

P68 RNA helicase is a nucleocytoplasmic shuttling protein

Haizhen Wang^{1,*}, Xueliang Gao^{1,*}, Yun Huang², Jenny Yang², Zhi-Ren Liu¹

¹Departments of Biology, Georgia State University, Atlanta, GA 30303, USA; ²Departments of Chemistry, Georgia State University, Atlanta, GA 30303, USA

P68 RNA helicase is a prototypical DEAD box RNA helicase. The protein plays a very important role in early organ development and maturation. Consistent with the function of the protein in transcriptional regulation and pre-mRNA splicing, p68 was found to predominately localize in the cell nucleus. However, recent experiments demonstrate a transient cytoplasmic localization of the protein. We report here that p68 shuttles between the nucleus and the cytoplasm. The nucleocytoplasmic shuttling of p68 is mediated by two nuclear localization signal and two nuclear exporting signal sequence elements. Our experiments reveal that p68 shuttles via a classical RanGTPase-dependent pathway.

Keywords: P68 RNA helicase, nucleocytoplasm shuttle, NLS, NES, DEAD box

Cell Research (2009) **19**:1388-1400. doi: 10.1038/cr.2009.113; published online 29 September 2009

Introduction

In eukaryotic cells, the nucleus is separated from the cytoplasm. Maintenance of cellular functions requires trafficking of many bio-macromolecules into and out of the nucleus. Proteins that are targeted to the nucleus are marked by one or more sequence elements termed nuclear localization signal (NLS) [1, 2], while proteins that are transported out of the nucleus carry one or more nuclear exporting signal (NES) sequence tags [3]. Nucleocytoplasmic trafficking occurs via the nuclear pore complex (NPC). Most protein transportation through the NPC is mediated by the interaction between specific cargos and a nuclear receptor system, importins and exportins [4-6]. Interestingly, many proteins often carry both NLS and NES signals. This characteristic usually leads to shuttling of these proteins between the nucleus and the cytoplasm [7].

The nuclear p68 RNA helicase (hereafter referred to as p68) is a prototypical member of the DEAD box family of RNA helicases [8, 9]. As an early example of a cellular RNA helicase, the ATPase and the RNA unwinding activities of p68 were previously documented [10-12].

Expression of p68 correlates with cell proliferation and early organ maturation [13]. P68 is suggested to function in DNA methylation/demethylation pathways [14]. Our laboratory has demonstrated *in vitro* and *in vivo* that p68 is an essential splicing factor that plays a role in unwinding the transient U1:5' splice site duplex [15, 16]. Interestingly, results from several laboratories including our laboratory suggest that p68 may be involved in transcriptional regulation of a number of genes [17-22, 23]. Studies appear to suggest that p68 is involved in the transcriptional regulation by different mechanisms of action dependent on each individual regulated gene and cognate biological processes [13, 21, 24-27]. Experiments in our laboratory also demonstrate that p68 is phosphorylated at multiple amino acid residues, including serine/threonine and tyrosine [28, 29]. Tyrosine phosphorylation of p68 correlates with tumor progression [25]. Phosphorylation of p68 at Y593 mediates the effects of growth factors in promoting epithelial-mesenchymal-transition (EMT). The phospho-p68 promotes EMT by facilitating β -catenin nuclear translocation [30]. In the present study, we demonstrate that p68 shuttles between the nucleus and the cytoplasm. P68 shuttling is mediated by two NLS and two NES sequence elements. Our data show that p68 shuttles via the classical RanGTPase-dependent pathway.

Results

P68 RNA helicase shuttles between the nucleus and the cytoplasm

*These two authors contributed equally to this work.

Correspondence: Zhi-Ren Liu

Tel: 404-413-5419; Fax: 404-413-5301

E-mail: biozrl@langate.gsu.edu

Received 9 March 2009; revised 25 May 2009; accepted 24 June 2009; published online 29 September 2009

We previously reported that Y593-phosphorylated p68 facilitates cytoplasmic β -catenin nuclear translocation by displacing the cytoplasmic β -catenin anchor protein axin [30]. We reasoned that cytoplasmic localization of p68 is due to p68 shuttling between the nucleus and the cytoplasm. A number of nuclear localized proteins have been shown to be nucleocytoplasmic shuttles [31, 32]. We thus employed a heterokaryon assay [33] using SW620

and NIH3T3 cells to test whether p68 shuttles between the nucleus and the cytoplasm. HA-tagged p68 proteins were exogenously expressed in SW620. After fusing the SW620 with NIH3T3 cells, the HA-p68 proteins were detected in the nucleus of NIH3T3 cells (Figure 1, upper panel). As a negative control, the non-shuttling protein MS2-DEK [34] expressed in SW620 cells could not be detected in the nucleus of NIH3T3 cells (Figure 1, bot-

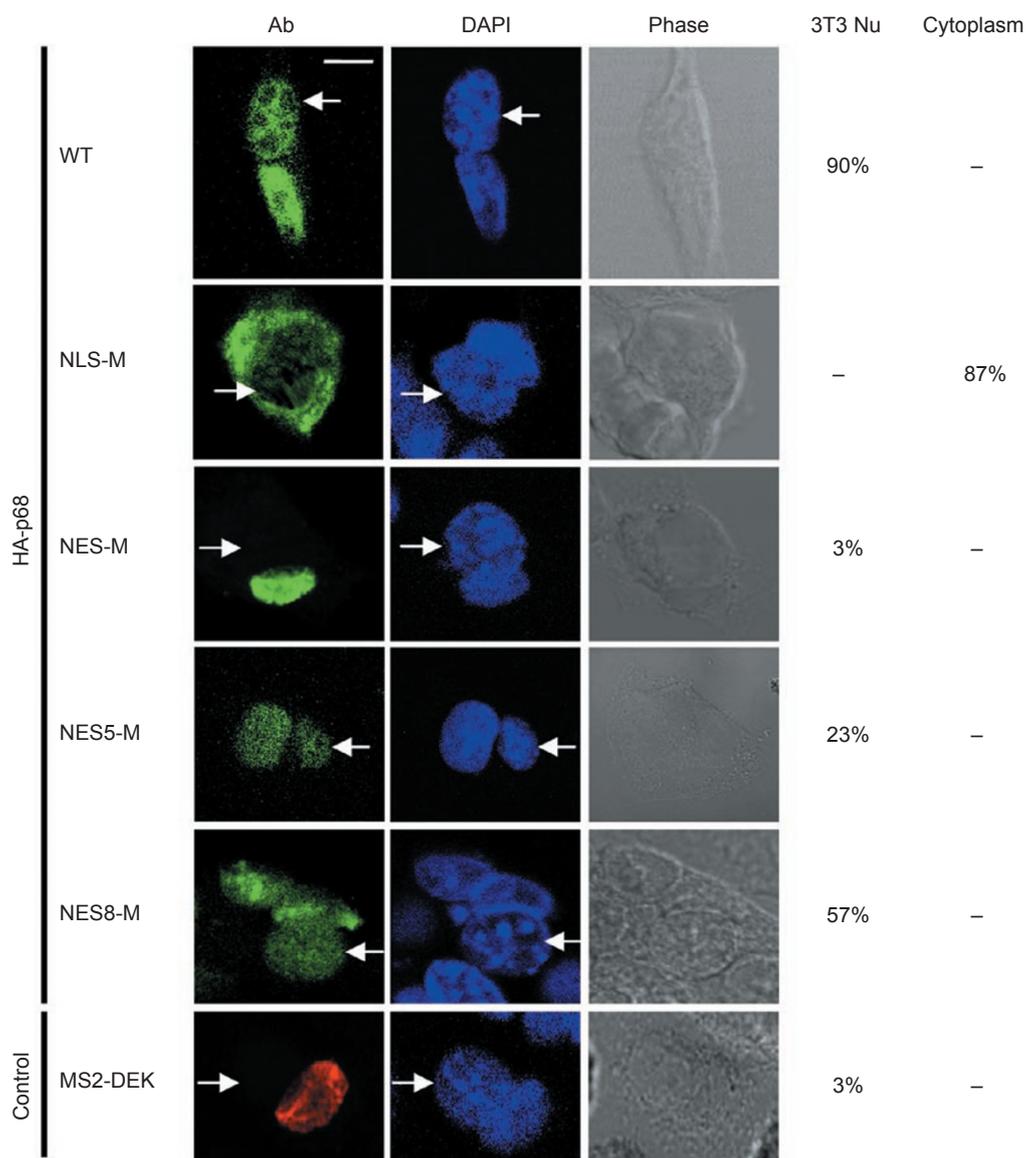


Figure 1 P68 shuttles between the nucleus and the cytoplasm. Representative images of SW620 cells expressing HA-p68s (WT, NLS-M, NES-M, NES5-M and NES8-M). After fusion with NIH3T3 cells, the HA-p68s were immunostained using anti-HA antibody (Ab). The green signal represents staining of HA-p68s. DAPI stains DNA in the cell nucleus of the fused cells (DAPI). The same treated cells were also revealed by phase contrast microscopy (Phase). MS2-DEK (immunostained by antibody against MS2) was a negative control for nucleocytoplasm shuttling assays. Arrows indicate the nucleus of mouse NIH3T3 cells. The numbers on the right side of images are the percentages of cells with the HA-p68s detected in NIH3T3 nucleus (HA-3T3/NE) or in the cytoplasm (HA-Cyto) of the fusion cells based on counting a random group of 30 cells. Scale bar = 10 μ m.

