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High-Content Classification of Nucleocytoplasmic Import or Export Inhibitors

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Transcription factors of the nuclear factor κ B family are the paradigm for signaling dependent nuclear translocation and are ideally suited to analysis through image-based chemical genetic screening. The authors describe combining high-content image analysis with a compound screen to identify compounds affecting either nuclear import or export. Validation in silico and in vitro determined an EC_{50} for the nuclear export blocker leptomycin B of 2.4 ng/mL (4.4 nM). The method demonstrated high selectivity ($Z' > 0.95$), speed, and robustness in a screen of a compound collection. It identified the IκB protein kinase inhibitor BAY 11 7082 as an import inhibitor, the p38 mitogen-activated protein (MAP) kinase inhibitor PD98509 as an import enhancer, and phorbol ester as an export inhibitor. The results establish a robust method for identifying compounds regulating nucleocytoplasmic import or export and also implicate MAP kinases in nuclear import of nuclear factor κ B. (*Journal of Biomolecular Screening* 2007:621-627)

Key words: NF-κB, nucleocytoplasmic transport, signal transduction, second messenger-activated kinases, high-content screening, automation, image recognition

INTRODUCTION

DEFECTS IN THE NUCLEOCYTOPLASMIC TRANSPORT of transcription factors are implicated in diseases ranging from cancer to inflammatory illnesses, such as asthma. Chemical genetic phenotypic screens offer insight into the mechanisms underlying these conditions when the disease phenotype can be examined and manipulated at a cellular level through the screening of collections of small-molecule modulators.

In the case of asthma, signal transduction plays a role in the recruitment and activation of inflammatory cells in the asthmatic airway. A large number of transcription factors, such as the signal transducers and activators of transcription, activator protein 1, nuclear factor of activated T cells, cyclic adenosine monophosphate response-element binding proteins, guanine-adenine and thymine-adenine repeats, Ets family proteins, and nuclear factor κ B (NF-κB) proteins, have been involved in the physiopathology of asthma.[1-3]

The NF-κB pathway is directly implicated in the pathogenesis of cancer, diabetes, and systemic inflammatory response syndrome. Cytoplasmic to nuclear translocation is a key step in NF-κB regulation. In unstimulated cells, NF-κB is retained in the cytoplasm through masking of the nuclear localization signals on NF-κB dimers by inhibitory proteins known as IκBs.[4,5] Upon exposure of cells to an NF-κB–activating stimuli, the IκB protein kinase (IKK) complex phosphorylates IκBs on 2 conserved N-terminal serine residues. Phosphorylated IκB is then ubiquitinated and degraded, thus releasing NF-κB, which is recognized by the nuclear import machinery and quickly shuttled into the nucleus to regulate NF-κB–dependent gene expression.

Cis-acting elements of IκBs govern its protein stability and subcellular localization.[6,7] IκBα comprises 3 domains: an N-terminal regulatory domain that controls signal-dependent degradation, a central ankyrin repeat domain that is necessary for NF-κB binding, and a C-terminal region rich in proline, glutamate/aspartate, serine, and threonine regulating basal turnover. An additional sequence, a leucine-rich nuclear export sequence (NES) within the last ankyrin repeat, is postulated to function during the termination of NF-κB activity.[8] Activated NF-κB stimulates the synthesis of IκBα mRNA,[9,10] and newly synthesized IκB proteins can enter the nucleus to bind to and remove NF-κB from gene promoters.[11] It is believed that the C-terminal NES of IκBα can actively export these IκBα NF-κB complexes out to the cytoplasm to restore the preinduction repression.[8]

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The leucine-rich NES is a highly conserved sequence used by a variety of proteins to facilitate their delivery from the nucleus to the cytoplasm and is important in regulating protein function through subcellular localization.[12] Nuclear export of proteins, such as HIV Rev,[13,14] cyclin B1,[14] and protein kinase A inhibitor,[15] can be inhibited by leptomycin B, a *Streptomyces* metabolite.[16] Several groups have reported that CRM1 (exportin 1), related to the β-importin family of nuclear proteins, is the receptor for the leucine-rich NES and that leptomycin B interferes with the interaction between CRM1 and NES by directly binding to CRM1.[17-21] Nuclear import and export of NF-κB are mediated through interactions with the importin and exportin proteins.[22]

High-content screens are growing in importance as a research and drug discovery tool, as the cell-based models and the technology to image these assays—automated image acquisition and measurement—proliferate.[23,24] High-content screens exploit image analysis algorithms to extract measurement of protein localization and concentration from image data. Our interest was to develop a highly robust algorithm for measuring nucleocytoplasmic transport independent of the requirement for image segmentation or cell recognition as part of a screen to discern nuclear import inhibitors from export inhibitors. This method has application in the identification of small-molecule modulators of transcription factor biology.

MATERIALS AND METHODS

Chemicals

All fine chemicals were purchased from Sigma-Aldrich (St. Louis, MO). DRAQ5 was from BioStatus (Shepshed, UK). Kinase and phosphatase inhibitors were purchased as 95% to 99% pure 10-mM stock solutions in dimethylsulfoxide or water (Biomol, Hamburg, Germany). Stock solutions and formatted assay plates were stored at –20 °C. Primary rabbit antibodies against NF-κB were from Santa Cruz Biotechnology (Santa Cruz, CA), secondary goat anti-rabbit Alexa 488 antibodies were purchased from Molecular Probes (Eugene, OR), and goat serum was from Gibco-BRL (Carlsbad, CA).

Cell lines and cell culture

HeLa cells (ATCC, Manassas, VA) were cultivated in high-glucose glutamax Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 110 mg/mL sodium pyruvate, 10% fetal calf serum (Gibco), and 1% penicillin streptomycin (Invitrogen). HEK 293 cells were cultivated in Dulbecco's Modified Eagle Medium/F12 (Invitrogen) supplemented with 10% fetal calf serum and 1% penicillin streptomycin. HeLa cells in 96-well plates (seeding density of 15,000 cells/well) were incubated for 16 h.

NF-κB immunofluorescent detection

Cells were washed twice with phosphate-buffered saline (PBS), fixed for 10 min with 4% (w/v) paraformaldehyde in PBS, and then washed with PBS. Permeabilization was performed with 0.1% TX-100 PBS for 10 min, and cells were washed in PBS and then incubated with a 1:200 dilution of rabbit anti–NF-κB in 10% goat serum–PBS overnight at 4 °C. Plates were washed 3 times with PBS for 10 min on an orbital rotator. Alexa 488 goat antirabbit secondary antibody (1:1000) was incubated with the cells for 60 min at room temperature, and cells were washed 3 times for 10 min with PBS on an orbital shaker before the addition of 5 μM of DRAQ5 in PBS for 10 min at 37 °C.

Cell imaging

The NF-κB nucleocytoplasmic transport was imaged using the Opera ultra-high-throughput confocal screening system (Evotec Technologies, Hamburg, Germany). The Opera is a fully automated, 4-color laser excitation confocal system (405, 488, 532, 637 nm) based on an inverted microscope architecture to image cells cultivated in 96- or 384-well coverslip-bottomed microplates (Greiner, Monroe, NC). Images were acquired with 0.7 NA 20 × water immersion or 40 × 0.7 NA water immersion lenses (Olympus Instruments, Tokyo, Japan) at room temperature with confocality generated by a nipkow disc system and image acquisition via 3 parallel 16-bit CCD cameras. Images were corrected for optical vignetting using the Opera acquisition software and a standard reference set of 1 to 10 μm multicolor fluorescent beads. Images were exported as 16 bit. TIFF files were scaled before export to Adobe Photoshop.

Cell-based screening

Cells were treated with 0.1 to 20 ng/mL leptomycin B in culture medium for 40 min to arrest nuclear export.

Compounds in DMSO or H_2O were diluted into culture medium just prior to screening at 10 μM for all compounds except bafilomycin (1 μM) in culture medium with or without 5 ng/mL leptomycin B before transfer to cells. Cells were fixed as above to arrest the assay. Plates were imaged by Opera using 488/637 nm excitation and 510 nm (50 nm bandpass) or 680nm (50 nm bandpass) filters, respectively. Typically, 3 image pairs were acquired per well.

RESULTS

Our rationale was to establish a high-content screen capable of identifying compounds affecting either nuclear import or export of transcription factors, such as NF-κB. High-throughput

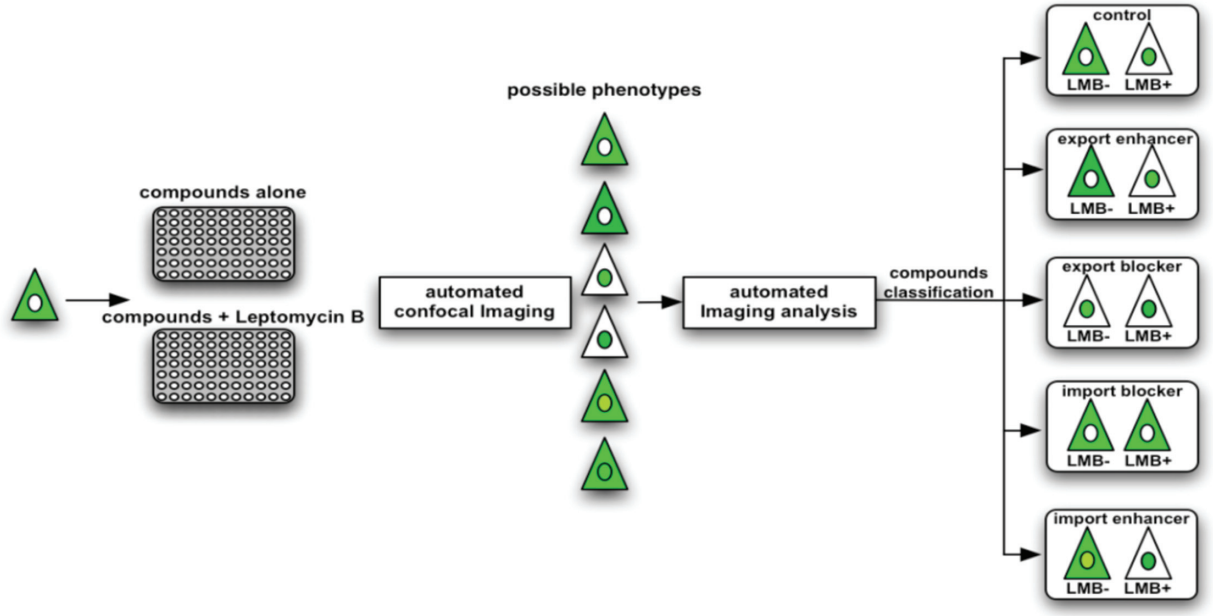

FIG. 1. Scheme of the symmetrical nucleocytoplasmic screen for import and export blockers. Nuclear factor κ B localization was measured in cells after compound treatment alone, which will identify export blockers, and after compound treatment when nuclear export is blocked by leptomycin B to identify nuclear import blockers.

immunofluorescent detection of NF-κB transport was quantified with a novel image analysis algorithm to screen a collection of kinase and phosphatase inhibitors. A symmetrical high-content imaging screen scheme was designed to discriminate between compounds affecting nuclear export or import of NF-κB (**Fig. 1**). In this scheme, we 1st screened nuclear localization of NF-κB after compound treatment and performed a 2nd screen in which the nuclear export blocker leptomycin B[19] (5 ng/mL) was added together with the compounds in parallel. Although nuclear export inhibitors can be easily identified in the 1st compound screen, cells in which nuclear import is arrested cannot be distinguished from mock-treated cells because the phenotype is identical. This would apply regardless of the normal distribution of the transcription factor between the nucleus and the cytoplasm. When the screen is performed in the presence of leptomycin B as a nuclear export blocker, nuclear import inhibitors or those promoting import can be discriminated because the control phenotype is blocked nuclear export.

