Purification of nuclear localization signal-containing proteins and its application to investigation of the mechanisms of the cell division cycle

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The GTP bound form of the Ran GTPase (RanGTP) in the nucleus promotes nuclear import of the proteins bearing nuclear localization signals (NLS). When nuclear envelopes break down during mitosis, RanGTP is locally produced around chromosomes and drives the assembly of the spindle early in mitosis and the nuclear envelope (NE) later. RanGTP binds to the heterodimeric nuclear transport receptor importin α/β and releases NLS proteins from the receptor. Liberated NLS proteins around chromosomes have been shown to play distinct, essential roles in spindle and NE assembly. Here we provide a highly specific protocol to purify NLS proteins from crude cell lysates. The pure NLS fraction is an excellent resource to investigate the NLS protein function and identify new mitotic regulators, uncovering fundamental mechanisms of the cell division cycle. It takes 2–3 days to obtain the NLS fraction.

Introduction

Eukaryotes are discriminated from prokaryotes by the presence of the nucleus, a membrane-enclosed organelle that isolates the genetic material from cytoplasmic components. The nuclear envelope, NE, is composed of inner and outer nuclear membranes fused at the nuclear pore complexes (NPCs). The NPCs are aqueous channels that mediate selective bidirectional transport of proteins and RNAs. Most transport is regulated by the GTPase Ran and nuclear transport receptors.[1] Nuclear import receptors bind to proteins containing NLSs (classical or non-classical) in the cytoplasm, mediate their transport through the NPC and release them in the nucleus upon RanGTP binding.[2]

The NE is important to maintain genome integrity and regulate gene expression in the cell,[3] but would be a physical obstacle when chromosomes segregate into daughter cells in mitosis. Therefore, higher eukaryotes disassemble the NE at the onset of mitosis and reassemble it after chromosome segregation at the end of mitosis. Recent studies uncovered that NLS-containing nuclear proteins play major roles during mitosis in the NE-disassembled mitotic cytoplasm. For example, NLS-containing microtubule-associated proteins (MAPs) drive spindle assembly in mitosis,[4] while in interphase they cannot regulate microtubules located in the cytoplasm. Separately, NLS proteins are required for NE reassembly at the end of mitosis.[5] The importin α/β heterodimer is the transport receptor for the proteins containing the classical, basic NLS. The receptor still binds the cargos during mitosis and inhibits their functions. RanGTP, locally produced around chromatin, binds importin β and releases NLS proteins from the receptor. The liberated NLS proteins locally function in spindle and NE assembly around chromatin. The identification of specific NLS proteins involved is a key to full understanding of spindle and NE assembly.

