Video Article

Adenofection: A Method for Studying the Role of Molecular Chaperones in Cellular Morphodynamics by Depletion-Rescue Experiments

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URL: <http://www.jove.com/video/54557>

DOI: [doi:10.3791/54557](https://doi.org/10.3791/54557)

Keywords: Molecular Biology, Issue 115, Adenovirus, cell transduction, RNA interference, cell transfection, live-cell imaging, cytoskeletal dynamics, LifeAct-GFP, tubulin-RFP, mitosis, muscle cell differentiation, HeLa cells, C2C12 cells

Date Published: 9/16/2016

Citation: Fuchs, M., Boulanger, M.C., Lambert, H., Landry, J., Lavoie, J.N. Adenofection: A Method for Studying the Role of Molecular Chaperones in Cellular Morphodynamics by Depletion-Rescue Experiments. *J. Vis. Exp.* (115), e54557, doi:10.3791/54557 (2016).

Abstract

Cellular processes such as mitosis and cell differentiation are governed by changes in cell shape that largely rely on proper remodeling of the cell cytoskeletal structures. This involves the assembly-disassembly of higher-order macromolecular structures at a given time and location, a process that is particularly sensitive to perturbations caused by overexpression of proteins. Methods that can preserve protein homeostasis and maintain near-to-normal cellular morphology are highly desirable to determine the functional contribution of a protein of interest in a wide range of cellular processes. Transient depletion-rescue experiments based on RNA interference are powerful approaches to analyze protein functions and structural requirements. However, reintroduction of the target protein with minimum deviation from its physiological level is a real challenge. Here we describe a method termed adenofection that was developed to study the role of molecular chaperones and partners in the normal operation of dividing cells and the relationship with actin remodeling. HeLa cells were depleted of BAG3 with siRNA duplexes targeting the 3'UTR region. GFP-tagged BAG3 proteins were reintroduced simultaneously into >75% of the cells using recombinant adenoviruses coupled to transfection reagents. Adenofection enabled to express BAG3-GFP proteins at near physiological levels in HeLa cells depleted of BAG3, in the absence of a stress response. No effect was observed on the levels of endogenous Heat Shock Protein chaperones, the main stress-inducible regulators of protein homeostasis. Furthermore, by adding baculoviruses driving the expression of fluorescent markers at the time of cell transduction-transfection, we could dissect mitotic cell dynamics by time-lapse microscopic analyses with minimum perturbation of normal mitotic progression. Adenofection is applicable also to hard-to-infect mouse cells, and suitable for functional analyses of myoblast differentiation into myotubes. Thus adenofection provides a versatile method to perform structure-function analyses of proteins involved in sensitive biological processes that rely on higher-order cytoskeletal dynamics.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54557/>

Introduction

Functional inactivation of gene expression in mammalian cells is the gold standard to dissect protein functions. Newly developed technologies of genome editing based on the use of site-specific nucleases such as Zinc-finger nucleases and clustered regularly interspaced short palindromic repeats (CRISPR)/CAS9 now allow the generation of cell lines with targeted gene deletion and mutation^{1,2}. These novel approaches should revolutionize the way we are studying protein function and our understanding of the genetics of human diseases. In some instances, however, long-term or complete gene knockout is not desirable and may provoke secondary cell compensation mechanisms. The generation of genetically modified cell lines can also be limiting when dealing with primary cell cultures with limited proliferation capacity, or when screening of a large set of mutations in various cell types is sought. This is often required for determining the dependence of a cell biological process on structural requirements of a protein. To that end, reversible knockdown by RNA interference that enables transient depletion-rescue experiments in various cellular backgrounds still remains a simple and powerful approach to perform structure-function analyses of a protein of interest³. However, a major drawback to this approach is the difficulty to achieve efficient silencing and to reintroduce the protein of interest or its variants at near physiological levels in a majority of the cell population. This is crucial to enable comprehensive studies that attempt to correlate functional effects seen at the level of single cells (hypomorphic phenotype) with those seen in cell population-based assays, for instance on protein-protein interactions.

Using classic transfection methods, one can hardly achieve homogenous and low expression of exogenous proteins in a large population of cells. Transduction of cells with recombinant viruses like adenoviruses often enables more normalized expression of exogenous proteins. Yet, adenovirus uptake is limited by the CAR receptor, which is absent in non-human cells or only weakly expressed in some human cell types. Furthermore, the cellular entry of adenoviruses activates signaling pathways that regulate cell shape and adhesion⁴⁻⁶. This is obviously not

desirable when studying regulatory mechanisms of cell morphodynamics. We were facing this problematic when we undertook functional analyses of a chaperone complex, BAG3-HSPB8, in cell division and actin dynamics. Pioneering work had described a role for this chaperone complex in protein quality control and autophagy during stress^{7,8}. Most of these studies, however, relied on protein overexpression, assuming that the chaperones are normally upregulated during stress. This has left open the question of whether BAG3, in complex with HSPB8, can contribute to the normal operation of dividing cells expressing these chaperones like many cancer cell types⁹. In particular, whether the chaperone complex contributes to the remodeling of actin-based structures that control mitotic progression was of great interest, given the emerging connections between HSPB chaperones and cytoskeletal dynamics¹⁰. To address this issue, we were seeking to develop an efficient method for depletion-rescue experiments that would not interfere with mitotic progression or cellular morphology, and which would preserve protein homeostasis to avoid secondary perturbation of the dynamics of macromolecular complexes regulating cell-shape changes. Thus ideally, depletion-add-back of the gene of interest should be performed simultaneously.

The use of complexes of adenovirus with a cationic polymer or lipids has been described to promote gene transfer *in vitro* and *in vivo*^{11,12}. For instance, calcium phosphate (CaPi) appears to form a precipitate with adenovirus that enhance virus binding-entry via a CAR-independent pathway¹³. Indeed, we found that combining adenovirus-based cell transduction and transfection with cationic compounds could enhance the efficiency of the depletion-rescue experiments. This allowed us to lower the amounts of virus by 3- to 20-fold, depending on the cell line and the gene of interest, and benefit from a wider window in order to adjust the expression of exogenous proteins at near endogenous levels in the majority of a cell population of interest with minimum impact on cellular morphology. Under such conditions, we could also achieve high efficiency knockdown of endogenous protein expression (>75%). We hereby describe the method step by step and provide evidence that protein homeostasis is not significantly perturbed as assessed by the unchanged levels of stress-induced chaperones of the Heat Shock Protein family, making the method suitable for functional analyses of the *physiological role* of molecular chaperones by time-lapse video microscopy. The protocol is amenable to cell synchronization procedures and to the use of commercially available baculoviruses for co-expression of low levels of fluorescent markers, with minimum interference with normal actin-based and spindle dynamics during mitotic progression. We further show the versatility of the method, which is applicable to "hard to transduce" mouse C2C12 cells, with no significant impact on myoblast differentiation into myotubes *in vitro*.

Protocol

1. Preparation of Medium and Solutions (all sterile filtered)

1. C2C12 mouse muscle cells (differentiation studies)

1. Prepare 500 ml of Growth Medium for C2C12 cell culture maintenance: DMEM High Glucose supplemented with 10 % FBS and 2mM L-Glutamine.
2. Prepare 100 ml Differentiation Medium (DM) for C2C12 differentiation: DMEM High Glucose supplemented with 2% Horse Serum.

2. HeLa cells (studies in mitotic cells)

1. Prepare 500 ml α MED for HeLa RFP-H2B cell culture maintenance and experiments: α MED supplemented with 10% FBS and 2 mM L-Glutamine (α MED^{10%}).
2. Prepare 500 ml α MED-minus (without deoxyribonucleosides/ribonucleosides) for HeLa RFP-H2B cell synchronization: α MED-minus supplemented with 10% FBS and 2 mM L-Glutamine (α MED-minus^{10%}).
3. Prepare 20 ml α MED without Phenol Red for HeLa-RFP-H2B live cell acquisition: α MED without Phenol Red supplemented with 10% FBS and 2 mM L-Glutamine.
4. Prepare 100 mM thymidine: dissolve 24.2 mg in 1 ml H₂O at 37 °C by vortexing. Filter sterilize and keep at 4 °C.

3. Common solutions

1. Prepare 0.05% Trypsin/EDTA: 0.05% Trypsin, 0.625 mM EDTA in 1x phosphate buffered saline (PBS). Filter sterilize and store aliquots at -20 °C. Once thawed, keep aliquot at 4 °C.
2. Prepare HBS2x: 280 mM NaCl, 50 mM HEPES, 1.5 mM Na₂HPO₄. Adjust pH exactly between 7.01-7.05 with 10 N NaOH. Filter sterile and keep at 4 °C.

2. Coating of Cell Culture Plates with Fibronectin and Plating of HeLa-RFP-H2B Cells

NOTE: Prior to the experiment, each manipulator should set up the optimal cell plating conditions to achieve a proper density of cells since variations may occur between each manipulator and each different cell line.

1. The day before the experiment, expand HeLa-RFP-H2B cells to make sure that they are within the exponential phase of growth on the day of plating. Make 2 successive 1/3 dilutions in 10 cm plates starting from an 80% confluent 1x10 cm plate.
NOTE: Plan the number of plates for the experiment. Calculate each condition as duplicates, one for short-term live-cell imaging and one for protein extracts to determine the efficacy of knockdown and exogenous protein expression by Western blot analysis. Plan one additional plate to determine the cell number prior to virus transduction.
2. Prior to cell plating, coat glass bottom dishes with 10 μ g/ml fibronectin. Add 1 ml of a 1:100 dilution of 1 mg/ml fibronectin (diluted in sterile 1x PBS) per 35 mm dish to well cover the whole surface and incubate for 1 hr at 37 °C, 5% CO₂.
3. During the incubation, pre-warm α MED supplemented with 10% FBS (α MED^{10%}) and 0.05% Trypsin/EDTA at 37 °C.
4. After 45 min of incubation, start preparing the cell solution. In the sterile hood, aspirate the medium from a 10 cm plate, rinse gently twice with 1.5 ml 0.05% Trypsin/EDTA, and leave 0.5 ml of Trypsin/EDTA at the last aspiration.
5. Incubate the cells for 2-3 min at 37 °C, 5% CO₂. By gently tapping the plate and observation under the microscope, verify that all cells have been detached. Add 10 ml of α MED^{10%} and pipette gently several times to separate the cells. Count a 10 μ l sample of the cell suspension using a haemocytometer.

6. Aspirate fibronectin solution from the glass bottom dishes. Do not allow fibronectin to dry out before cell plating.
7. Plate 1.5×10^5 cells per 35 mm dish in 2 ml α MEM^{10%} and incubate at 37 °C, 5% CO₂. Verify after 30-45 min under the microscope that the cells are well separated, since they have the tendency to accumulate in the center of the plate.
8. If necessary, agitate the plates gently cross-wise to redistribute the cells. Plate one 35 mm plate additional to the number of conditions in order to count the cell number prior to virus transduction.
9. Grow cells until the next day to achieve a cell density of at least 50%. A cell density lower than 50% results in a significant decrease of virus transduction efficiency, increased variations of protein expression per cell, and increased cell toxicity.
NOTE: Coating of glass dishes with fibronectin improves cell growth and morphology and promotes proper progression of cells through mitosis. It can generally be replaced by commercially available gelatin that is cheaper.

3. Adenovirus Transduction and Endogenous Protein Knockdown by siRNA Transfection in HeLa-RFP-H2B Cells Using CaPi Precipitates

Caution! Working with viruses requires special precautions and a proper disposal of all material that has been in contact with the virus.

Caution! In our hands, CaPi precipitates often have more undesirable effects, for instance on biological processes involving vesicle trafficking (e.g., autophagy). Accordingly it is recommended to use a cationic lipid transfection reagent (see below) and to wait at least 48 hr before the analyses.

NOTE: A control adenovirus carrying an unrelated gene (i.e., LacZ) or no gene is used to reach a minimal MOI in all Adenofections (10-20 pfu/cell) using the lowest amount of recombinant adenovirus carrying the gene of interest.

NOTE: This procedure has been shown to help normalizing expression per cell in a large cell population.

1. One day after cell plating, count the number of cells from the additional plated dish. Aspirate the medium and rinse once with 1 ml 0.05% Trypsin/EDTA. Add again 1 ml of 0.05% Trypsin/EDTA and incubate at 37 °C, 5% CO₂ until all cells have detached. Separate the cells well using a 1ml pipette and count the number of cells per ml using a haemocytometer.
2. Determine the quantity of virus needed to transduce cells at a multiplicity of infection (MOI) of 2 plaque-forming units (PFU) of the protein of interest (POI) per cell and 18 PFU per cell of an empty vector (e.g., LacZ) to have a total of 20 PFU per cell. At the same time transduce 4 PFU of each baculovirus (Actin and α Tubulin, GFP and RFP-tagged respectively). Transduce the cells using the following adenofection protocol. Viruses were used as described in Fuchs *et al.*¹⁴
3. Prepare in a sterile hood a 1.5 ml plastic tube for each condition and add 400 μ l of warm α MEM-minus^{10%}.
4. Thaw an aliquot of the viruses slowly on ice and, if necessary, dilute the virus stock in order to pipet a volume greater than 1 μ l to minimize pipetting errors.
5. Add the calculated virus quantity per condition to each 1.5 ml plastic tube containing 400 μ l α MEM-minus^{10%} and mix gently by pipetting. Place the virus stock immediately back at -80 °C to keep its activity.
6. Aspirate the medium from the cells and gently pipette the virus mix drop-wise. Incubate the cells at 37 °C, 5% CO₂ and agitate the plates carefully under the sterile hood each 15 min for 1 hr to well cover the cells with the virus. Using a small amount of medium facilitates the contact of the virus with the cells.
7. After incubation, gently pipette 1.6 ml α MEM-minus^{10%} to each plate to obtain a total volume of 2 ml, start cell synchronization by adding 2 mM thymidine and incubate the cells for a further 2 hr at 37 °C, 5% CO₂.
8. Meanwhile, prepare the siRNA transfection mix following the CaPi transfection method (see **Figure 1**). If no siRNA is necessary, replace the amount of siRNA by sterile water to perform an *empty transfection*.
9. Calculate 200 μ l mix for each condition, resulting in 400 μ l per duplicate. The following example is given for a final siRNA concentration of 50 nM and has to be adapted for each protein of interest. In a 1.5 ml plastic tube, pipette 50 μ l 1 M CaCl₂ and 11 μ l 20 μ M siRNA into 139 μ l sterile H₂O and mix by vortexing. After a quick spin, add carefully drop-wise 200 μ l HBS2x (280 mM NaCl, 50 mM HEPES, 1.5 mM Na₂HPO₄, pH 7.01-7.05).
NOTE: The smaller the drops the smaller are the precipitates, resulting in better transfection efficiency. Gently mix three times by air injection using a 200 μ l-pipette.
10. Incubate the mix for 30 min at RT. Add slowly drop-wise 200 μ l of transfection mix to each plate and agitate cross-wise. Transfer the plates at 37 °C, 5% CO₂ for 16 hr.
11. The next day, rinse cells twice with 2 ml warm HEPES (6.7 mM KCl, 150 mM NaCl, 10 mM HEPES, pH 7.3) and add 2 ml α MEM-minus^{10%}. Do not proceed with more than four cell plates at a time since variations in the temperature affect the length of the cell cycle.
12. Visualize the infection efficiency under an inverted fluorescent microscope at a magnification of 20-40x (air) and acquire three representative images per condition in both fluorescent and transmission channels for documentation. Seven hours later, add 2 mM thymidine and incubate for a further 16 hr at 37 °C, 5% CO₂.
13. The following day, 48 hr after siRNA transfection and virus infection, rinse the cells twice with 2 ml warm phosphate buffer saline (PBS) and release for 7 hr in 2 ml α MEM^{10%} w/o Phenol Red for live cell imaging or with Phenol Red for protein extraction.
14. Harvest cells for each condition 48 hr post-transfection for preparation of protein extracts and Western blot analysis to determine the efficiency of knockdown and the expression of the endogenous protein¹⁵.
NOTE: Use antibodies against the protein of interest as well as appropriate antibodies serving as loading controls. The efficiency of knockdown should be determined by loading decreasing amounts of control cell lysates (transfected with control siRNA), to provide a titration curve (i.e., 1, 1/2, 1/4, 1/6).

4. