Chapter 30

Analysis of CRM1-Dependent Nuclear Export in Permeabilized Cells

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Abstract

Nuclear protein import and export assays in permeabilized cells have been instrumental for the identification of transport factors and for the molecular characterization of nucleocytoplasmic transport pathways. Our original assay to quantitatively analyze CRM1-dependent export was based on stably transfected cells expressing GFP-NFAT. We now present a simplified version of the assay using transiently transfected cells expressing GFP-NFAT or GFP-snurportin1 as a fluorescent export cargo and mCherry-emerin as a marker protein for transfected cells. CRM1- and Ran-dependent export is recapitulated in digitonin-permeabilized cells and quantified by flow cytometry. The assay should be applicable to other combinations of cargo and marker proteins.

Key words Nucleus, Nuclear transport, Export, CRM1, Ran, NFAT, Snurportin 1, Digitonin, Flow cytometry

1 Introduction

Twenty years after the identification of nuclear export sequences (NESs) in the HIV-1 Rev protein [1] and in PKI, the heat stable inhibitor of the catalytic subunit of cAMP-dependent protein kinase [2], the number of proteins carrying characteristic hydrophobic NESs is still growing. CRM1, a member of the importin β-family of nuclear transport receptors, has been recognized as the major factor that mediates transport of proteins out of the nucleus, although other family members are involved in export of selected cargoes as well (for reviews *see* refs. 3–5). More recently, CRM1 was identified as a potential target in anti-cancer therapy (for review *see* ref. 6), based on the observation that it is upregulated in many tumor cells. This probably relates to the fact that many tumor suppressors like p53 [7] or p27 [8] are bona fide CRM1 cargoes.

We initially designed an assay to analyze nuclear export in digitonin-permeabilized cells that was based on similar principles as the classic nuclear import assay developed by Adam and Gerace [9].

Sue Shackleton et al. (eds.), *The Nuclear Envelope: Methods and Protocols*, Methods in Molecular Biology, vol. 1411,
DOI 10.1007/978-1-4939-3530-7_30,

The assay used GFP-NFAT (nuclear factor of activated T cells) as a CRM1-dependent reporter protein [10]. In vivo, nuclear trafficking of GFP-NFAT can easily be controlled by adding the calcium ionophore ionomycin to cells, a treatment that leads to rapid nuclear import of the protein [11, 12]. Nuclear export, on the other hand, occurs upon medium exchange and can be recapitulated in digitonin-permeabilized cells in vitro. After such an export reaction in permeabilized suspension cells, residual nuclear fluorescence can be quantified by flow cytometry, allowing the analysis of a large number of samples in a short period of time. Again, the method for quantification was based on a previous approach [13]. Although this assay proved very powerful for the identification of CRM1 [10], as well as for the characterization of co-factors like Ran [14], RanBP1 [15], RanGAP [16], and Nup214 [17], it has not widely been used by other researchers, because it requires a cell line stably transfected with the GFP-NFAT plasmid. We therefore set out to develop a similar assay that now uses transiently transfected cells expressing GFP-NFAT or GFP-snurportin1 (GFP-SPN1) as CRM1-dependent export cargoes and mCherry-emerin as a marker that allows gating for transfected cells in the subsequent flow cytometry analysis. Other combinations of cargoes and markers are possible. Nuclear accumulation of the export cargoes is achieved by treating the cells with the selective CRM1-inhibitor leptomycin B (LMB). For GFP-NFAT, ionomycin is used as an additional import-inducing agent (*see* **Note 1**). The marker protein should remain associated with the cells upon digitonin permeabilization. Emerin is a protein that localizes to the inner nuclear membrane. In our transfection assays, it is also found in stable association with other membranes, e.g., the endoplasmic reticulum. Alternatively, other membrane proteins can be used that should, of course, not interfere with nucleocytoplasmic transport (*see* **Note 2**).

Using the same principles described here, it should be possible to monitor nuclear export in vitro of virtually any CRM1-dependent export cargo (*see* **Note 3**). Several aspects of the methods are similar to those described in a previous chapter in *Methods in Molecular Biology* [18], where we presented the method using cells that stably express GFP-NFAT.

2 Materials

2.1 Plasmids and Cells

1. NFAT reporter: for the specific construct we use, the coding sequence of human NFAT was amplified from pSH107c (kindly provided by G.R. Crabtree, Stanford, CA) and cloned via *Hind*III and *Bam*HI into a pS65T-C1 (Clontech)-based eukaryotic expression vector, behind the coding sequence of a modified version of GFP (S65T; [10]).

2. SPN1 reporter: for the specific construct we use, the coding sequence of human SPN1 was amplified from GST-SPN1 [19] and cloned via *Xho*I and *Eco*RI into the pEGFP-C1 vector [20].
3. Emerin reporter: for the specific construct we use, the coding sequence of human emerin was amplified from emerin-GFP (kindly provided by Eric Schirmer, Edinburgh) and cloned via *Xho*I and *Bam*HI into the pmCherry-C1 vector (Clontech), yielding mCherry-emerin.
4. Ran expression plasmid: we use human wild-type Ran in pET11d.
5. CRM1 expression plasmid: we use a His-tagged human CRM1 in the pQE60 vector (kindly provided by I. Mattaj, EMBL, Heidelberg, Germany).
6. Cells for transport assay: HeLa p4 cells (P4.R5 MAGI) [21] were obtained through the NIH AIDS Reagent Program (Division of AIDS, NIAID, NIH, Dr. Nathaniel Landau; *see* **Note 4**).
7. Growth medium for assay cells: Dulbecco's Modified Eagle's medium (DMEM), containing 10 % fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL).
8. Cells for cytosol preparation: HeLa suspension cells (CSH HeLa strain, obtained from Dr. Frauke Melchior, Heidelberg).
9. Growth medium for cytosol preparation: Joklik's modified S-MEM containing 10 % fetal bovine serum (FBS), 100 U/mL and 100 μg/mL penicillin streptomycin.

2.2 Buffers for Assay and Preparation of Cytosol

1. Lysis buffer: 5 mM HEPES-KOH, pH 7.3, 10 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM dithiothreitol (DTT), 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 μg/mL of each leupeptin, pepstatin, and aprotinin.
2. Transport buffer: 20 mM HEPES-KOH, pH 7.3, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA, DTT, and protease inhibitors as above (*see* **Note 5**).

2.3 Buffers and Reagents for Preparation of Recombinant Transport Factors

1. Transport buffer: as above.
2. Ran buffer: 50 mM HEPES-NaOH, pH 8.0, 500 mM NaCl, 5 mM $MgCl_2$, 0.1 mM PMSF, and 1 μg/mL of each leupeptin, pepstatin, and aprotinin.
3. CRM1 buffer: 50 mM HEPES-NaOH, pH 7.4, 500 mM NaCl, 2 mM $MgCl_2$, 20 mM β-mercaptoethanol, 0.1 mM PMSF, and 1 μg/mL of each leupeptin, pepstatin, and aprotinin.

   CRM1 low salt buffer: 50 mM HEPES-NaOH, pH 7.4, 50 mM NaCl, 2 mM $Mg(OAc)_2$, 2 mM DTT.

4. CRM1 high salt buffer: 50 mM HEPES-NaOH, pH 7.4, 500 mM NaCl, 2 mM $Mg(OAc)_2$, 2 mM DTT.
5. PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4.
6. Imidazole 5 M stock.
7. Standard bacterial culture reagents.
8. DEAE Sepharose.
9. Preparative S75 column (see GE Healthcare for specifications to match if another is used).
10. PD-10 Desalting Columns or a HiPrep 26/10 Desalting (Sephadex G-25) type column (e.g., GE Healthcare, though any similar desalting column will do).
11. MonoQ anion exchange column (see GE Healthcare for specifications to match if another is used).
12. Isopropylthio-β-Δ-galactoside (IPTG) 1 M stock.
13. Ammonium sulfate.
14. GDP 100 mM stock.

2.4 Additional Reagents for Transport Assays

1. ATP-regenerating system: (A) 100 mM ATP in 20 mM HEPES, 100 mM $Mg(OAc)_2$ (*see* **Note 6**). Adjust pH to 7.4 with NaOH. (B) 80 mg/mL creatine phosphate in H_2O. (C) 2000 U/mL creatine phosphokinase in 50 % glycerol, 20 mM HEPES, pH 7.4. Store all components at −20 °C and mix 2A:2B:1C (ATP:creatine phosphate:creatine phosphokinase) before use.
2. Leptomycin B (LMB; Enzo Life Sciences): prepare 370 μM stock (for preincubation of permeabilized cells) and 10 μM working dilution in ethanol (for treatment of intact cells); store at −20 °C.
3. Ionomycin: prepare stock (1 mM in DMSO) and store at −80 °C.
4. Digitonin: prepare stock (1 % in DMSO) and store at −20 °C.
5. Trypan blue: use 1:1 with cell suspension.
6. Oligonucleotide (*see* **Note 7**): dissolve desalted oligonucleotides (5′AGAG GAAAATTTGTTTCATA and 5′TATGAAACAAA TTTTCCTCT), each at 200 μM, in 40 mM Tris–HCl, pH 7.4, 20 mM $MgCl_2$, 50 mM NaCl, combine equal volumes and anneal by heating to 65 °C for 5 min and slow cooling to room temperature. Freeze in aliquots and store at −20 °C.
7. Wheat germ agglutinin (WGA): WGA, which inhibits most nucleocytoplasmic transport pathways, is dissolved in transport buffer at 2 mg/mL and stored at −80 °C.

3 Methods

3.1 Preparation of Cytosol

1. Grow HeLa suspension cells in spinner flasks to mid-log phase and collect them by centrifugation at 300 × *g* for 15 min (3–10 L can easily be handled). All of the following steps are performed on ice or at 4 °C.
2. Wash twice with PBS and once with transport buffer.
3. Resuspend in 1 volume of lysis buffer and swell for 10 min on ice.
4. Lyse the cells in a dounce homogenizer (*see* **Note 8**). After centrifugation at 1500 × *g* for 15 min, the supernatant is cleared by centrifugation at 120,000 × *g* for 1 h.
5. Dialyze overnight against transport buffer (10,000 K_d cutoff), freeze the resulting cytosol (~10 mg/mL) in aliquots in liquid nitrogen, and store at −80 °C.

3.2 Preparation of Recombinant Transport Factors

3.2.1 Ran

Various methods for the preparation of Ran have been described in the literature. We typically use a modified version of the one described by Melchior et al. [22]. Human wild-type Ran is expressed from pET11d in BL21-(DE3) cells.

1. Grow 2 L of a bacterial culture to an OD_{600} of 0.6 and induce expression with 0.5 mM isopropylthio-β-Δ-galactoside (IPTG) for 3 h at 37 °C. All subsequent steps are performed on ice or at 4 °C.
2. Harvest cells and resuspend them in 100 mL of Ran buffer, and lyse them. We find that the use of an EmulsiFlex-C3 (Avestin) for lysing produces reproducibly good protein, but other lysing methods should be able to be applied.
3. Clear lysate by centrifugation at 100,000 × *g* for 30 min.
4. Incubate the supernatant in batch with 70 mL DEAE Sepharose, equilibrated in Ran buffer, for 1 h with gentle agitation. Pour the slurry into a column and collect the flowthrough and approximately 100 mL of the subsequent wash (Ran buffer; wash until no protein can be detected).
5. Precipitate Ran from the DEAE flowthrough with ammonium sulfate: add salt gradually to 55 % saturation, stir for 2 h, and centrifuge for 20 min at 100,000 × *g*. Resuspend precipitate in 6–8 mL transport buffer containing 250 μM GDP, and clarify by centrifugation at 14,000 × *g* for 15 min.
6. Load onto a preparative S75 column, equilibrated in transport buffer. Collect 5-mL fractions and analyze them by SDS-PAGE.
7. Pool Ran-containing fractions, freeze in aliquots in liquid nitrogen, and store at −80 °C.

The final concentration of Ran (ca. 95 % pure) should be 2–3 mg/mL.

3.2.2 CRM1

The preparation of His-tagged CRM1 from pQE60-CRM1 (kindly provided by I. Mattaj, EMBL, Heidelberg, Germany) was originally described in [23].

1. Express the protein at 37 °C overnight in TG1 cells without induction by IPTG.
2. Harvest bacteria, wash them in PBS, and store cell pellets at −80 °C until purification.
3. Thaw and resuspend bacteria in cold CRM1 buffer, and lyse them. As for Ran we recommend an EmulsiFlex-C3 (Avestin). All following steps are performed on ice or at 4 °C.
4. Clear the lysate by centrifugation at 100,000 × *g* for 30 min.
5. Incubate the supernatant with Ni-NTA sepharose equilibrated in CRM1 buffer at 4 °C for 1 h.
6. Wash beads four times with 50 mL CRM1 buffer, each supplemented with 30 mM imidazole.
7. Transfer beads to an empty column and elute with CRM1 buffer containing 300 mM imidazole.
8. Change to CRM1 low salt buffer using a desalting column attached to an FPLC system.
9. Load the protein onto a MonoQ anion exchanger column equilibrated in CRM1 low salt buffer and elute with an increasing concentration of CRM1 high salt buffer.
10. Pool the peak fractions containing CRM1, change into transport buffer with desalting columns, and freeze in aliquots in liquid nitrogen. Store at −80 °C.

3.3 Transfections and Treatment of Cells

1. Grow HeLa p4 cells in DMEM medium in 24-well dishes.
2. Transfect cells with Lipofectamine (Life Technologies) or the calcium phosphate method [24], using 0.8 μg plasmid per ~10^5 cells per 24-well, with or without coverslips (*see* **Note 9**).
3. Incubate overnight and induce nuclear import of the reporter protein with 5 nM LMB for 90 min. For GFP-NFAT, also add 1 μM ionomycin for the last 30 min. The localization of GFP-NFAT and mCherry-emerin before and after treatment of cells with ionomycin/LMB is shown in Fig. 1. Continue as described in Subheading 3.4 or 3.5.

3.4 Analysis of Transport by Fluorescence Microscopy

Although the major advantage of our approach is the quantitative analysis by flow cytometry (*see* Subheading 3.5), we briefly outline the method for microscopic analysis as well.

1. Wash cells grown on coverslips with cold transport buffer and permeabilize with digitonin (final concentration ~70 μg/mL in transport buffer) on ice (*see* Fig. 1). The required amount of the detergent should be tested on a separate slide for each cell type, using trypan blue (*see* **Note 8**).

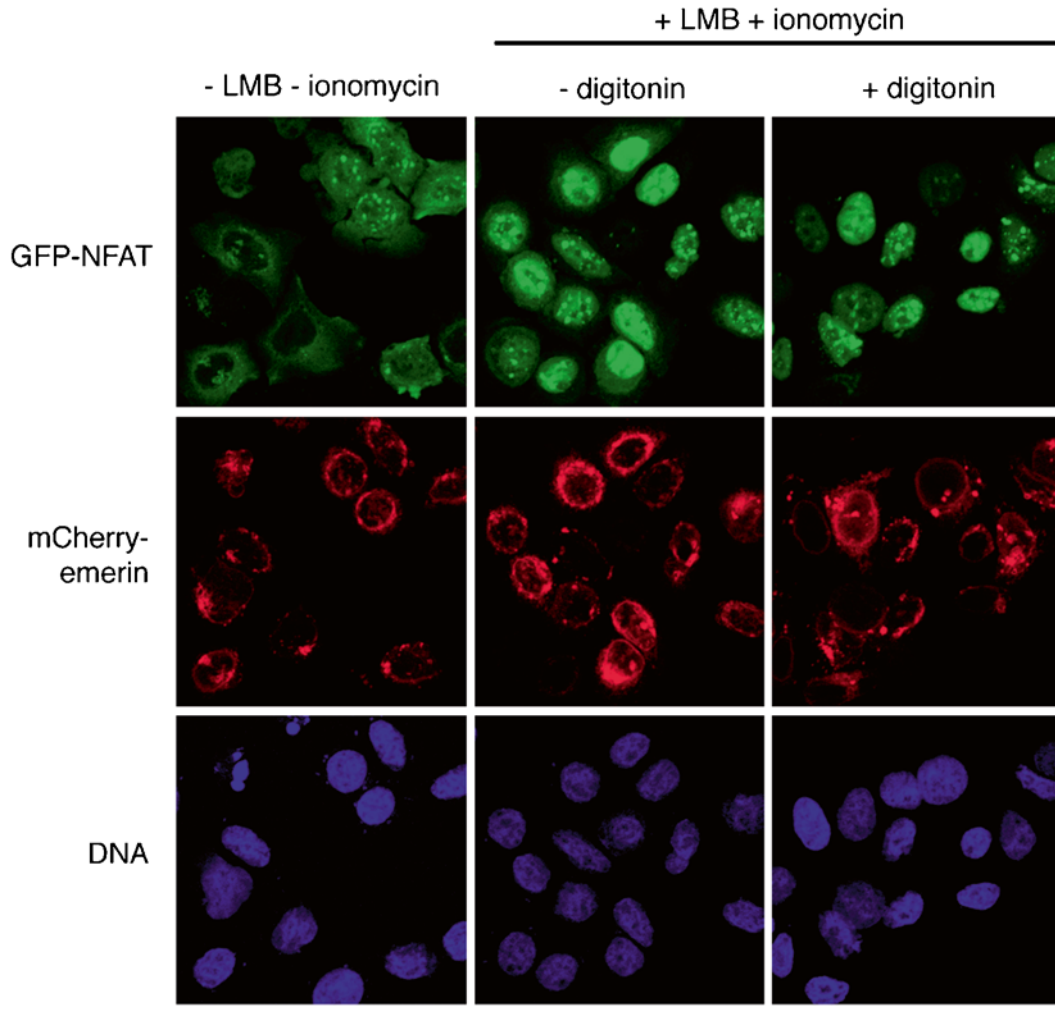

Fig. 1 Subcellular localization of GFP-NFAT and mCherry-emerin. HeLa p4 cells were transfected with plasmids coding for GFP-NFAT (*green*) and mCherry-emerin (*red*) and analyzed by confocal microscopy. GFP-NFAT localized to the cytoplasm or was homogenously distributed in the cells. Nuclear import of GFP-NFAT was induced by LMB and ionomycin. The nuclear localization of GFP-NFAT did not change upon permeabilization of the cells with digitonin. Note that the membrane association of mCherry-emerin did not vary in any of the tested conditions, confirming its suitability as a marker protein

2. Subject cells to preincubation and transport reaction, using reagents as outlined in Subheading 3.5, **steps 3** and **4**. Use ~40 μL solution per coverslip and incubate at 30 °C in a moisturized chamber.
3. Wash with cold transport buffer. Fix cells with 3.7 % formaldehyde in PBS.
4. Analyze cells by fluorescence microscopy and quantify images using local software.

3.5 Analysis of Transport by Flow Cytometry

1. Trypsinize cells: wash with PBS, add trypsin. Remove most of the trypsin solution immediately to minimize carryover of the protease. Incubate at 37 °C until cells detach. Collect cells in cold transport buffer containing 10 % fetal bovine serum (FBS) to inactivate the trypsin. The following steps are performed on ice or at 4 °C unless otherwise indicated. Centrifuge for 5 min at 300 × *g* and wash once in transport buffer.
2. Resuspend cells in transport buffer at 10^7/mL. Add digitonin to 70 μg/mL (0.7 μL of a 1 % stock per 10^6 cells). Leave on ice for 3 min and check permeabilization with trypan blue, using 5 μL of cells. Add more digitonin if less than 90–95 % of the nuclei appear blue. Wash with transport buffer and centrifuge as in **step 1**.
3. Preincubation: Resuspend cells at 10^7/mL in transport buffer and add 100 nM LMB and 25 μL ATP-regenerating system per mL of cell suspension. Incubate for 15 min in a 30 °C water bath. Wash cells twice with transport buffer. The preincubation results in depletion of transport factors like CRM1 that initially remain associated with the permeabilized cells. Furthermore, endogenous CRM1 will be modified by LMB. Without the preincubation, Ran will be the only rate-limiting factor for CRM1-dependent export [10].
4. Resuspend cells in transport buffer at 2×10^7/mL. The transport reaction (40 μL in a 5-mL flow cytometry tube) should contain: 10 μL of cells (200,000), 1 μL ATP-regenerating system, 0.5 μL annealed oligonucleotide for cells transfected with GFP-NFAT (final concentration: 1.25 μM). Add cytosol (~1 mg/mL usually yields optimal transport) and/or recombinant transport factors.
5. Incubate on ice or in a 30 °C water bath for 30 min.
6. Add 4 mL cold transport buffer. Centrifuge for 10 min at 300 × *g* and 4 °C. Remove most of supernatant by aspiration, leaving ~200 μL behind.
7. Analyze export of the GFP-cargo by flow cytometry. We use a FACSCanto II Cell Analyzer (Becton Dickinson), and typically count 5000–10,000 cells. For measuring GFP-fluorescence, gate the cells such that only mCherry-positive ones are analyzed. In most cases, the median fluorescence is best suited for statistical analysis of transport. Export reactions may be standardized by assigning a GFP-cargo fluorescence value of 100 to a 4 °C control (*see* **Note 10**).

Typical results for nuclear transport reactions as analyzed by flow cytometry are shown in Figs. 2 and 3. With respect to the efficiency of nuclear export of GFP-NFAT, they are very similar to those obtained using stably transfected cells [10, 18]. Cytosol, which should contain all relevant transport factors, strongly