Biochemically, nuclear extracts are prepared from cultured cells by isolating the nucleus and extracting proteins with high salt.[6] However, such nuclear extracts are highly contaminated with cytoplasmic proteins, which interfere with the identification of nuclear proteins involved in spindle and NE assembly. To
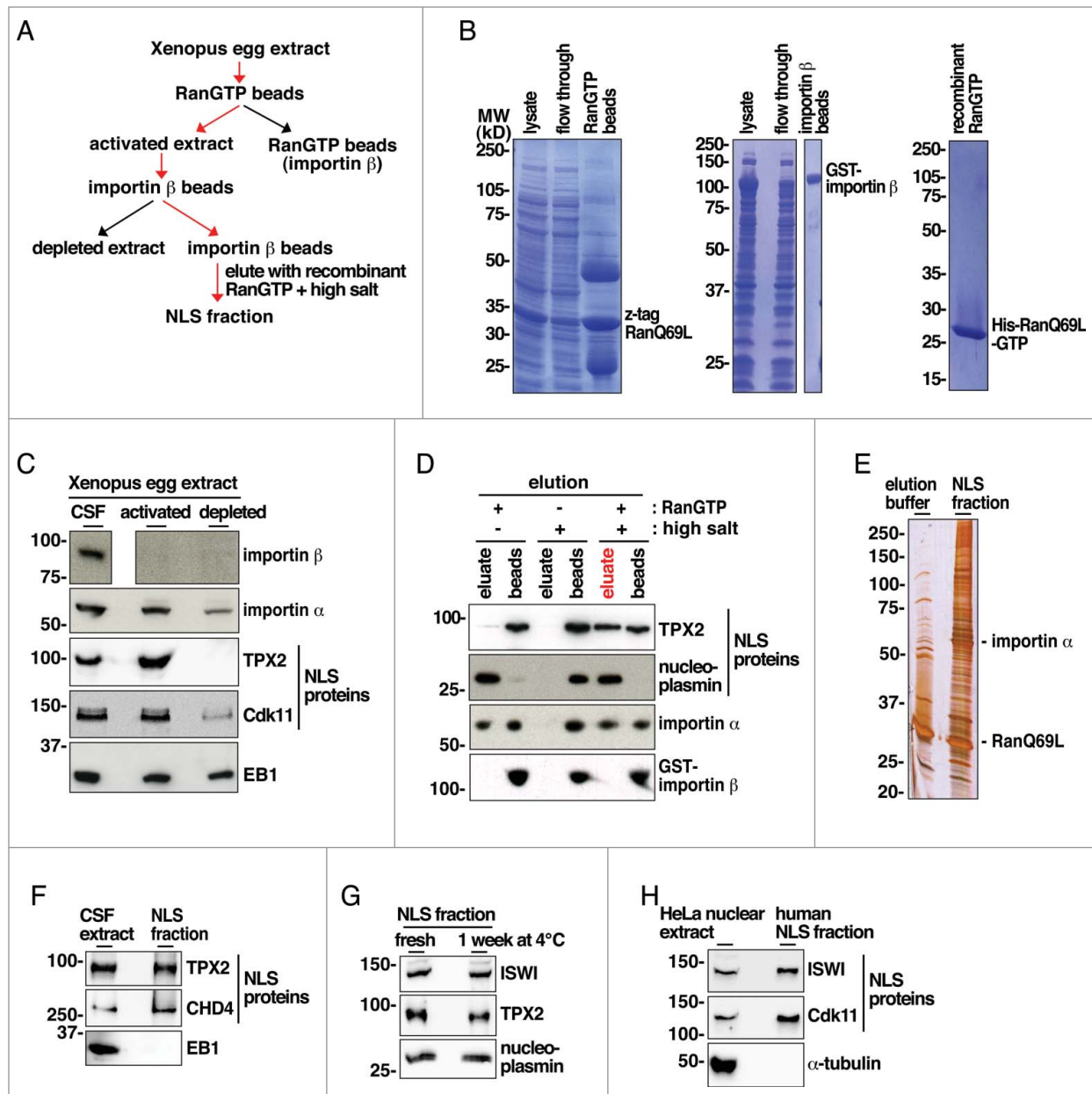
Figure 1. NLS purification method. (**A**) Purification strategy. Xenopus egg extract, either CSF or interphase, is incubated with z-tag RanQ69L (RanGTP) beads. Endogenous importin β binds to the beads. The activated extract, containing free NLS proteins, is incubated with GST-fused importin β beads. The proteins bind to the beads either directly or via endogenous importin α. NLS proteins are eluted from the beads by recombinant His-RanQ69L-GTP (RanGTP) in the presence of 0.5 M NaCl. (**B**) Coomassie staining of the affinity beads and recombinant RanGTP required for the NLS purification. (**C**) Extract after bead treatment. Each extract defined in **Fig. 1A** is immunoblotted. Importin β is depleted from the activated extract and NLS proteins are depleted from the depleted extract. EB1, a microtubule regulator without NLS, is not depleted and serves as a control. (**D**) Elution of NLS proteins in the presence or absence of 20 μM RanGTP and 0.5 M NaCl. Immunoblots were done for eluate and beads after elution. Note that RanGTP efficiently elutes some NLS proteins such as nucleoplasmin but others such as TPX2 only in the presence of high salt. (**E**) Elution buffer containing 20 μM RanGTP and 0.5 M NaCl, and eluate containing additionally NLS proteins and importin α. (**F**) The recovery of NLS proteins from Xenopus egg extract. From 5 ml CSF extract, 3.75 ml NLS fraction was obtained. 0.5 μl CSF extract and 5 μl NLS fraction were analyzed by SDS-PAGE and Western blot. The recovery of NLS proteins was quantified from the gels (TPX2, 16 ± 8%, n = 4; CHD4, 12 ± 7%, n = 3; ISWI, 29 ± 14%, n = 3; nucleoplasmin, 4 ± 1%, n = 4). Importantly, a cytoplasmic protein EB1 was not included in the NLS fraction (recovery 0 ± 0%, n = 3). Errors represent SD from the indicated number of independent experiments. (**G**) The Xenopus NLS fraction was kept at 4°C for 1 week and immunoblotted for NLS proteins. The level of the tested proteins was not affected after 1 week. (**H**) Purification of human NLS proteins. 5 ml HeLa nuclear extract (from ~1.5 x 10⁹ cells) produced 0.75 ml NLS fraction. 5 μl of the extract and NLS fraction were immunoblotted. The recovery of NLS proteins was quantified (ISWI, 24 ± 8%, n = 3; Cdk11, 65 ± 22%, n = 3; TPX2, 18 ± 9%, n = 4; MTA2, 6 ± 2%, n = 3). α-tubulin, a cytoplasmic protein contaminated in HeLa nuclear extracts, was not recovered in the NLS fraction (0 ± 0%, n = 3). Errors represent SD from the indicated number of experiments.

overcome this problem, we developed an affinity purification method to purify NLS proteins using RanGTP and importin β (**Fig. 1A**). It was previously shown that endogenous NLS proteins in Xenopus egg extracts bind an importin β column,[7] but elution of the NLS proteins from the column was difficult. We achieved this elution by using recombinant RanGTP in the presence of high salt.[8] Using the pure NLS fraction, 3 essential spindle assembly factors have been identified,[8-10] and NE assembly was reconstituted in Xenopus egg extracts (see below). Moreover, we showed that ISWI, an NLS-containing MAP, was not necessary for spindle assembly, but specifically required for spindle maintenance in anaphase,[11] showing that nuclear NLS-containing proteins regulate multiple mitotic events.