Cell-based assays of nucleocytoplasmic transport

NF-κB nucleocytoplasmic transport requires nuclear export via CRM1 to maintain its distribution, and NF-κB accumulates in the nucleus after stimulation of the tumor necrosis factor receptor or treatment of cells with leptomycin B.[25] Leptomycin B covalently modifies CRM1 at cysteine 528 and blocks its interaction with nuclear export signals, thus inhibiting NF-κB nuclear export.[19,26] Cells treated with 0.1 to 20 ng/mL leptomycin B for 40 min showed NF-κB accumulation in the nucleus via indirect immunofluorescent detection of NF-κB (**Fig. 2A**). Nuclei were stained with the DNA stain DraQ5, which gave well-demarcated nuclei with no detectable staining outside the extranuclear staining (**Fig. 2A**).

Image analysis of nucleocytoplasmic transport

NF-κB nucleocytoplasmic transport is spatially well defined within the rims of the nuclear envelope, but there are limitations on methods available for analyzing nucleocytoplasmic transport within the constraints of high-throughput biology in terms of speed, reliability, cost, academic availability, and robustness. We devised a stable and highly robust algorithm that gave a measure of the level of the NF-κB label within the nucleus, as defined by co-localization with the nuclear staining. The algorithm relied on the assumptions that 1) the fluorescence intensity of the nucleus (stained with DRAQ5) and the