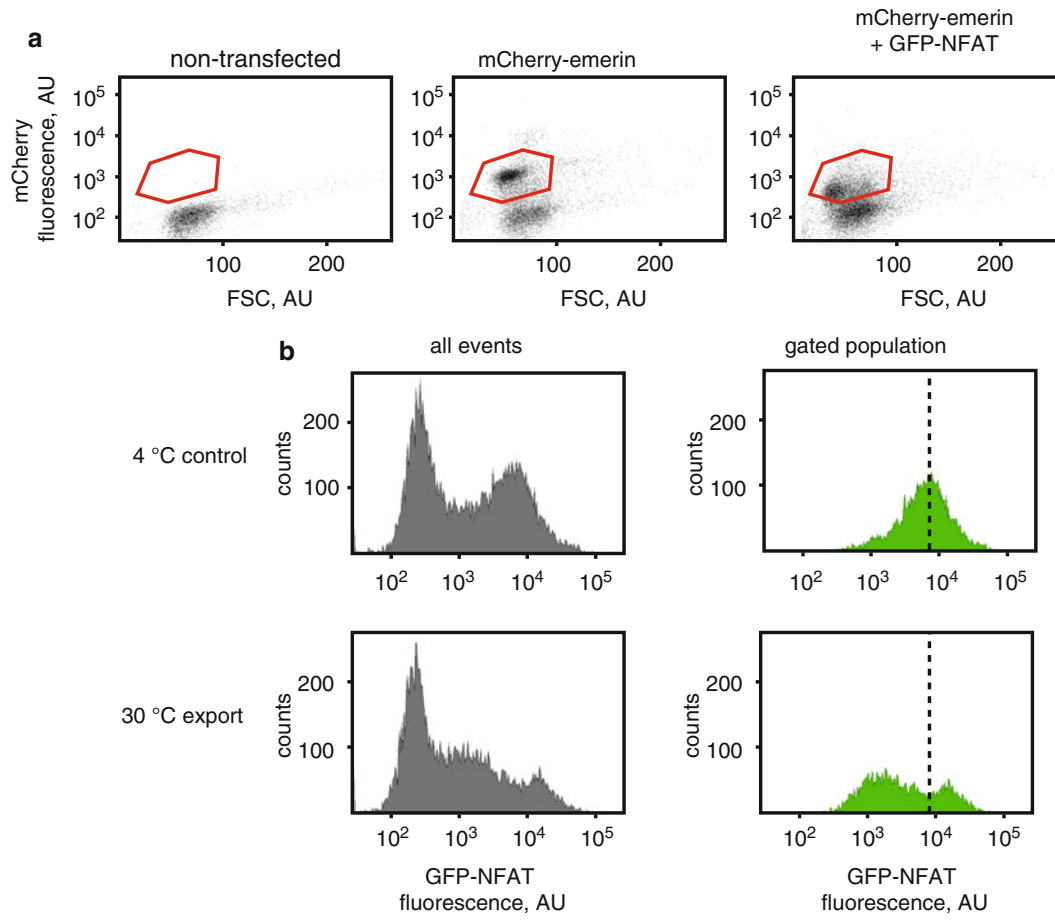

Fig. 2 Analysis of nuclear export by flow cytometry. HeLa p4 cells were transfected with plasmids coding for GFP-NFAT and/or mCherry-emerin. (**a**) Cell populations negative or positive for mCherry-emerin could be clearly distinguished by analyzing non-transfected cells and cells transfected with mCherry-emerin, alone or in combination with GFP-NFAT. A gate (*red area*) was set in a way that only mCherry-emerin-positive cells were included in further analysis. *FSC* forward scatter, *AU* arbitrary units. (**b**) Comparison of GFP-NFAT fluorescence in the total and the gated population. A prominent peak for non-transfected cells can be observed on the *left side* of the fluorescence intensity profile in the total population (*left panel*). Gating for the transfection marker mCherry-emerin (as described above) led to a simplified fluorescence intensity profile of GFP-NFAT, as most non-transfected cells were excluded from the analysis (*right panel*). The fluorescence intensity of nuclei was normalized to a 4 °C control sample (indicated by the *dashed line*) and was clearly reduced upon export of GFP-NFAT. Note the small remaining peak with a high GFP-NFAT fluorescence in the export reaction, which could result from cells that had not been sufficiently permeabilized and were therefore not accessible for export factors

promoted nuclear export (i.e., resulted in low levels of fluorescence). CRM1 and Ran were only active when added together, indicating that both factors are rate-limiting under our experimental conditions. The physiological significance of the results was validated using several control conditions. First, we included wheat germ agglutinin (WGA) in our reaction. WGA is a lectin that binds

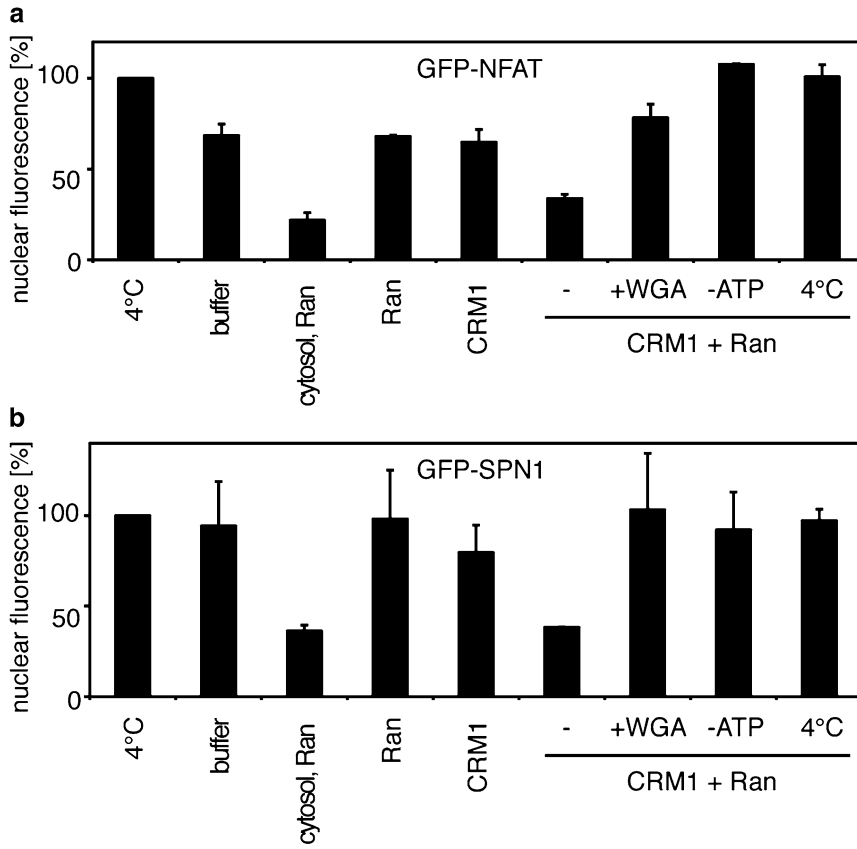

Fig. 3 CRM1 and Ran promote nuclear export of GFP-NFAT and GFP-SPN1. HeLa p4 cells were transfected with plasmids coding for GFP-NFAT or GFP-SPN1 and mCherry-emerin and subjected to nuclear export reactions in vitro. Nuclear fluorescence of GFP-NFAT (**a**) and GFP-SPN1 (**b**) was analyzed by flow cytometry. Total populations were gated for mCherry-emerin-positive cells and the median fluorescence intensities of samples incubated at 4 °C were set as 100 %. Reactions contained 0.7 mg/mL cytosol, 2 μM Ran, 300 nM CRM1, and/or 150 μg/mL WGA, as indicated. The average and standard deviation of two independent experiments are shown

to various *O*-glycosylated nucleoporins [25] and is known to inhibit the majority of nuclear transport pathways [26, 27]. Here, it clearly inhibited CRM1-dependent export of GFP-NFAT and GFP-SPN1. Next, reactions were performed in the absence of an ATP-regenerating system (–ATP) or at 4 °C. Under both conditions, the cells retained a high level of fluorescence. Taken together, these controls validate our in vitro assay as a transport system that faithfully reconstitutes nuclear export in transiently transfected, permeabilized cells.

4 Notes

1. For GFP-NFAT, ionomycin alone is sufficient to induce nuclear import. If LMB is used, cells must be washed thoroughly to prevent the drug from modifying CRM1 that is added to the reaction.
2. In our experiments, we did not observe an obvious influence of mCherry-emerin on CRM1-dependent export. New combinations of cargoes and transfection markers should be analyzed by fluorescence microscopy to verify efficient nuclear import of the cargo and unaltered localization of the marker upon LMB treatment as well as their unchanged localization upon permeabilization of the plasma membrane by digitonin.
3. Depending on the size of the CRM1-cargo, the protein might leak out of the nucleus in digitonin-permeabilized cells. This is not a problem for GFP-NFAT, which is >100 kDa in size. Smaller proteins can be fused to larger tags (e.g., GST or double-GFP) to minimize passive diffusion.
4. We typically use HeLa p4, because the transfection rate in these cells is very high.
5. EGTA inhibits the cellular phosphatase calcineurin, which dephosphorylates NFAT, resulting in nuclear import of the protein [12]. Thus, re-import of the reporter protein, which would complicate the analysis, is prevented under our conditions.
6. Use sodium-ATP. Lithium-ATP may interfere with export of GFP-NFAT [10].
7. This double-stranded oligonucleotide mimics the DNA-binding site of NFAT. It can stimulate export of GFP-NFAT about twofold, probably by promoting the release of GFP-NFAT from chromatin.
8. Check permeabilization with trypan blue, which stains the nuclei. As an alternative to dounce homogenization, the plasma membrane can be permeabilized with digitonin. The nuclear membrane remains intact, because of its lower level of cholesterol, as compared to the plasma membrane. Add 0.5–1 μL of a 10 % solution of digitonin in DMSO for 10^7 cells.
9. Transfection conditions are chosen such that essentially all mCherry-positive cells are also positive for our GFP-export cargo. In the final analysis by flow cytometry, this allows gating on mCherry-positive cells and measuring the residual GFP-fluorescence, assuming that even GFP-negative cells did contain the export cargo at the beginning of the reaction. Depending on the efficiency of the transfection, we obtain 15–30 % of total cells that express both, the red and the green

fluorescent protein (*see* Fig. 1). The calcium phosphate method should not be used for proteins like NFAT, whose intracellular localization is controlled by the cellular calcium level.

10. It is also possible to simultaneously monitor nuclear import of fluorescently labeled import substrates (e.g., Cy5-BSA-NLS; [10]). Furthermore, one may use propidium iodide to determine the DNA content of the cells. This can be done for nuclear import or export, allowing analysis of nuclear transport with respect to the cell cycle [28].