Here, we provide a protocol for the purification of NLS proteins from Xenopus egg extracts and human HeLa cells. In principle, lysates from other cell lines or tissues can be used as an NLS-protein source. Importin β is a major nuclear transport receptor, but other transport receptors can be used as affinity columns to purify their specific cargos. The pure NLS fractions (either classical or non-classical) are excellent resources to investigate the NLS protein function and to identify new mitotic regulators.

Materials

Reagents
- IgG Sepharose (GE)
- Glutathione Sepharose (GE)
- Talon Metal Affinity Resins (BD)
- Ni-NTA (Qiagen)
- Mono S (GE)
- Affi-Gel 15 Activated Affinity Media (Bio-Rad)
- pQE70 z-tag Xenopus RanQ69L[12]
- pGEX mouse importin β[13]
- pQE32 Xenopus RanQ69L[1]
- pQE70 -Xenopus ΔIBB-importin α[15]
- pQE70 Xenopus ΔIBB-ED importin α mutant
- BL21 pREP4 E. coli strain
- HeLa cells nuclear extracts, from 5×10^9 cells (~6 mg protein/ml × ~17 ml = ~100 mg protein) (Ipracell)

Equipment
- Tubes, 15 ml (Sarstedt)
- Tubes, 50 ml (Sarstedt)
- Mobicol columns (MoBiTec), 0.5 ml and 10 ml columns
- Benchtop centrifuge (Heraeus)
- High speed centrifuge (Sorvall or Beckman)
- Microfluidizer (Microfluidics)
- AKTA purification system (GE)
- Rotating wheel
- Dialysis membrane Spectra/Por 4, MWCO 12-14,000 Da (Spectra)

Reagent set up
20x XB (Extract Buffer) salts 2 M KCl, 20 mM MgCl$_2$, 2 mM CaCl$_2$. Autoclave and store at RT.

CSF-XB 10 mM K–HEPES, 100 mM KCl, 3 mM MgCl$_2$, 0.1 mM CaCl$_2$, 50 mM sucrose and 5 mM EGTA, pH 7.7. To prepare 1 l, dissolve 2.85 g HEPES and 17.15 g sucrose in dH$_2$O, and add 50 ml 20x XB salts, 2 ml 1 M MgCl$_2$, and 10 ml 0.5 M EGTA, adjust pH with KOH to 7.7, and fill up with dH$_2$O to 1 l. Store at 4°C.

Wash buffer CSF-XB, 100 mM NaF (1000 mM stock in dH$_2$O, 4°C), 80 mM β-glycerophosphate (1000 mM stock in dH$_2$O, 4°C), 0.1 mM sodium vanadate (200 mM stock,[16] −20°C), 0.1% digitonin (5% stock in dH$_2$O, −20°C), and 1 mM DTT (1000 mM stock in dH$_2$O, −20°C). Store at 4°C.

Elution buffer Wash buffer, 20 μM His-RanQ69L-GTP, 500 mM NaCl, 1 mM GTP, 1 mM ATP, and 10% glycerol. Prepare before use.

Dialysis buffer CSF-XB, 10% glycerol, and 1 mM DTT. Prepare before use.

S250 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl$_2$, 10 mM HEPES pH 7.5 with KOH. Store at 4°C.

Procedure

Recombinant proteins
z-tag RanQ69L (Ran mutant that cannot hydrolyze GTP and thus maintains it) Express in BL21 pREP4 E. coli strain at 18°C overnight, resuspend cell pellet on ice with 15 ml of PBS supplemented with 150 mM NaCl, 2 mM MgCl$_2$, and complete EDTA-free (Roche) per 1 l culture. Lyse the cells using Microfluidizer, and centrifuge the lysate at 20,000 × g for 15 min at 4°C using a high speed centrifuge. The supernatant (~20 ml from 1 l culture) is aliquoted, frozen in liquid N$_2$ and stored at −80°C.

GST-importin β Express in BL21 pREP4 E. coli strain at 30°C for 4 h, resuspend cell pellet on ice with 15 ml of PBS supplemented with 10% glycerol, 1 mM DTT, and complete EDTA-free per 1 l culture. Lyse the cells using Microfluidizer, and centrifuge at 20,000 × g for 15 min. The supernatant (~20 ml from 1 l culture) was aliquoted, frozen in liquid N$_2$ and stored at −80°C.

His-RanQ69L-GTP Express in BL21 pREP4 E. coli strain at 18°C overnight, resuspend cell pellet at 4°C with 15 ml of PBS supplemented with 150 mM NaCl, 2 mM MgCl$_2$, complete EDTA-free (Roche) and 8 mM imidazole per 1 l culture. Lyse the cells using Microfluidizer and centrifuge at 20,000 × g for 15 min. Purify the supernatant (~20 ml from 1 l culture) with TALON beads (BD), and subsequently with Mono S using AKTA system (GE). Check the fractions by Coomassie staining and dialyze the pooled fractions to PBS containing 5 mM MgCl$_2$ and 10% glycerol. Incubate the His-RanQ69L fraction with 1 mM GTP and 10 mM EDTA at 4°C for 2 h, and add 10 mM MgCl$_2$ drop by drop. Freeze the GTP bound RanQ69L (**Fig. 1B**) with aliquots in liquid N$_2$ and store at −80°C.

