cell pellets were gently resuspended in hypotonic buffer [20 nM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 0.1 mM Na3VO4, 10% glycerol, 0.2% NP-40, 20 mM NaF, 1 mM DTT, 1 mM PMSF, complete proteinase inhibitors (Roche Molecular Biology): 1 tablet/8 ml]. The cell suspension was rocked at 4°C for 10 min and then centrifuged at 13,000 rpm at 4°C for 10 min, and the supernatant saved as the cytoplasmic extract. The nuclear pellet was washed in hypotonic buffer once and centrifuged as above. The crude nuclei was incubated with high salt buffer (hypotonic buffer without NP-40, 420 mM NaCl, 20% glycerol) and rocked at 4°C for 30 min. The lysate was then centrifuged at 13,000 rpm at 4°C for 10 min and the supernatant was collected as the nuclear extract.

Western blot analysis

Subcellular fractionation and extracts from SB or Cos-7 cells expressing EGFP or EGFP fusion proteins were separated by 12% SDS–PAGE and transferred to Hybond nitrocellulose membranes (Bio-Rad, USA). The membranes were blocked with 5% skimmed milk in PBST for 1 h at room temperature, washed with PBST once, and incubated with antibodies against GFP or protein α at room temperature for 1 h, then washed with PBST three times and incubated with secondary antibody conjugated with HRP at room temperature for 40 min. After rinsing three times, the specific proteins were visualized by enhanced chemiluminescence (Pierce, USA).

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