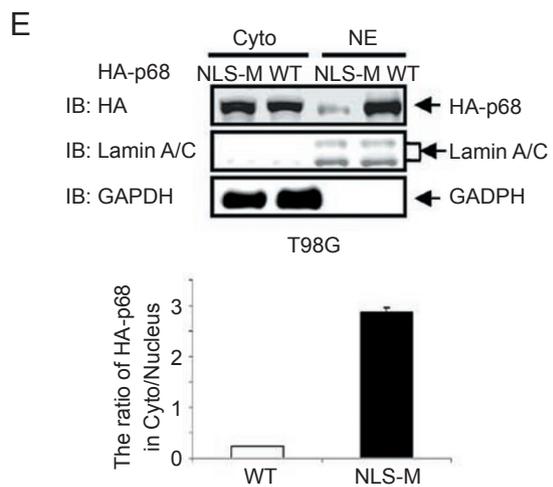
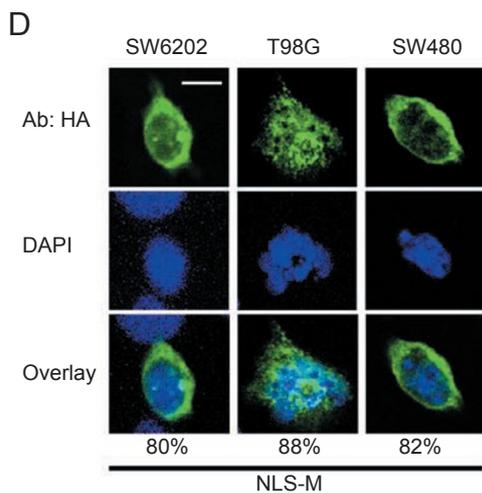
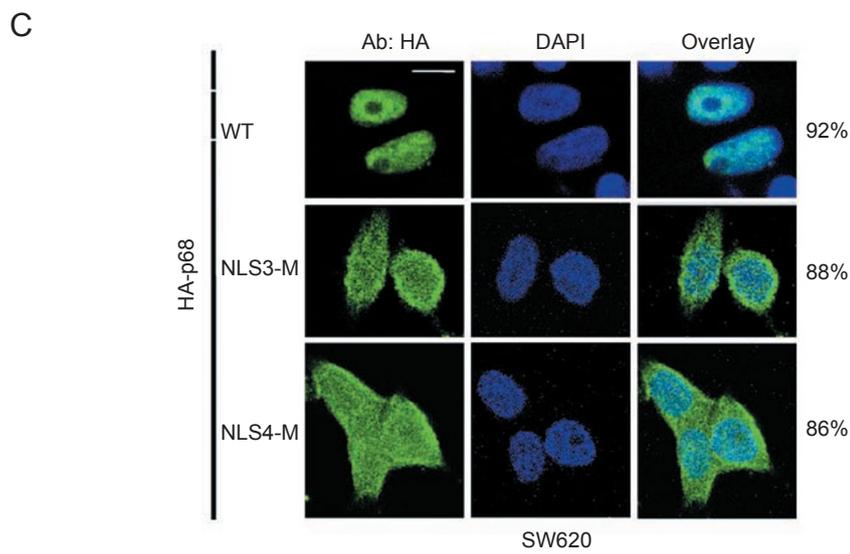
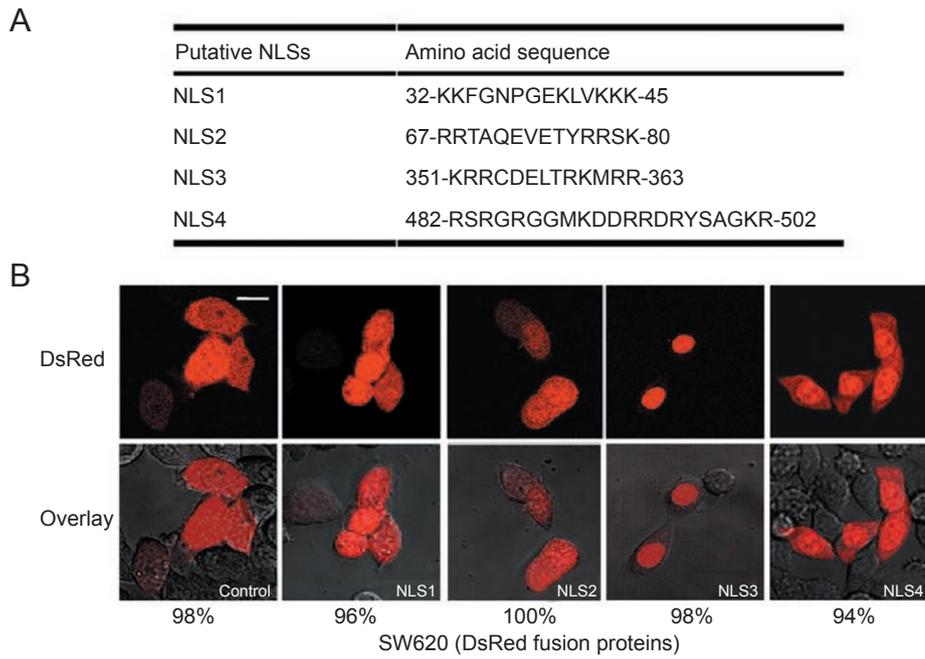
tom panel). These experimental results suggest that p68 is a nucleus-cytoplasm shuttling protein with a much longer residence time in the nucleus.

Identification of NLSs and NESs of p68

Most nucleocytoplasmic shuttling proteins carry sequence elements of both NLS and NES. We analyzed the amino acid sequence of p68 and found a number of sequence segments that resemble NLSs and NESs (Figure 2A and 3A). The NLS sequences were selected based on similarity to the classical SV40 and bipartite NLS sequences [35, 36], while the NES sequences were selected based on similarity to the consensus hydrophobic residue rich NES sequence, $\phi X_{2-3}\phi X_{2-3}\phi X\phi$, where ϕ is a hydrophobic residue and X is any amino acid residue [37]. To test the functionality of these putative NLSs and NESs in p68, we first fused each individual putative NLS or NES with a fluorescent protein DsRed. The fusion proteins were expressed in SW620 cells. It was clear that only NLS3 and NLS4 led to a substantial nuclear accumulation of the fluorescent protein (Figure 2B). To verify the functionality of NLS3 and NLS4, we made mutations in NLS3 (R352A, R353A, K360A and R362A) or NLS4 (R484A, R494A and K501A) in the context of full-length p68. The HA-tagged mutants were expressed in SW620 cells. Immunostaining of the exogenously expressed HA-p68, wt and the mutants, indicated that nuclear localization of the HA mutants was dramatically reduced (Figure 2C). Quantification of fluorescence intensity in the nucleus and the cytoplasm of a random group of cells confirmed the reduction of nuclear HA-p68 (the mean average of Cyto/Nu fluorescence intensity ratio were 0.038 ± 0.026 for wt, 1.477 ± 0.029 for NLS3-M and 1.489 ± 0.097 for NLS4-M). The results suggested that NLS3 and NLS4 indeed functioned as NLSs of p68. Fusion of NES2 and NES5 with the fluorescent protein