His-ΔIBB-importin α (lacking the importin β binding domain) and His-ΔIBB-ED mutant (ED: the double point mutants of importin α that cannot bind NLS[17]) Express in E. coli and purify with Ni-NTA beads (Qiagen), as described for

importin α.[14] Dialyze to 20 mM HEPES pH 7.4, 200 mM NaCl, and store at −80°C with aliquots.

Affinity column preparation

RanGTP beads Incubate excess amount of the z-tag RanQ69L lysate with IgG Sepharose (in our case, 3.5 ml lysate per 1 ml wet bed volume of beads) in a tube at 4°C for 1 h. Centrifuge the tube at 2,000 rpm for 1 min at 4°C using a benchtop centrifuge, and remove the supernatant as much as possible. Wash the beads with CSF-XB buffer, centrifuge the tube at 2,000 rpm for 1 min at 4°C, and discard the buffer using vacuum. Repeat the washing 3 more times. Adjust the beads to 50% v/v slurry with CSF-XB, and use them immediately or store at 4°C for ~1 week. Analyze the beads, lysate and flow through by SDS-PAGE and Coomassie staining to ensure that beads are saturated with recombinant RanQ69L (**Fig. 1B**).

Importin β beads Incubate excess amount of the GST-importin β lysate with Glutathione Sepharose (in our case, 1.7 ml of the lysate per 1 ml wet bed volume of beads) in a tube at 4°C for 1 h. Wash the beads with CSF-XB, adjust them to 50% v/v slurry, and check them by SDS-PAGE and Coomassie staining (**Fig. 1B**).

ΔIBB-importin α and ΔIBB-ED mutant beads Incubate 30 mg of the purified His-ΔIBB-importin α or His-ΔIBB-ED mutant with 1ml of wet bed volume Affi-Gel 15 for 2 h at 4°C. Centrifuge the tube at 2,000 rpm for 1 min at 4°C, and remove the supernatant. Add 3 ml 1 M ethanolamine-HCl pH 8.0 and incubate for 1h at 4°C to block any active esters on the beads. Wash the beads 3 times with 200 mM glycine, 500 mM NaCl pH 2.2 to remove any protein not covalently bound on the beads and 3 times with PBS. Adjust the beads to 50% v/v slurry with PBS, 0.1% NaN₃, 0.1 mM PMSF, and use them immediately or store at 4°C for ~1 week.

Cell extract preparation

Xenopus cytostatic factor (CSF)-arrested mitotic egg extract Prepare from Xenopus laevis eggs as described.[18] Use extracts directly to purify NLS proteins, or freeze them in liquid N₂ and store at −80°C. For the NLS purification, it seems not important whether extracts remain in mitosis or change to interphase.

Cell extracts from cultured cells or tissues Instead of Xenopus egg extracts, prepare cell extracts from cultured cells or tissues.[19] Cell extracts are also available commercially. In this paper, we used nuclear extracts from HeLa cells[6] (Ipracell) to purify human NLS proteins.

Xenopus interphase high-speed egg extract and floated membranes Prepare as described previously.[20]

Purification of NLS-containing proteins (see strategy Fig. 1A)

Here, we provide a protocol with 5 ml Xenopus egg extracts (obtained from ~5 frogs), which purifies sufficient NLS proteins to optimize/establish an assay. The purification can be scaled up or down (e.g., start from 50 ml egg extracts to identify new spindle assembly factors and 0.5 ml for Western blot).

1. Incubate 5 ml of Xenopus egg extracts with 2 ml wet bead volume of RanGTP beads (drained) in a 10 ml Mobicol column with cap at 4°C for 1 h. Frozen extracts seems to be sufficient to purify NLS proteins. However, if you use the bead-treated extract for functional assays afterwards, use fresh CSF extract. 'Problem' (**Table 1**)

2. Place the column on a 50 ml centrifuge tube and centrifuge it at 3,000 rpm for 1 min at 4°C using a benchtop centrifuge. Repeat the centrifugation in the same tube. The extract is recovered in the tube (~5 ml) without dilution. Endogenous importin β is depleted in this step (**Fig. 1C**), and endogenous NLS proteins are in principle liberated. 'Problem' (**Table 1**)

3. Incubate the activated extract (~5 ml) with 6 ml wet bead volume of importin β beads (drained) in a 10 ml Mobicol column with cap at 4°C for 1 h.

4. Place the column on a new 50 ml tube and centrifuge it at 3,000 rpm for 1 min. Repeat the centrifugation in the same tube. The extract is recovered in the tube (~5 ml) without dilution, which is important if it is used for assays. Endogenous NLS proteins are depleted from the extract (**Fig. 1C**). When needed, freeze the depleted extract with aliquots in liquid N₂ and store at −80°C.

5. Wash the importin β beads with the wash buffer in the Mobicol column with cap. Place the column in a 50 ml tube and

Table 1. Problem table.