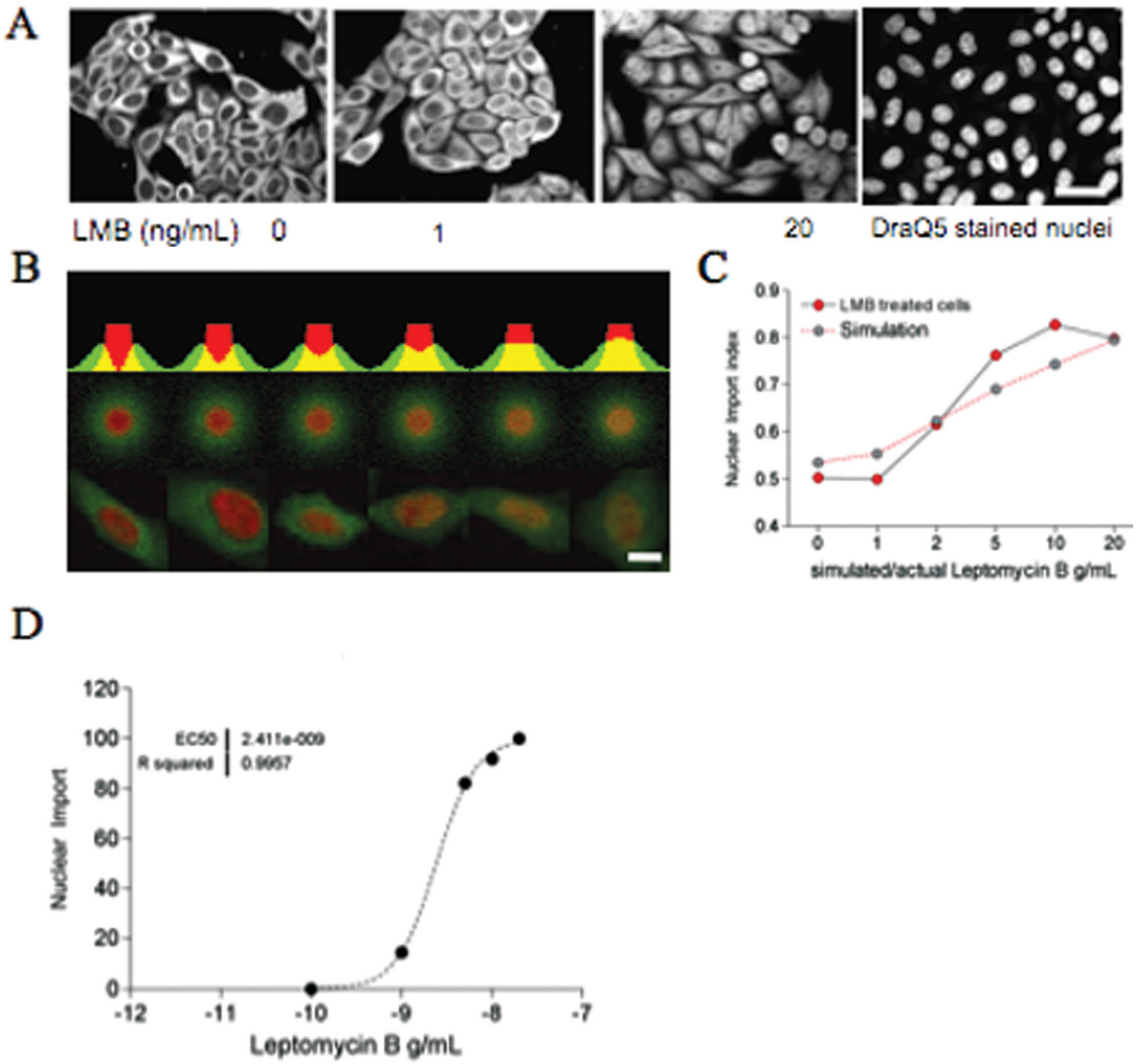

FIG. 2. Quantitation of nuclear factor κ B (NF-κB) nucleocytoplasmic transport high-throughput immunofluorescence assay. (**A**) Cytoplasmic NF-κB accumulates in the nucleus of HeLa cells treated with leptomycin B (LMB) for 40 min prior to immunofluorescent detection of NF-κB and nuclear staining with 10 μM DRAQ5. Scale bar = 20 μm. (**B**) Simulated nuclear localization images, with red representing the nucleus and green NF-κB localization where a cytoplasmic distribution of NF-κB is morphed onto the nucleus in an XZ (upper row) and XY (center row) simulated image series from left to right. Images of cells after LMB treatment and nuclear staining. Scale bar = 5 μM. (**C**) Quantitation of nuclear import on the simulation (upper row) and cell images (lower row). (**D**) EC_{50} determination for LMB in terms of nuclear localization of NF-κB after incubation with 0 to 20 ng/mL LMB, detection of NF-κB, and nuclear staining in microplates. The fitted EC_{50} for LMB was 2.4 ng/mL, within a 95% confidence interval of 1.9 to 3 ng/mL with an R^2 of 0.9957, and is representative of more than 5 assays. All images were acquired on the automated confocal system.

expression level of NF-κB are independent of leptomycin B concentration and 2) that as NF-κB enters the nucleus, the co-localization of the NF-κB signal with nuclear staining increases. Based on these 2 assumptions, we developed a quantitative measure of NF-κB localization in the nucleus.

In a typical experiment, three 2-color images were independently analyzed per condition and used to compute the standard deviation. A gray-scale image was defined as the function G: $\Omega \subset N2 \rightarrow \square$. The function G represents the NF-κB signal and R the nuclear stain. In terms of image processing, each image

is normalized as follows: The mean of pixel intensity is computed by $G = \frac{1}{n} \sum_{(x, y) \in \Omega} G(x, y)$ and the standard deviation by $\sigma_G = \frac{1}{n} \sum_{(x, y) \in \Omega} (G(x, y) - G)^2$, and the pixels are normalized by $G_{\sigma(x, y)} = \frac{G(x, y) - G}{\sigma_G}$.