resulted in high levels of cytoplasmic fluorescence protein (Figure 3B). Interestingly, fusion of NES8 with DsRed led to slightly higher levels of fluorescent protein localization in the cytoplasm (Figure 3B), indicating that NES8 is a weak nuclear export signal. Treatment of cells with leptomycin B (LMB) abolished the cytoplasmic localization patterns of DsRed fusion proteins (NES2, NES5 and NES8) (Figure 3C). The results suggested that NES2, NES5 and NES8 potentially function as nuclear export signals of p68. To further test the functions of NLSs and NESs of p68, we constructed several p68 deletion mutants (Figure 4A). These deletion mutants were expressed in SW620 cells either as HA-tagged proteins or as eGFP-fusion proteins (due to small sizes of truncates). Locations of each putative NLSs and NESs of p68 in the p68 truncation proteins are indicated. The localizations of these p68 truncates were analyzed by immunostaining with anti-HA antibody or imaging of eGFP. DII and DIII localized to the nucleus, indicating that NLS4 is a functional NLS, while the NES1 and NES7-8 are not functional NES. Strong cytoplasmic DIV and DV were observed, suggesting that one or more of NES2-6 are functional NES. The stronger fluorescence in DV than that of DIV and certain levels of cytoplasmic localization of DVI indicate a function of NLS3. In summary, these results support that NLS3 and NLS4 may function as NLSs of p68, while NES2 and NES5 may function as NESs of p68. Consistent with our experimental results, NLS3 is located in an exposed helical secondary structure, while NLS4 is located in an exposed loop that is flanked by two α -helices in a computer-simulated p68 model structure (Figure 4B). Both NES5 and NES8 are also located on the exposed surface in the model structure, while NES2 is buried under an α -helix in the model structure. This structure model provided additional support for the identification of the NLSs and NESs of p68.

Figure 2 NLSs of p68. **(A)** Putative sequence segments of p68 that resemble NLS. **(B)** Representative of confocal fluorescent microscopy images show the localizations of p68 putative NLSs (N-terminal NLS1, NLS2, NLS3, NLS4) fused with DsRed in SW620 cells. The fused proteins were expressed in the cells. The red signal represents the expressed fusion proteins. The control is the expressed DsRed protein without the NLS fusion. **(C)** Examples of confocal images show subcellular localizations of the exogenously expressed HA-p68s in SW620 cells by immunostaining using anti-HA antibody (HA-p68). The green signal represents staining of HA-tag. The blue signal represents staining of DNA. HA-p68s, wild-type (WT) or NLS mutant (NLS3-M, NLS4-M), were expressed in the cells. The right panels are overlay of anti-HA and DAPI stains. **(D)** HA-p68 NLS mutant (NLS-M) was expressed in different cell lines (indicated). Examples of confocal images of the cells show subcellular localizations of the NLS-M by immunostaining using anti-HA antibody. The top panel is the immunostaining using anti-HA antibody. The middle panel is DAPI stain of cell nucleus. The bottom panel is overlay of anti-HA and DAPI stains. In **(B)**, **(C)** and **(D)**, the numbers are percentage of cells showing similar image pattern (nuclear vs cytoplasm staining) in a random group of 50 cells. Scale bar = 10 μ m. **(E)** The levels of exogenously expressed HA-p68s, wild-type (WT) or NLS mutant (NLS-M), in the extracts made from the cytoplasm or the nucleus of T98G cells were examined by immunoblot using anti-HA antibody (IB:HA) (upper panel). The IBs with antibodies against Lamin A/C and GAPDH are the loading controls. The bottom panel is the quantization of an average four separate experiments of the IB signals and presented as ratio of cytoplasmic over nuclear HA-p68s (WT or NLS-M).



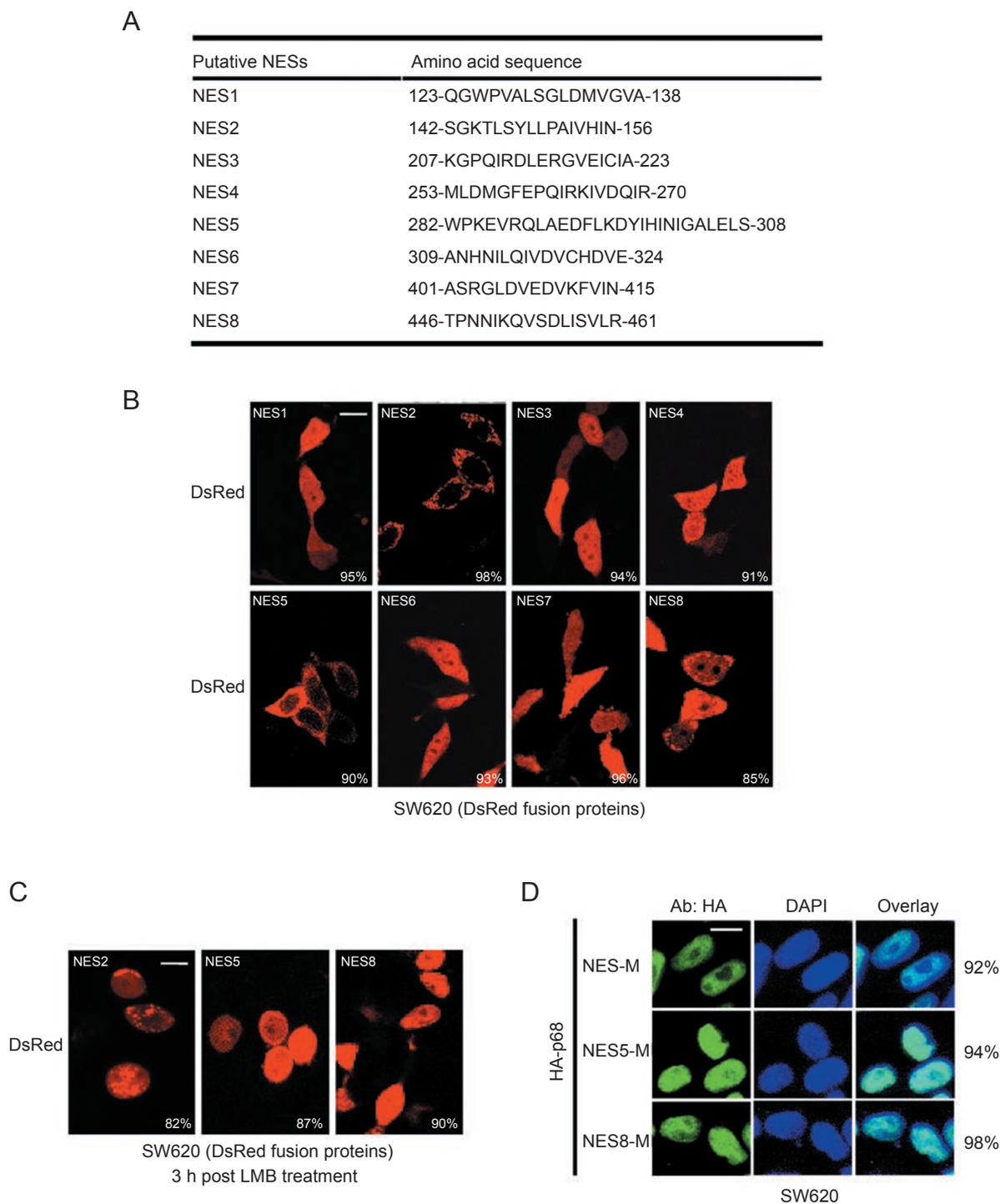


Figure 3 NESs of p68. **(A)** Putative sequence segments of p68 that resemble NES. **(B)** Examples of confocal fluorescent microscopy images of SW620 cells show localizations of p68 putative NESs (N-terminal fusion) fused DsRed. The fused proteins were expressed in the cells. The red signals are the fluorescence of the expressed fusion proteins. **(C)** Inhibition of the exporting of selected putative p68 NESs (NES2, NES5 and NES8) fused with DsRed protein by LMB. The fusion proteins were expressed in SW620 cells. The cells were treated with LMB 24 h post fusion protein expression. The confocal fluorescence images were taken 3 h post drug treatments. **(D)** Examples of confocal fluorescent microscopy images of SW620 cells show the subcellular localizations of the p68 NES mutants (NES-M, NES5-M and NES8-M) by immunostaining using anti-HA antibody. The green signal represents staining of HA-tag. The blue signal represents staining of DNA. In **(B)**, **(C)** and **(D)**, the numbers are percentage of cells showing similar localization pattern in a random group of 50 cells. Scale bar = 10 μ m.

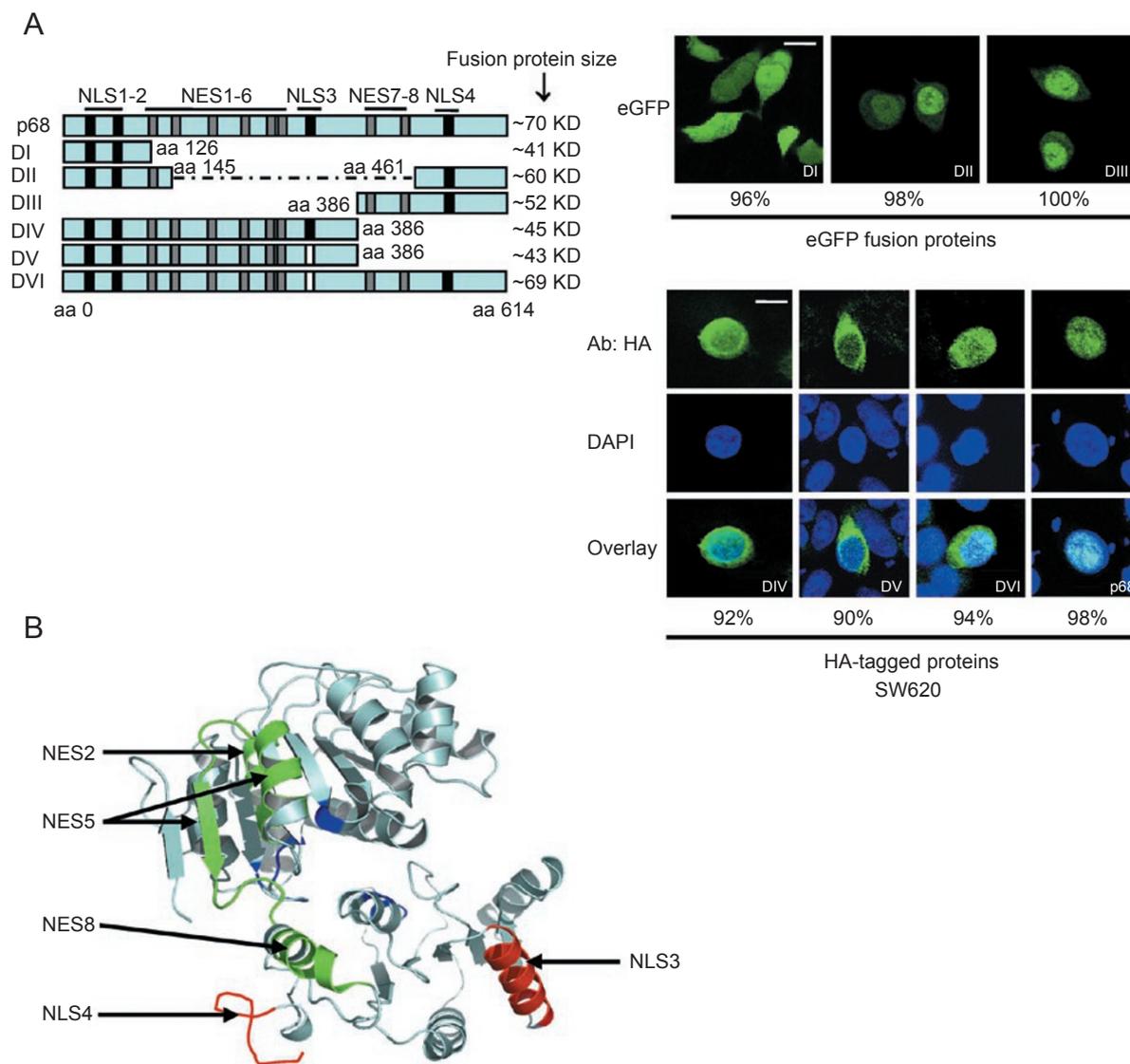
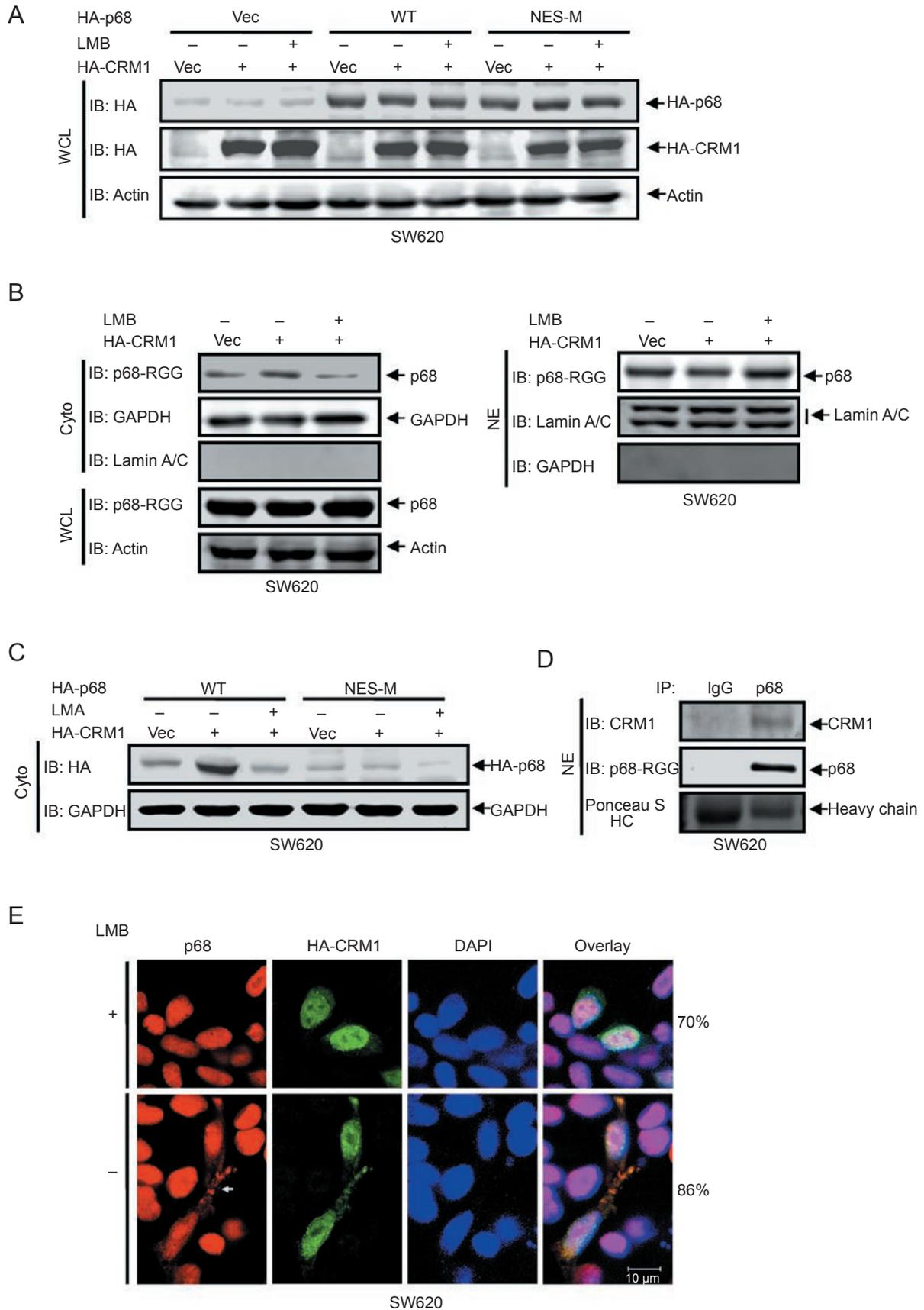


Figure 4 NESs and NLSs of p68. **(A)** P68 truncates (illustrated in the left panel) containing different putative NLSs and NESs, either fusion C-terminal flank eGFP or N-terminal HA-tag, were expressed in SW620 cells. The numbers indicate the molecular weight of each fusion protein. The black bars indicate the location of NLSs. The gray bars indicate NESs. The white bars indicate that the corresponding NLSs were deleted. (Right panel) Examples of confocal fluorescent microscopy images show the localizations of eGFP-fused (up) or HA-tagged p68 truncates (down) in SW620 cells. The HA-tagged p68 truncates in SW620 cells were immunostained with anti-HA antibody (green). Blue is the DAPI stain of the cell nucleus and overlay of anti-HA and DAPI stains. The numbers are percentage of cells showing similar localization pattern in a random group of 50 cells. Scale bar = 10 μ m. **(B)** Model structure of p68 RNA helicase core domain. The model structure was constructed based on the X-ray crystal structure of another DEAD box RNA helicase *Drosophila vasa*. The positions of NLS3 and NLS4, NES2, NES5, and NES8 are indicated by arrows.

We next mutated both NLS3 and NLS4 by the same mutations described above (hereafter referred to as NLS-M). The mutant was expressed in SW620, T98G and SW480 cells. Immunofluorescence staining demonstrated that no significant levels of p68 mutant localized in the cell nucleus (Figure 2D). The results were further verified by immunoblot analyses of exogenously expressed

HA-p68s (wt and the mutant) in the cytoplasmic and nuclear extracts made from HA-p68s-expressing T98G cells (Figure 2E). It was also clear that the mutant no longer shuttled between the nucleus and the cytoplasm as demonstrated by the heterokaryon assay (Figure 1, 2nd panel from top). The distribution pattern did not change significantly when the heterokaryon cells were treated



with LMB (data not shown). In our structure model, NES5 and NES8 are well exposed, while NES2 is buried. Thus, we constructed the p68 NES mutant by mutations at both NES5 and NES8, with charges of F293A, L294A, L298A, L305A, L456A and I457A (hereafter referred to as NES-M). Immunofluorescence staining of SW620 cells that expressed the mutant showed an exclusive nuclear localization of the mutant (Figure 3D). Heterokaryon assay demonstrated that the nucleocytoplasmic shuttling of p68 was almost completely abolished by the mutations (Figure 1, 3rd panel from top). In contrast, mutations separately on either NES5 or NES8 (same mutations) did not completely abolish but certainly reduced the shuttling of HA-p68 to the NIH3T3 cell nucleus (Figure 1, 4th and 5th panels from top). Our mutational analyses confirmed that the NLS3/NLS4 and NES5/NES8 elements were functional NLSs and NESs, and that these NLSs and NESs were required for p68 nucleocytoplasmic shuttling.

P68 shuttles via a RanGTPase-dependent pathway

CRM1 is an export receptor mediating nuclear export of proteins that carry leucine-rich NESs [38, 39]. We demonstrated that p68 RNA helicase carries NLS and NES sequences that mediate nucleocytoplasmic shuttling. We thus asked whether overexpression of CRM1 would affect the localization of p68. We exogenously expressed CRM1 in SW620 cells (Figure 5A). Immunoblot of p68 indicated that there were significantly higher levels of cytoplasmic p68 after CRM1 was exogenously expressed in the cells, and the increases in cytoplasmic p68 were inhibited by LMB treatment (Figure 5B). Increases in cytoplasmic p68 by expression of CRM1 were not observed with p68 mutant that carries mutations at both NES5 and NES8 (Figure 5C). To further confirm

the effects of CRM1 on export of p68, we probed the interaction between p68 and endogenous CRM1 via co-immunoprecipitation. It was clear that p68 co-immunoprecipitated with endogenous CRM1 (Figure 5D). The results suggest that p68 RNA helicase was exported from the nucleus mediated by the exportin pathway. The effects of exogenous expression of CRM1 on p68 export were further confirmed by immunostaining analyses. It was evident that staining of p68 in the cytoplasm was significantly increased upon the expression of CRM1, and the increases were inhibited by LMB (Figure 5E). To further verify that the p68 nucleocytoplasmic shuttling is mediated by the RanGTPase pathway, we tested whether p68 interacts with importin *in vitro*. We used a commercially available his-tag importin $\alpha 2$. When his-importin $\alpha 2$ was incubated with recombinant GST-p68, GST-p68 could not be pulled down by his-importin $\alpha 2$ (Figure 6A). It is known that importin $\alpha 2$ functions as a heterodimer with importin β [40, 41]. Therefore, we added another commercially available recombinant importin $\beta 1$. It was clear that GST-p68 co-precipitated with his-importin $\alpha 2$ when importin $\beta 1$ was also present, but the p68 NLS mutant (NLS-M) did not co-precipitate with his-importin $\alpha 2$ under any condition (Figure 6A). We also carried out co-precipitation experiments with immunopurified HA-p68 from HEK293 cells. The his-importin $\alpha 2$ /importin $\beta 1$ co-immunoprecipitated with the purified HA-p68 using anti-HA antibody. However, the importins did not co-immunoprecipitate with HA-p68 NLS mutant (Figure 6B). The interaction of p68 with importins was also verified by co-immunoprecipitation experiment with endogenous importin $\alpha 2/\beta 1$ (Figure 6C). The observations suggest that p68 interacts with the importin $\alpha 2/\beta 1$ dimer, providing additional support for the nucleocytoplasmic shuttling of p68 via the RanGTPase pathway.

Figure 5 P68 nucleocytoplasm shuttle is RanGTPase pathway dependent. **(A)** Exogenous expression of HA-p68s, wild-type (WT) and mutant (NES-M), and HA-CRM1 in SW620 cells were analyzed by immunoblot of whole cell lysate (WCL) using anti-HA antibody (IB: HA). **(B)** (Left panel) Cytoplasmic (Cyto) p68 levels of SW620 cells were analyzed by immunoblot using the antibody p68-rgg (IB: p68-RGG). Immunoblots of p68 in whole cell lysate (IB:p68-RGG) indicate cellular levels of p68. (Right panel) Nuclear (NE) levels of p68 in SW620 cells were analyzed by immunoblot using the antibody p68-rgg (IB: p68-RGG). **(C)** The cytoplasmic (Cyto) levels of exogenously expressed HA-p68s (WT and NES-M) were analyzed by immunoblot of cytoplasmic extracts using anti-HA antibody (IB: HA). In **(A)**, **(B)** and **(C)**, IBs of β -actin (IB: actin), GAPDH (IB:GAPDH), Lamin A/C (IB: Lamin A/C) are controls. The SW620 cells were transfected with vector alone (Vec), the vector for CRM1 (HA-CRM1), and the vector for CRM1 plus treatment with LMB (HA-CRM1 + LMB). **(D)** Co-immunoprecipitation of p68 with endogenous CRM1 in nuclear extracts (NE) of SW620 cells was analyzed by immunoprecipitation using the antibody Pabp68 (IP, p68). The immunoprecipitates were examined by immunoblot using antibody against CRM1 (IB: CRM1) and the antibody p68-RGG (IB:p68-RGG). Ponceau S staining the IgG heavy chain is the loading control (Ponceau S HC). Immunoprecipitation using rabbit IgG (IP, IgG) is negative control for co-immunoprecipitation. **(E)** Examples of confocal fluorescent microscopy images of SW620 cells show the cellular localizations of endogenous p68 (P68) and exogenously expressed HA-CRM1 (HA-CRM1). The cells were treated (+) or untreated (-) with LMB. Red signals are staining of p68. The green signal represents staining of HA-tag. The blue signal represents staining of DNA. Overlay images are overlay of staining of p68, HA-CRM1 and DNA. The numbers are percentage of cells showing similar image pattern in a random group of 50 cells.

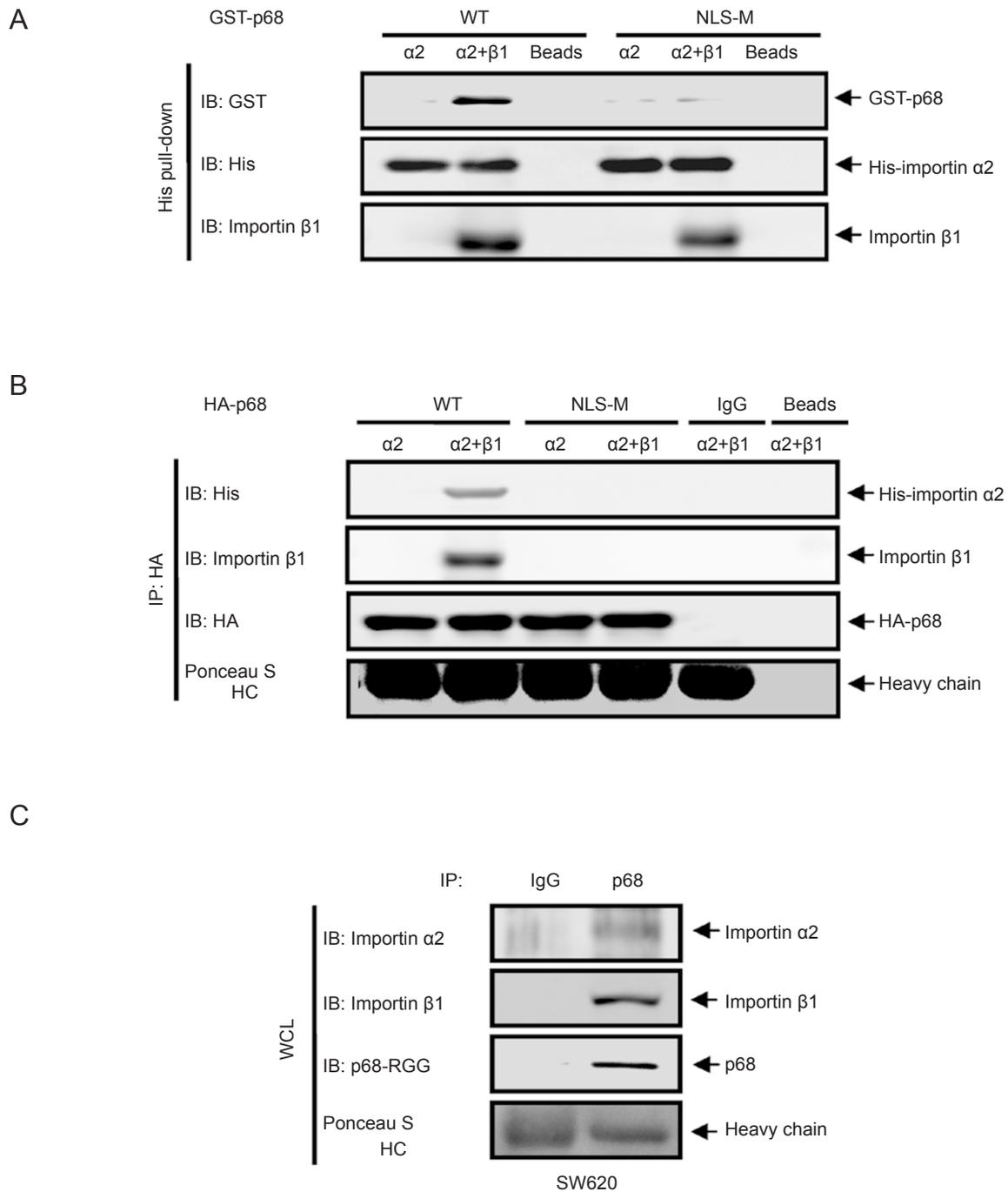


Figure 6 P68 interacts with importins *in vitro*. **(A)** Immunoblot analyses of GST-p68 (IB: GST) and his-importin $\alpha 2$ (IB: His) in His-pull-down proteins (His Pull-down) from a protein mixture of GST-p68 wild-type (WT) or GST-p68 NLS-M with; his-importin $\alpha 2$ ($\alpha 2$), his-importin $\alpha 2$ and importin $\beta 1$ ($\alpha 2 + \beta 1$), and Ni-TED beads alone (Beads). Importin $\beta 1$ is visualized by immunoblot using antibody against importin β (IB: Importin β) **(B)** Immunoblot analyses of his-importin $\alpha 2$ (IB: His) and HA-p68 (IB: HA) in co-immunoprecipitates (IP: HA) from a protein mixture of HA-p68 wild-type (WT), HA-p68 mutant NLS-M (NLS-M), rabbit IgG (IgG) or protein G beads (Beads) with; his-importin $\alpha 2$ ($\alpha 2$), his-importin $\alpha 2$ and importin $\beta 1$ ($\alpha 2 + \beta 1$). **(C)** Co-immunoprecipitation of p68 with endogenous importin $\alpha 2$ and $\beta 1$ in whole cell lysate (WCL) of SW620 cells was analyzed by immunoprecipitation using the antibody Pabp68 (IP, p68). The immunoprecipitates were examined by immunoblot using antibodies against importin $\alpha 2$ (IB: Importin $\alpha 2$), importin $\beta 1$ (IB: Importin $\beta 1$), and the antibody p68-RGG (IB: p68-RGG). Immunoprecipitation using rabbit IgG (IP, IgG) is negative control for co-immunoprecipitation. In **(B)** and **(C)**, Ponceau S staining IgG heavy chain (Ponceau S: HC) are the loading controls.

Discussion

P68 RNA helicase was shown to predominately localize in the cell nucleus [42]. However, recent experiments carried out in Janknecht's and our laboratories showed a transient cytoplasmic localization of the protein [19], (Gao and Liu, unpublished observations). In this report, we presented data demonstrating the nucleocytoplasmic shuttling of p68 RNA helicase. Our experiments showed that p68 shuttling is mediated by two NLS and two NES sequence elements. The p68 nuclear export and import follow a RanGTPase-dependent pathway. Interestingly, a number of DEAD/DExH box RNA helicases have also been shown to shuttle between the nucleus and cytoplasm, including eIF-4AIII, An3, GRTH/Ddx25 and RNA helicase A [34, 43-45]. P68 RNA helicase was previously shown to interact with mRNA/mRNP [16]. Thus, one possibility is that the p68 export and shuttling is associated with mRNA export. It was shown that shuttling of GRTH/Ddx25 is dependent upon mRNP exporting [43]. However, the interaction of p68 with CRM1 is RNA-independent, as p68 still co-immunoprecipitated with CRM1 in the nuclear extracts treated by RNase (data not shown). Although the result did not prove that p68 nuclear export is mRNA export-independent, the result does suggest a possibility that mRNA exporting is not necessarily required for the p68 nuclear exporting.

The cellular function(s) of the p68 RNA helicase nucleocytoplasmic shuttling is an open question. In general, proteins that shuttle between the nucleus and the cytoplasm usually have distinct cellular functions in these two separated subcellular compartments. They often re-localize to the nucleus or the cytoplasm to fulfill specific functional role(s) in response to particular cellular signal stimuli [46, 47]. Alternatively, nucleocytoplasmic shuttling of a protein can function as 'chaperons' to help nuclear import or export of other proteins or RNAs [48, 49]. Cellular functions of p68 helicase in the cell nucleus are well documented. The protein is functionally involved in gene transcription [21-23, 50], pre-mRNA [15, 16], pre-rRNA and pre-miRNA processing [51, 52]. Whether p68 RNA helicase has any potential function(s) in the cytoplasm is currently not very clear. Goh *et al.* [53] detected the interaction of p68 with HCV-NS5B in the cytoplasm in the viral-infected or NS5B-expressing 293 cells and the interaction is essential for viral replication. In another independent study, Harris *et al.* [54] found that the 3'-nontranslated region of HCV interacts with p68 in an RNA affinity capture experiment using the cytoplasmic extracts of 293 cells that express the viral replicon. These observations are consistent with our experimental results indicating that p68 localizes both in the nucleus and the

cytoplasm. However, despite the demonstration of the function of p68 in HCV replication in the cytoplasm of virus-infected cells, evidence suggesting the involvement of p68 in any cellular process in the cytoplasm is currently lacking. We have observed a significant increases in cytoplasmic p68 levels upon treatment of cells with several growth factors and chemokines [30], (Wang, unpublished observations), indicating that p68 may function in the cytoplasm under specific cellular conditions, such as abnormal growth or cell migration in response to growth factor or chemokine stimulations.

Localization of p68 is intriguing. The protein is shuttling between the nucleus and the cytoplasm, while the protein predominantly resides in the cell nucleus, and is nearly undetectable outside of the nucleus by immunostaining or by immunoblot analyses of cytoplasmic extracts. The phenomenon suggests that the function of the NESs of p68 is tightly regulated. It is possible that an interacting partner(s) in the nucleus masks the p68 NES sequences. Nuclear export of p68 may thus depend on the dissociation of the interaction partner(s). Our data demonstrate that p68 has three sequence elements that potentially function as NESs. On the other hand, structural modeling of p68 helicase core showed that one of the potential NES (NES2) is buried under an α -helix. Therefore, it is possible that a specific post-translational modification drives conformational changes, which subsequently expose this buried NES sequence for p68 export [55]. Very similar regulatory mechanisms were observed in nuclear exporting and importing of Dok1 and NF-AT1 [56, 57]. Consistent with this notion, we observed a substantially increased cytoplasmic p68 level when the cells were treated with several growth factors and chemokines [30], (data not shown), suggesting a possibility that the treatments of growth factors and chemokines may trigger the exposure of the NES sequences for nuclear export of p68. There are two NLS sequences. Mutation of either NLS reduced nuclear localization (increased cytoplasmic p68, Figure 2C), indicating that both NLSs contribute partly to the nuclear localization of p68.

Materials and Methods

Reagents, antibodies and cells

LMB, PEG3350, cycloheximide, HA peptide and protease inhibitor cocktail were purchased from Calbiochem, Sigma and Roche. Antibodies against HA and His tags, GAPDH, Lamin A/C, β -actin, and Histone 2A were purchased from Roche, Cell signaling, Upstate and Chemicon. The monoclonal antibody P68-RGG and polyclonal antibody Pabp68 against p68 were produced in our laboratory. Cell lines SW480, SW620, HT29, NIH3T3 and T98G were purchased from ATCC and cultured by following the vendor's instructions.