Step	Problem	Possible reason	Solution
1	CSF extracts change to interphase	With bead treatment as well as freezing and thawing, some preparations of extracts cannot maintain the mitotic state	Add recombinant cyclin B Δ90 (~0.1 mg/ml extract)[24]
2	Inefficient depletion of importin β from other cell extracts	GTP is loaded to RanQ69L beads by endogenous RCC1 in Xenopus egg extracts. This loading may be inefficient in the diluted cell extracts	Do not worry. Optimal amount of RanQ69L bead treatment increases the recovery of NLS proteins, but this does not need the complete depletion of importin β.
6	Many inclusion of recombinant RanQ69L-GTP	Excess recombinant RanQ69L-GTP	Elution with 10 μM RanQ69L-GTP shows similar level of elution
7	Inclusion of NLS proteins that directly bind to importin β	Importin β beads bind to NLS proteins via importin α or directly	Prepare an affinity column with GST-importin β and His-importin α[14]

centrifuge at 3,000 rpm for 1 min to remove the buffer. Repeat the washing 4 more times.

6. Incubate the importin β column with 3.75 ml of the elution buffer with cap at 4°C for 2 h or overnight. Place the column on a new 50 ml tube and centrifuge it at 3,000 rpm for 1 min. Centrifuge again in the same tube to recover all the eluate (~3.75 ml). 'Problem' (**Table 1**).

7. The eluate contains NLS proteins, importin α, and recombinant RanGTP, but not importin β (**Fig. 1D and E**). The recovery of NLS proteins from egg extracts is approximately 15%, although it depends on the respective proteins (**Fig. 1F**). Dialyze the NLS fraction (~3.75 ml) using the membrane Spectra to the dialysis buffer at 4°C for >4 h. Repeat the dialysis one more time. Centrifuge the sample at 20,000 g for 10 min, and store the supernatant (~2 ml, protein concentration ~1 mg/ml) in aliquots at −80°C. The NLS proteins are in general stable at 4°C for at least 1 week (**Fig. 1G**). 'Problem' (**Table 1**)

8. Strip the residual proteins from the IgG Sepharose and glutathione Sepharose following their instructions and store at 4°C for next use.

Other cell extracts can also be used as NLS sources, although extract and bead ratio needs to be optimized. We have optimized condition to purify human NLS proteins from HeLa nuclear extracts, contaminated with cytoplasmic proteins (**Fig. 1H**). Incubate 5 ml of HeLa nuclear extract (from ~1.5 × 10⁹ cells, ~6 mg protein / ml, total ~30 mg) with 0.25 ml RanGTP beads. Incubate the activated extract with 0.75 ml importin β beads. Elute NLS proteins from the beads with 0.75 ml of the elution buffer. The recovery of NLS proteins is roughly 28% (**Fig. 1H**).

Application using the pure NLS fraction

The NLS fractions are useful to identify new spindle and NE assembly factors. For example:

(a) Microtubule nucleation and stabilization activities

The mitotic spindle is assembled by distinct regulation of microtubules, including microtubule nucleation (de novo formation) and stabilization (elongation of pre-existing microtubules).[21, 22]

To detect microtubule nucleation activity, the Xenopus NLS fraction or the elution buffer (**Fig. 1E**) are concentrated ~10 times by Amicon Ultra 10 kDa cut off (Millipore). The concentrated fractions (1 μl) are incubated in 10 μl of the depleted extract (**Fig. 1A and C**) supplemented with 1 μM Cy3-labeled tubulin[23] at 20°C for 30 min. Fix each sample (1 μl) with the fixative (1 μl; 0.15× CSF-XB, 11% formaldehyde, 48% glycerol, 10 μg/ml Hoechst 33342) on a slide and squash it with a coverslip. Analyze the samples under fluorescence microscopes (**Fig. 2A**). The NLS fraction induces de novo microtubule aster formation.

To examine microtubule stabilization activity,[8] incubate the concentrated NLS fraction or elution buffer (1 μl) at 20°C for 30 min in 10 μl of the depleted extract supplemented with 1 μM Cy3-tubulin, 2000 purified centrosomes /μl, and 0.15 mg/ml anti-TPX2 antibody that inhibits the microtubule nucleation described above. Fix samples with 0.5 ml 0.25% glutaraldehyde, 10% glycerol, and 0.1% Triton X-100 in BRB80 (80 mM K-PIPES, 1 mM $MgCl_2$, and 1 mM EGTA, pH 6.8), and spin down onto 12 mm round coverslips through a cushion of 25% glycerol in BRB80.[18] Coverslips are postfixed with methanol at −20°C for 10 min, washed with PBS, mounted on slides, and analyzed by microscopy (**Fig. 2B**). The NLS fraction stabilizes and elongates microtubules nucleated by centrosomes.

The NLS fraction is separated by Mono Q column, and each fraction is dialyzed and tested in the above assays (**Fig. 2C**), showing microtubule nucleation and stabilization activities are distinct. Western blot of the Mono Q fractions shows that the nucleation activity matches to TPX2 distribution. Further purification of the microtubule stabilization activity and mass spectrometry identify the microtubule stabilization factor Cdk11.[8]

(b) Microtubule-associated proteins (MAPs)

To comprehensively identify new RanGTP-regulated spindle assembly factors, we purified MAPs from the Xenopus NLS fraction using taxol-stabilized pure microtubules (**Fig. 2D, lane 1**). Mass spectrometry identified 168 NLS proteins.[9] Importantly, no cytoplasmic MAPs (such as EB1) are included in this NLS-MAP purification. Among the identified proteins, CHD4 and MEL-28 have been shown to be essential for spindle assembly in early mitosis,[9, 10] while ISWI is specifically required for spindle maintenance in anaphase.[11] Characterization of the other NLS proteins will certainly identify additional microtubule regulators toward the full understanding of spindle assembly.

(c) Nuclear envelope assembly (**Fig. 3**)

In Xenopus egg extracts, importin α binds and negatively regulates NLS proteins in NE assembly. The correct balance between importin α and NLS proteins is crucial for this process.[5] The purified NLS fraction can be used to identify factors involved in NE formation.

First, NLS proteins, including the NE formation factors, are depleted from Xenopus egg extracts. Specifically, freshly prepared interphase high-speed egg extracts were incubated with 1:1 vol of ΔIBB-importin α or ΔIBB-ED mutant (that does not bind the NLS, a negative control) beads in 0.5 ml Mobicol columns on a rotating wheel for 45 min at 4°C, and extracts were collected by centrifugation. The treatment was repeated one more time with fresh beads, and extracts were frozen in liquid nitrogen. The depletion of NLS proteins was confirmed by Western blot (**Fig. 3A**).

Next, we tested the ability of rich source of importin α-binding proteins to restore NE assembly in the depleted extracts (**Fig. 3B and C**). 1 μl of HeLa nuclear extracts, interphase Xenopus high-speed egg extracts or the Xenopus NLS fractions were added to 9 μl of the depleted extracts and incubated with 150 sperm heads/ μl at 20°C for 20 min. Then, energy mix (1 mM ATP, 10 mM creatine phosphate, 0.2 mg/ml creatine kinase), glycogen (0.4 mg/ml) and 0.5 μl floated membranes were added, and the reaction was incubated for 90 min at 20°C.[20] To label membranes, DiIC18 (1 μg/ml) was added 10 min before fixation.[20] Reactions were fixed with 0.5 ml fixative (0.5% glutaraldehyde, 2% PFA, 50 mM K-PIPES pH 6.8, 1 mM $MgCl_2$,
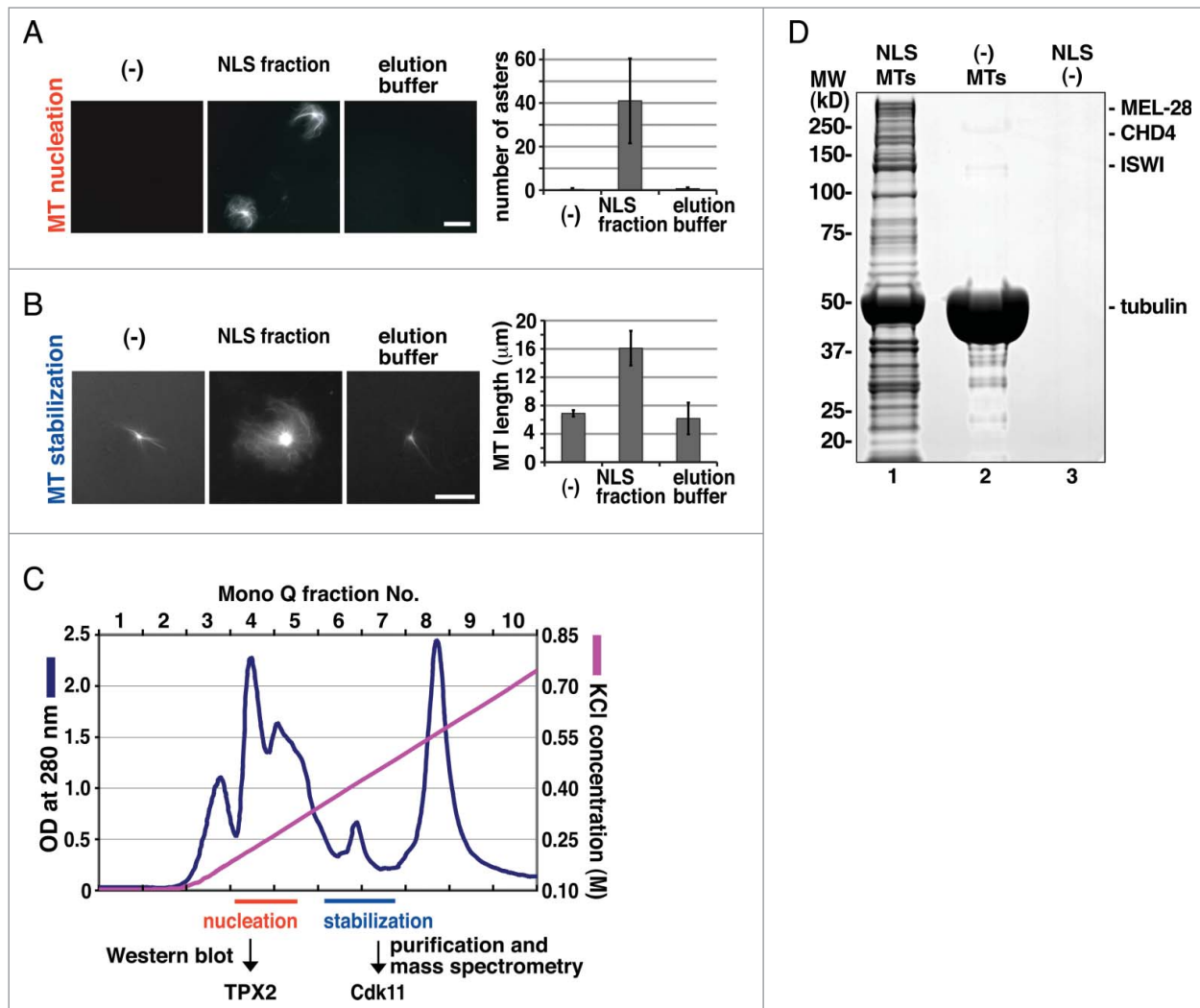
Figure 2. The pure NLS fraction is valuable to investigate the mechanisms of spindle assembly and function. (**A**) Microtubule (MT) nucleation induced by the NLS fraction. The Xenopus NLS fraction or elution buffer was concentrated ~10 times and dialyzed to the dialysis buffer. The fractions were incubated in the depleted extract containing Cy3-labeld tubulin, and fixed on coverslips. Number of asters was counted in 10 randomly selected fields with 40x objective. Error bars, SD from 3 independent experiments. Scale bar, 20 μm. (**B**) MT stabilization induced by the NLS fraction. The concentrated, dialyzed fractions were incubated in the depleted extract containing centrosomes, Cy3-tubulin, and anti-TPX2 antibody that inhibits the MT nucleation shown in A. Samples were fixed, spun down on coverslips, and mounted. MT length of centrosomal asters was quantified.[8] Error bars, SD from >20 asters. n > 3 experiments. Scale bar, 20 μm. (**C**) The Xenopus NLS fraction was applied to Mono Q column and eluted with the salt gradient. Each fraction was dialyzed and tested as in A, showing MT nucleation activity in fraction No. 4 and 5. Western blot showed the peak of TPX2 also in the No. 4 and 5. Each fraction was tested as in B, showing MT stabilization activity in fraction No. 6 and 7. Further purification of the activity and subsequent mass spectrometry identified Cdk11.[8] (**D**) Microtubule-associated protein (MAP) preparation from the NLS fraction. The Xenopus NLS fraction was incubated with or without taxol-stabilized pure MTs. MAPs were eluted from the MTs with high salt (0.5 M NaCl), and were analyzed by SDS-PAGE and Coomassie staining. Mass spectrometry identified new spindle assembly factors (MEL-28 and CHD4) and maintenance factor (ISWI) in the first lane.