After similar normalization of the nuclear intensity R, a correlation value between R and G is computed via $Corr(R, G) = \sum_{(x, y) \in \Omega} R_\sigma(x, y) G_\sigma(x, y)$. Thus, the degree of co-localization between NF-κB labeling and the nuclear stain is quantified, irrespective of the geometry and the number of cells.

Validation of the image analysis algorithm

A simulation of NF-κB entry into the nucleus was used to validate the image analysis algorithm. NF-κB distribution in the cell was represented by a subtraction of 2 evolving Gaussian shapes, and the nuclear stain was represented by a nonevolving thresholded Gaussian (**Fig. 2B**). This permitted the volume of the shape(s) to remain constant and thus respected assumption 1 that leptomycin B treatment would not alter intensities of NF-κB or the nucleus. The simulation is depicted in a vertical and XY image in **Figure 2B** and **C**. The green intensity was linearly evolved to fill the center and thus tended to increase the number of pixels that had a high value of G and R, satisfying assumption 2.

Figure 2B shows the evolution between the 2 situations: where the NF-κB simulation (green) shape was entirely absent from the nucleus and where it morphed linearly from an NF-κB–free empty nucleus to a filled nucleus. The identical simulation is shown in **Figure 2B** but in 2 dimensions and with additive white Gaussian noise added to mimic the predicted real experimental situation. These simulations were conceived to represent the repression of nuclear export of NF-κB through an increasing exposure of cells to leptomycin B. The measurement algorithm extracted a linearly increasing coefficient for nuclear entry, as was expected (**Fig. 2C**). It was then applied to images of HeLa cells that were treated with increasing concentrations of leptomycin B for 40 min before fixation, indirect immunofluorescent detection of the NF-κB distribution, and nuclear staining with DRAQ5 (**Fig. 2A**). The nuclear localization coefficient across a logarithmic gradient of leptomycin B was fitted to a variable slope model and gave an EC_{50} for leptomycin B of 2.4 ng/mL (4.4 nM), within the 95% confidence limits of 1 ng/mL and 3 ng/mL.

Nucleocytoplasmic import/export screen

A symmetrical screen was devised as a suitable method to allow the discrimination between compounds affecting nuclear import or nuclear export (**Fig. 3A**). It comprised 2 parallel screens: In the 1st screen, compounds were screened for their effects on endogenous NF-κB localization in wild-type cells, allowing the identification of compounds that caused nuclear localization of NF-κB through an inhibition of export. In the 2nd screen, cells were treated with compounds and leptomycin B, and in this case, inhibitors of nuclear import could be distinguished from compounds that had a null phenotype in the 1st screen (**Fig. 3A**).

A collection of kinase and phosphatase inhibitors covering most of the kinase families in the genome were used in this screen, and the collection also contained leptomycin B as an internal control. Leptomycin B was identified as a pure export blocker in the screens, and the collected screen data are presented in **Figure 3A**. Molecules acting on nuclear import or export were identified using cluster analyses of the effect of a compound on both screens, shown in the XY plot in **Figure 3A**. This resolved an import blocker, export blocker, and import enhancer compound well outside the distribution of the control.

Three compounds were defined as disrupting the nucleocytoplasmic transport of NF-κB: BAY 11 7082, phorbol ester (phorbol 12 myristate 13 acetate), and PD98059. BAY 11 7082 is an IκB-α kinase inhibitor and gave the phenotype of blocking the nuclear import of NF-κB (**Fig. 3B**). In contrast, the effect of PD 98059 was to enhance nuclear import and was demonstrated only in cells treated with leptomycin B to block NF-κB export (**Fig. 3**), whereas PD 98059 increased the NF-κB nuclear localization coefficient 1.15-fold (**Fig. 3B**) over the highest concentration of leptomycin B. We determined the concentration dependence (AC50) of the selected compounds on nucleocytoplasmic transport. BAY 11 7082 had an AC50 of 5 μM for the inhibition of nuclear import when measured in the presence of leptomycin B. In contrast, phorbol ester promoted nuclear localization of NF-κB 1.3-fold at 10 nM irrespective of the addition of leptomycin B, and it is classified as a nuclear export inhibitor. The AC50 for PD98059 was 1 μM, with a strong nuclear localization phenotype observed when NF-κB export was blocked, implying that its target (p38 mitogen-activated protein [MAP] kinase) may regulate the rate of nuclear import (**Fig. 3B**).

DISCUSSION

A method is presented and validated for the identification of small-molecule disruptors of nuclear import or export. This method may have applications in the chemical genomic identification of molecules involved in nucleocytoplasmic transport and their evaluation as therapeutic drugs.

Chemical biology—the application of high-throughput and high-content methods for identifying small molecules that are suitable to assign function to the genome and its protein complement—is emerging as a tool for both cell biology and drug discovery.[23,24] In this context, image-based, high-content methods are gaining interest for their combination of image-based analyses, automation, and compound library screening, and they provide a means

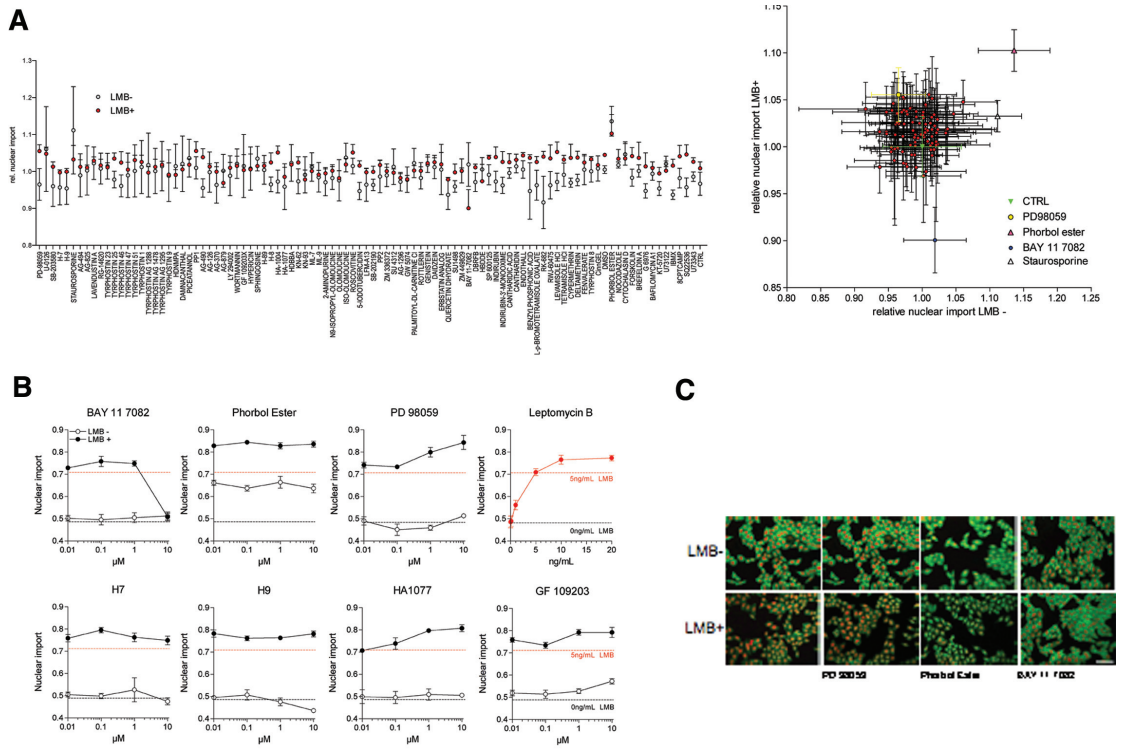

FIG. 3. High-content symmetrical screening of a compound collection reveals nucleocytoplasmic import and export blockers. Cells were treated with either a compound collection alone or with 5 ng/mL leptomycin B (LMB) to block nuclear export. (**A**) Compound effects were scored relative to the untreated control (left panel, •) or, in the case of cells screened with compounds and 5 ng/mL LMB, relative to the leptomycin-treated cells (left panel, •). The normalized values were plotted against one another and then clustered using the XYZ algorithm. (**B**) Dose dependence/AC50 titrations for BAY11 7082, PD98059, and phorbol ester in the NF-κB transport assay. (**C**) Effects of cell treatment with BAY11 7082, PD98059, and phorbol ester on NF-κB nucleocytoplasmic transport in controls or cells treated with LMB to block nuclear export, using indirect immunofluorescent detection of NF-κB (green) and nuclear staining (red). Scale bar = 20 µm.

to exploit cell biological methods to modulate protein function and ultimately manipulate disease states. In contrast to the in vitro methods used in high-throughput drug discovery, high-content screening seems ideally suited to evaluate the effect of compounds on spatially resolved events that are related to disease at a cellular level. Nucleocytoplasmic transport of transcription factors was readily analyzed and successfully screened using the method established here.

The method relies on a robust, well-characterized mathematical principle for image analysis of high-throughput immunofluorescence images coupled to a symmetrical cell-based screening rationale that gave significantly more information on nucleocytoplasmic transport than a single screen. In terms of speed and robustness, the algorithm presented here analyzed 180 images in 60 s with a high Z′ factor. This speed is based