References

1. Fischer U, Huber J, Boelens WC, Mattaj IW, Lührmann R (1995) The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell 82:475–483
2. Wen W, Meinkoth JL, Tsien RY, Taylor SS (1995) Identification of a signal for rapid export of proteins from the nucleus. Cell 82:463–473
3. Görlich D, Kutay U (1999) Transport between the cell nucleus and the cytoplasm. Annu Rev Cell Dev Biol 15:607–660
4. Hutten S, Kehlenbach RH (2007) CRM1-mediated nuclear export: to the pore and beyond. Trends Cell Biol 17:193–201
5. Pemberton LF, Paschal BM (2005) Mechanisms of receptor-mediated nuclear import and nuclear export. Traffic 6:187–198
6. Turner JG, Dawson J, Cubitt CL, Baz R, Sullivan DM (2014) Inhibition of CRM1-dependent nuclear export sensitizes malignant cells to cytotoxic and targeted agents. Semin Cancer Biol 27:62–73
7. Santiago A, Li D, Zhao LY, Godsey A, Liao D (2013) p53 SUMOylation promotes its nuclear export by facilitating its release from the nuclear export receptor CRM1. Mol Biol Cell 24:2739–2752
8. Ishida N, Hara T, Kamura T, Yoshida M, Nakayama K, Nakayama KI (2002) Phosphorylation of p27Kip1 on serine 10 is required for its binding to CRM1 and nuclear export. J Biol Chem 277:14355–14358
9. Adam SA, Marr RS, Gerace L (1990) Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J Cell Biol 111:807–816
10. Kehlenbach RH, Dickmanns A, Gerace L (1998) Nucleocytoplasmic shuttling factors including Ran and CRM1 mediate nuclear export of NFAT in vitro. J Cell Biol 141:863–874
11. Flanagan WM, Corthesy B, Bram RJ, Crabtree GR (1991) Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature 352:803–807
12. Shibasaki F, Price ER, Milan D, McKeon F (1996) Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. Nature 382:370–373
13. Paschal BM, Gerace L (1995) Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. J Cell Biol 129:925–937
14. Kehlenbach RH, Assheuer R, Kehlenbach A, Becker J, Gerace L (2001) Stimulation of nuclear export and inhibition of nuclear import by a Ran mutant deficient in binding to Ran-binding protein 1. J Biol Chem 276:14524–14531
15. Kehlenbach RH, Dickmanns A, Kehlenbach A, Guan T, Gerace L (1999) A role for RanBP1 in the release of CRM1 from the nuclear pore complex in a terminal step of nuclear export. J Cell Biol 145:645–657
16. Hutten S, Flotho A, Melchior F, Kehlenbach RH (2008) The Nup358-RanGAP complex is required for efficient importin alpha/beta-dependent nuclear import. Mol Biol Cell 19:2300–2310
17. Roloff S, Spillner C, Kehlenbach RH (2013) Several phenylalanine-glycine motives in the nucleoporin Nup214 are essential for binding of the nuclear export receptor CRM1. J Biol Chem 288:3952–3963
18. Kehlenbach RH, Gerace L (2002) Analysis of nuclear protein import and export in vitro using fluorescent cargoes. Methods Mol Biol 189:231–245
19. Strasser A, Dickmanns A, Schmidt U, Penka E, Urlaub H, Sekine M, Lührmann R, Ficner R (2004) Purification, crystallization and preliminary crystallographic data of the m3G cap-

binding domain of human snRNP import factor snurportin 1. Acta Crystallogr D Biol Crystallogr 60:1628–1631

20. Waldmann I, Spillner C, Kehlenbach RH (2012) The nucleoporin-like protein NLP1 (hCG1) promotes CRM1-dependent nuclear protein export. J Cell Sci 125:144–154
21. Charneau P, Mirambeau G, Roux P, Paulous S, Buc H, Clavel F (1994) HIV-1 reverse transcription. A termination step at the center of the genome. J Mol Biol 241:651–662
22. Melchior F, Sweet DJ, Gerace L (1995) Analysis of Ran/TC4 function in nuclear protein import. Methods Enzymol 257:279–291
23. Guan T, Kehlenbach RH, Schirmer EC, Kehlenbach A, Fan F, Clurman BE, Arnheim N, Gerace L (2000) Nup50, a nucleoplasmically oriented nucleoporin with a role in nuclear protein export. Mol Cell Biol 20:5619–5630
24. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1994) Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York
25. Hanover JA, Cohen CK, Willingham MC, Park MK (1987) O-linked N-acetylglucosamine is attached to proteins of the nuclear pore. Evidence for cytoplasmic and nucleoplasmic glycoproteins. J Biol Chem 262:9887–9894
26. Dargemont C, Kuhn LC (1992) Export of mRNA from microinjected nuclei of Xenopus laevis oocytes. J Cell Biol 118:1–9
27. Yoneda Y, Imamoto-Sonobe N, Yamaizumi M, Uchida T (1987) Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. Exp Cell Res 173:586–595
28. Kehlenbach RH, Gerace L (2000) Phosphorylation of the nuclear transport machinery down-regulates nuclear protein import in vitro. J Biol Chem 275: 17848–17856