Plasmids construction

The HA-tagged p68s expression plasmids (wild-type, and Y593F, Y595F, LGLD and NLS-M mutants) were constructed in pHM6 vectors as previously reported [30]. The vectors for expression of eGFP and DsRed fusion proteins were constructed using eGFP-pcDNA-3.1(+) or pDsRed1-N1 vectors. Full-length p68 or deletion mutants of p68 variants were subcloned into the vector by EcoR321 and NotI restriction sites. The p68 or deletion mutants were fused at the C-terminal of the fluorescent protein tag. Different putative NLS- and NES-tagged DsRed were constructed by cloning into pDsRed-N1 vectors at 5' (*Bam*HI and *Eco*RI sites) with the addition of a starting methionine at the N-terminal of each NLS and NES, respectively. Site-directed mutagenesis was performed using QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene). All the DNA clones and mutations were verified by auto-DNA sequencing at GSU. The procedures for transfection of expression vectors of HA-p68, fluorescent proteins, and Crm1 and duplex RNAi were similar to our previous reports [30, 50].

Expression and purification of recombinant GST-p68

Recombinant GST-p68 was expressed and purified as described in our previous report [58].

Interactions between p68 and importins

Recombinant GST-p68 (8 µg) was incubated with commercial his-importin α2 (8 µg) and/or importin β1 (Calbiochem) overnight at 4 °C in 500 µl in PBS buffer. After incubation, the protein complex was pulled down by Ni-TED silica beads. The pull-down proteins were separated by 10% SDS-PAGE, and were analyzed by immunoblot using anti-GST or anti-his-tag antibodies. The presence of importin β1 was visualized by ponceau S staining. For co-immunoprecipitation of HA-p68 with importin α2, HA-p68s, wt or mutant, were expressed in HEK cells and were immunoprecipitated using 30 βl anti-HA antibody. Protein G agarose (50 µl) was added to the mixture. After extensive washing, HA-p68s were eluted from the beads by competition using HA peptide (100 µg/ml). The eluted HA-p68s were dialyzed against PBS. The recombinant importin α2 and/or β1 was incubated with the purified HA-p68 in PBS for 4 h. The protein mixtures were immunoprecipitated using anti-HA antibody. The precipitated complex was separated in 10% SDS-PAGE followed by immunoblot using anti-his-tag antibody. The presence of HA-p68 was detected by immunoblot using the antibody p68-rgg.

Heterokaryon analyses and immunofluorescence imaging

The experimental procedures for immunofluorescence staining and imaging were similar to our previous reports [30, 59]. For heterokaryon analyses, SW620 cells were first transfected with HA-p68s (wt or mutants) or MS2-DEK. The cells were mixed with an equal number of mouse NIH3T3 cells 24 h post-transfection and reseeded in four-well chambers. Subsequently, 50 µg/ml of cycloheximide was added to the culture medium to inhibit protein synthesis. After 3 h, the co-cultured cells were fused using 50% PEG3350 for 2 min, washed and incubated with the medium containing 75 µg/ml cycloheximide for 3 h. The treated cells were then fixed and immunostained as described above. Rabbit polyclonal anti-MS2 antibody was employed to stain MS2-DEK, followed by Alex Fluor 555 goat anti-rabbit IgG antibody. In a random group of 30 cells, the numbers of cells with both NIH3T3

and SW620 nucleus and with HA-p68 in NIH3T3 nucleus or in the cytoplasm of fused cells were counted. The percentage of HA-p68 in the nucleus or cytoplasm of fused cells equals fused cells with HA-p68 in NIH3T3 nucleus or cytoplasm/total fused cells with expression of HA-p68.

Computational homology structure modeling the helicase core of p68

The sequence alignment of p68 RNA helicase core with homologous RNA helicase cores of several DEAD box family proteins (e.g., vasa, eIF4A, Dhhlp and UAP56) was performed by the program ClustalW. The secondary structure elements were predicted based on the consensus analysis using computational programs JPRED, PHD and PSIPRED [60, 61]. The homology modeling of the p68 RNA helicase structure was constructed using the homology-modeling server SWISS-MODEL based on X-ray crystal structure of *Drosophila* vasa [62], which has the highest alignment score with p68 RNA helicase core. The putative NLSs and NESs were indicated in the modeling structure.

Acknowledgments

We thank Drs Joan A Steitz (Yale University School of Medicine), Melissa J Moore (University of Massachusetts medical school) and Hung-Ying Kao (Case Western Reserve University) for providing the vectors for expression of MS2-DEK and human CRM1. We are grateful to Professor Peter Stockley (University of Leeds) for providing antibody against MS2-DEK. We also thank Birgit Neuhaus (Georgia State University) for assistance in confocal imaging. This manuscript is greatly improved by comments from Christie Carter, Michael Kirberger and Heena Dey (Georgia State University). This work is supported in part by research grants from National Institutes of Health (GM063874 and CA118113) and Georgia Cancer Coalition to ZR Liu. X Gao is supported by an MBD fellowship, GSU.

References

- 1 Goldfarb DS, Garipey J, Schoolnik G, *et al.* Synthetic peptides as nuclear localization signals. *Nature* 1986; **322**:641-644.
- 2 Schneider J, Schindewolf C, van Zee K, *et al.* A mutant SV40 large T antigen interferes with nuclear localization of a heterologous protein. *Cell* 1988; **54**:117-125.
- 3 Wen W, Meinkoth, JL, Tsien, RY, *et al.* Identification of a signal for rapid export of proteins from the nucleus. *Cell* 1995; **82**:463-473.
- 4 Nakielny S, Dreyfuss G. Transport of proteins and RNAs in and out of the nucleus. *Cell* 1999; **99**:677-690.
- 5 Gorlich D, Kutay U. Transport between the cell nucleus and the cytoplasm. *Annu Rev Cell Dev Biol* 1999; **15**:607-660.
- 6 Hill CS. Nucleocytoplasmic shuttling of Smad proteins. *Cell Res* 2009; **19**:36-46.
- 7 Lusk CP, Blobel G, King MC. Highway to the inner nuclear membrane: rules for the road. *Nat Rev Mol Cell Biol* 2007; **8**:414-420.
- 8 Crawford L, Leppard K, Lane D, *et al.* Cellular proteins reactive with monoclonal antibodies directed against simian virus 40 T-antigen. *J Virol* 1982; **42**:612-620.

- 9 Lane DP, Hoeffler WK. SV40 large T shares an antigenic determinant with a cellular protein of molecular weight 68,000. *Nature* 1980; **288**:167-170.
- 10 Iggo RD, Lane DP. Nuclear protein p68 is an RNA-dependent ATPase. *EMBO J* 1989; **8**:1827-1831.
- 11 Ford MJ, Anton IA, Lane DP. Nuclear protein with sequence homology to translation initiation factor eIF-4A. *Nature* 1988; **332**:736-738.
- 12 Hirling H, Scheffner M, Restle T, *et al.* RNA helicase activity associated with the human p68 protein. *Nature* 1989; **339**:562-564.
- 13 Stevenson RJ, Hamilton SJ, MacCallum DE, *et al.* Expression of the 'dead box' RNA helicase p68 is developmentally and growth regulated and correlates with organ differentiation/maturation in the fetus. *J Pathol* 1998; **184**:351-359.
- 14 Jost JP, Schwarz S, Hess D, *et al.* A chicken embryo protein related to the mammalian DEAD box protein p68 is tightly associated with the highly purified protein-RNA complex of 5-MeC-DNA glycosylase. *Nucleic Acids Res* 1999; **27**: 3245-3252.
- 15 Liu ZR. p68 RNA helicase is an essential human splicing factor that acts at the U1 snRNA-5' splice site duplex. *Mol Cell Biol* 2002; **22**:5443-5450.
- 16 Lin C, Yang L, Yang JJ, *et al.* ATPase/helicase activities of p68 RNA helicase are required for pre-mRNA splicing but not for assembly of the spliceosome. *Mol Cell Biol* 2005; **25**:7484-7493.
- 17 Fujita T, Kobayashi Y, Wada O, *et al.* Full activation of estrogen receptor alpha activation function-1 induces proliferation of breast cancer cells. *J Biol Chem* 2003; **278**:26704-26714.
- 18 Watanabe M, Yanagisawa J, Kitagawa H, *et al.* A subfamily of RNA-binding DEAD-box proteins acts as an estrogen receptor alpha coactivator through the N-terminal activation domain (AF-1) with an RNA coactivator, SRA. *EMBO J* 2001; **20**:1341-1352.
- 19 Rossow KL, Janknecht R. Synergism between p68 RNA helicase and the transcriptional coactivators CBP and p300. *Oncogene* 2003; **22**:151-156.
- 20 Wilson BJ, Bates GJ, Nicol SM, *et al.* The p68 and p72 DEAD box RNA helicases interact with HDAC1 and repress transcription in a promoter-specific manner. *BMC Mol Biol* 2004; **5**:11.
- 21 Endoh H, Maruyama K, Masuhiro Y, *et al.* Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor alpha. *Mol Cell Biol* 1999; **19**:5363-5372.
- 22 Bates GJ, Nicol SM, Wilson BJ, *et al.* The DEAD box protein p68: a novel transcriptional coactivator of the p53 tumour suppressor. *EMBO J* 2005; **24**:543-553.
- 23 Buszczak M, Spradling AC. The Drosophila P68 RNA helicase regulates transcriptional deactivation by promoting RNA release from chromatin. *Genes Dev* 2006; **20**:977-989.
- 24 Kahlina K, Goren I, Pfeilschifter J, *et al.* p68 dead box RNA helicase expression in keratinocytes: regulation, nucleolar localization, and functional connection to proliferation and VEGF gene expression. *J Biol Chem* 2004; **279**:44872-44882.
- 25 Yang L, Lin C, Liu ZR. Phosphorylations of DEAD box p68 RNA helicase are associated with cancer development and cell proliferation. *Mol Cancer Res* 2005; **3**:355-363.
- 26 Wei Y, Hu MH. [The study of P68 RNA helicase on cell transformation]. *Yi Chuan Xue Bao* 2001; **28**:991-996.
- 27 Warner DR, Bhattacharjee V, Yin X, *et al.* Functional interaction between Smad, CREB binding protein, and p68 RNA helicase. *Biochem Biophys Res Commun* 2004; **324**:70-76.
- 28 Yang L, Liu ZR. Bacterially expressed recombinant p68 RNA helicase is phosphorylated on serine, threonine, and tyrosine residues. *Protein Expr Purif* 2004; **35**:327-333.
- 29 Yang L, Lin C, Liu ZR. Signaling to the DEAD box-Regulation of DEAD-box p68 RNA helicase by protein phosphorylations. *Cell Signal* 2005; **17**:1495-1504.
- 30 Yang L, Lin C, Liu ZR. P68 RNA helicase mediates PDGF-induced epithelial mesenchymal transition by displacing axin from beta-catenin. *Cell* 2006; **127**:139-155.
- 31 Cartwright P, Helin K. Nucleocytoplasmic shuttling of transcription factors. *Cell Mol Life Sci* 2000; **57**:1193-206.
- 32 Zhu, J and McKeon, F, Nucleocytoplasmic shuttling and the control of NF-AT signaling. *Cell Mol Life Sci* 2000; **57**:411-420.
- 33 Fan XC, Steitz JA. Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the *in vivo* stability of ARE-containing mRNAs. *EMBO J* 1998; **17**:3448-3460.
- 34 Shibuya T, Tange TO, Sonenberg N, *et al.* eIF4AIII binds spliced mRNA in the exon junction complex and is essential for nonsense-mediated decay. *Nat Struct Mol Biol* 2004; **11**:346-351.
- 35 Kalderon D, Roberts BL, Richardson WD, *et al.* A short amino acid sequence able to specify nuclear location. *Cell* 1984; **39**(Part 2):499-509.
- 36 Robbins J, Dilworth SM, Laskey RA, *et al.* Two interdependent basic domains in nucleoplasmic nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* 1991; **64**:615-623.
- 37 Neumann G, Hughes MT, Kawaoka Y. Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. *EMBO J* 2000; **19**:6751-6758.
- 38 Formerod M, Ohno M, Yoshida M, *et al.* CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* 1997; **90**:1051-1060.
- 39 Watanabe M, Fukuda M, Yoshida M, *et al.* Involvement of CRM1, a nuclear export receptor, in mRNA export in mammalian cells and fission yeast. *Genes Cells* 1999; **4**:291-297.
- 40 Goldfarb DS, Corbett AH, Mason DA, *et al.* Importin alpha: a multipurpose nuclear-transport receptor. *Trends Cell Biol* 2004; **14**:505-514.
- 41 Lindsay ME, Plafker K, Smith AE, *et al.* Np60/Nup50 is a tri-stable switch that stimulates importin-alpha:beta-mediated nuclear protein import. *Cell* 2002; **110**:349-360.
- 42 Nicol SM, Causevic M, Prescott AR, *et al.* The nuclear DEAD box RNA helicase p68 interacts with the nucleolar protein fibrillar and colocalizes specifically in nascent nucleoli during telophase. *Exp Cell Res* 2000; **257**:272-280.
- 43 Sheng Y, Tsai-Morris CH, Gutti R, *et al.* Gonadotropin-regulated testicular RNA helicase (GRTH/Ddx25) is a transport protein involved in gene-specific mRNA export and protein translation during spermatogenesis. *J Biol Chem* 2006; **281**:35048-35056.
- 44 Aratani S, Oishi T, Fujita H, *et al.* The nuclear import of RNA helicase A is mediated by importin-alpha3. *Biochem Biophys*

- Res Commun* 2006; **340**:125-133.
- 45 Askjaer P, Bachi A, Wilm M, *et al.* RanGTP-regulated interactions of CRM1 with nucleoporins and a shuttling DEAD-box helicase. *Mol Cell Biol* 1999; **19**:6276-6285.
- 46 Xu L, Massague J. Nucleocytoplasmic shuttling of signal transducers. *Nat Rev Mol Cell Biol* 2004; **5**:209-219.
- 47 Kau TR, Way JC, Silver PA. Nuclear transport and cancer: from mechanism to intervention. *Nat Rev Cancer* 2004; **4**:106-117.
- 48 Loyola A, Almouzni G. Histone chaperones, a supporting role in the limelight. *Biochim Biophys Acta* 2004; **1677**:3-11.
- 49 Fried H, Kutay U. Nucleocytoplasmic transport: taking an inventory. *Cell Mol Life Sci* 2003; **60**:1659-1688.
- 50 Yang L, Lin C, Zhao S, *et al.* Phosphorylation of p68 RNA helicase plays a role in platelet-derived growth factor-induced cell proliferation by up-regulating cyclin D1 and c-Myc expression. *J Biol Chem* 2007; **282**:16811-16819.
- 51 Davis BN, Hilyard AC, Lagna G, *et al.* SMAD proteins control DROSHA-mediated microRNA maturation. *Nature* 2008; **454**:56-61.
- 52 Fukuda T, Yamagata K, Fujiyama S, *et al.* DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. *Nat Cell Biol* 2007; **9**:604-611.
- 53 Goh PY, Tan YJ, Lim SP, *et al.* Cellular RNA helicase p68 relocalization and interaction with the hepatitis C virus (HCV) NS5B protein and the potential role of p68 in HCV RNA replication. *J Virol* 2004; **78**:5288-5298.
- 54 Harris D, Zhang Z, Chaubey B, *et al.* Identification of cellular factors associated with the 3'-nontranslated region of the hepatitis C virus genome. *Mol Cell Proteomics* 2006; **5**:1006-1018.
- 55 Wrighton KH, Lin X, Feng XH. Phospho-control of TGF-beta superfamily signaling. *Cell Res* 2009; **19**:8-20.
- 56 Niu Y, Roy F, Saltel F, *et al.* A nuclear export signal and phosphorylation regulate Dok1 subcellular localization and functions. *Mol Cell Biol* 2006; **26**:4288-4301.
- 57 Okamura H, Aramburu J, Garcia-Rodriguez C, *et al.* Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol Cell* 2000; **6**:539-550.
- 58 Huang Y, Liu ZR. The ATPase, RNA unwinding, and RNA binding activities of recombinant p68 RNA helicase. *J Biol Chem* 2002; **277**:12810-12815.
- 59 Wang H, Liu Y, Gao X, *et al.* The recombinant beta subunit of C-phycocyanin inhibits cell proliferation and induces apoptosis. *Cancer Lett* 2007; **247**:150-158.
- 60 Cuff JA, Clamp ME, Siddiqui AS, *et al.* JPred: a consensus secondary structure prediction server. *Bioinformatics* 1998; **14**:892-893.
- 61 McGuffin LJ, Bryson K, Jones DT. The PSIPRED protein structure prediction server. *Bioinformatics* 2000; **16**:404-405.
- 62 Sengoku T, Nureki O, Nakamura A, *et al.* Structural basis for RNA unwinding by the DEAD-box protein Drosophila Vasa. *Cell* 2006; **125**:287-300.