on several factors. First, we favored a nonsegmentation approach because of the perceived advantages in accuracy when a generally error-prone segmentation was avoided. Each step of an image analysis algorithm introduces bias that can subsequently reduce the accuracy of the assay. Geometric segmentation methods require robust algorithms to detect cells and subcellular compartments that require approximations of cell shape.[27] The method used here avoids these limitations because it applies a strict measure of the co-localization of NF-κB with the nucleus based on a pixel-by-pixel—nongeometric—co-localization.

The screening method was successfully used for the identification of inhibitors of nuclear import or export. Three compounds were defined as disrupting the nucleocytoplasmic transport of NF-κB: BAY 11 7082, phorbol ester (phorbol 12 myristate 13 acetate), and PD98059. BAY 11 7082 is an IκB kinase inhibitor and gave the phenotype of blocking the nuclear import of NF-κB (**Fig. 3B**), consistent with the known inhibitory effect on I-κBα phosphorylation and NF-κB activation.[28] Phorbol ester–induced activation of NF-κB has been reported to act via activating the IKK kinase,[29] which supports the nuclear accumulation observed here and can be interpreted as increased nuclear import of NF-κB.

In contrast, the effect of PD 98059 was enhanced nuclear import in cells treated with leptomycin B to block NF-κB export (**Fig. 3**) and an increase of the NF-κB nuclear localization coefficient 1.15-fold, a greater effect than seen at the highest concentration of leptomycin B (**Fig. 3B**). PD 98059 is a cell-permeable inhibitor of MAP kinase that blocks phosphorylation and subsequent activation of p38 MAP kinase. PD 98059 does not directly inhibit phosphorylation of the NF-κB p65 subunit.[30] The observed increase in nuclear import occurs when nuclear export is blocked

through the covalent inactivation of CRM1 with leptomycin B, indicating that a PD 98059–sensitive kinase may regulate the rate of nuclear import.

Thus, we demonstrate that cell-based high-content screening of nucleocytoplasmic transport successfully identifies small molecules affecting import or export and will therefore be of use as a robust method to discover molecules that will identify new players in this transport pathway (i.e., novel inhibitors) and as a tool in transcription factor drug discovery. This adds to work on cell-based screening of nucleocytoplasmic transport, in which the advance is that compounds affecting import or export can be readily discriminated.

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