150 mM sucrose) for 30 min on ice and spun down onto poly-L-lysine-coated 12 mm round coverslips through a cushion of 30% (w/v) sucrose in S250 buffer. Coverslips were washed with PBS, and mounted for microscopy analysis of fluorescence-labeled membranes.

Depletion of NLS proteins from interphase Xenopus egg extracts inhibited fusion of the membranes attached on chromatin and prevented closed NE formation (**Fig. 3B and C**). Addition of HeLa nuclear extracts, Xenopus egg extracts or Mono Q-purified NLS fractions 6 and 7 restored closed NE formation (**Fig. 3B and C**). The original NLS fraction didn't restore NE formation possibly because it was too diluted.

Further fractionation of the active Mono Q fractions may lead to the identification of NLS proteins that interact with importin α and are important for NE formation.

Problem Solving

Please see **Table 1** for problem solving advice.
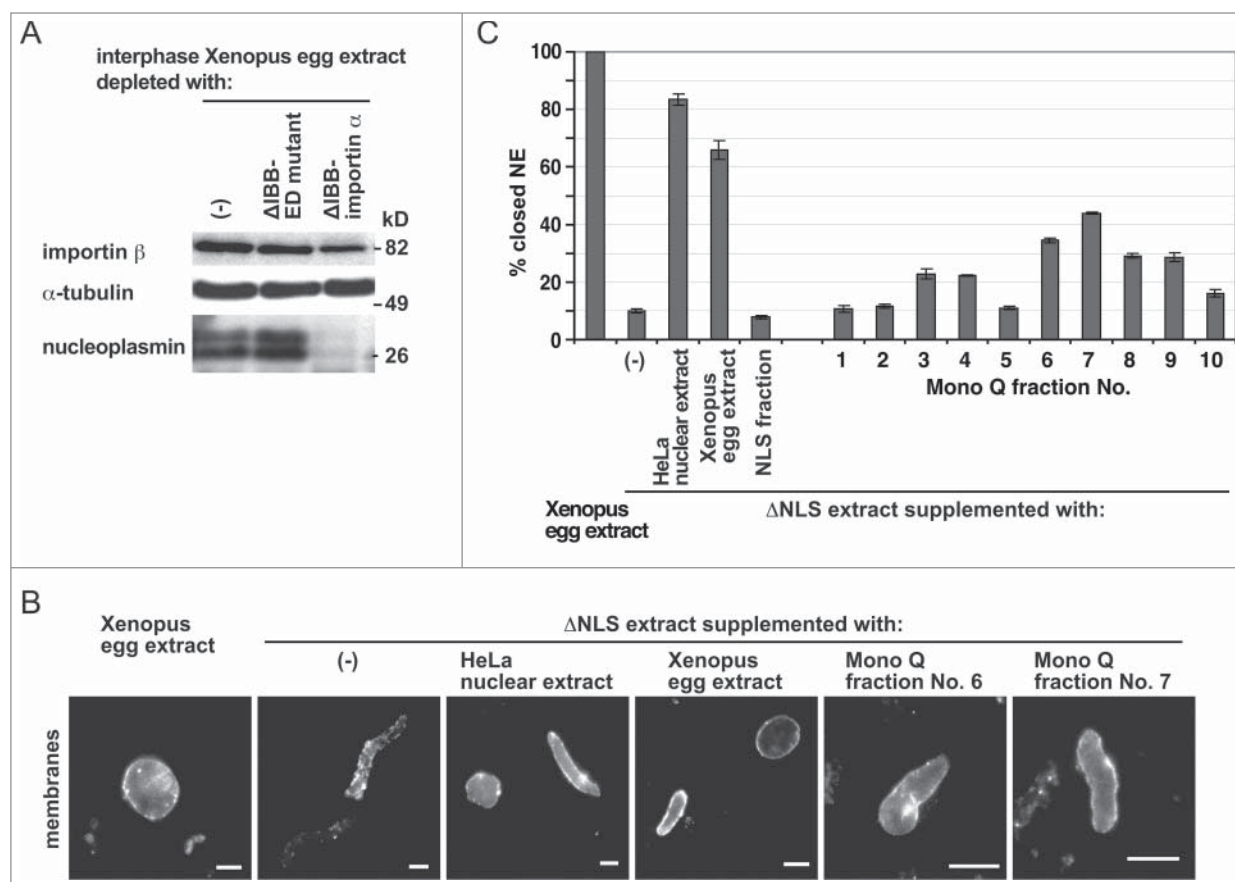
Figure 3. The NLS fractions promote NE formation. (**A**) Interphase Xenopus high-speed egg extracts were treated with ΔIBB-importin α or ΔIBB-ED mutant beads, and analyzed by Western blot. Nucleoplasmin (one of the most abundant NLS proteins in egg extracts) was depleted efficiently by ΔIBB-importin α but not by the ΔIBB-ED mutant. The levels of importin β and α-tubulin were unaffected. (−): no bead treatment. Note that we used ΔIBB-importin α to deplete NLS proteins but not importin β which is also important for NE assembly.[30] (**B**) NE assembly is inhibited by the NLS depletion and is restored by addition of 1:10 volume of a rich source of importin α binding proteins (HeLa nuclear extracts, Xenopus high-speed egg extracts or the Mono Q Xenopus NLS fractions 6 and 7 (**Fig. 2C**)). Non-treated egg extracts were used as a control. Nuclei were analyzed by membrane staining (DilC18). Scale bars, 10 μm. (**C**) Quantification of the inhibition and restoration of the NE formation. More than 100 randomly chosen chromatin substrates were measured for closed NE and normalized to the control. NE assembly is inhibited by the NLS depletion and is restored by addition of 1:10 volume of HeLa nuclear extracts and Xenopus high-speed egg extracts. The purified Xenopus NLS fraction did not restore the NE assembly probably because it is not enough concentrated. On the other hand, addition of the Mono Q fractions 6 and 7 significantly restored the NE assembly. Error bars represent SD from 3 different experiments.

Perspectives

Many nuclear proteins contain the basic, classical NLS that is recognized by importin β and its adaptor importin α. There are, however, ~20 importin β-family transport receptors.[25] Their specific cargos and the recognized NLS or nuclear export signal (NES) sequences are not well defined as for importin β. The purification method described here can be applied for other transport receptors to clarify their specific cargos. Among the receptors, addition of recombinant transportin to Xenopus egg extracts inhibits spindle and NE assembly independently of importin β,[26,27] suggesting that transportin sequesters its cargos essential for either assemblies. Using transportin beads, we could purify its specific cargos and identify new spindle and NE assembly factors beyond the classical NLS proteins.

It has been shown that actin or lamin filaments are assembled around mitotic chromatin, and are required for chromosome alignment and spindle assembly, respectively.[28,29] The assembly of lamin filaments requires RanGTP.[29] The NLS fraction shown here can be used to purify the proteins that bind actin or lamin filaments, thus to identify assembly factors of these filaments.

In summary, here we provide a highly specific method to purify NLS proteins from crude cell lysates. The NLS fraction has proven to be an excellent resource to identify new mitotic regulators. Not only for mitosis, this protocol should be widely applied for investigating the function of NLS proteins and uncovering fundamental mechanisms of the cell division cycle.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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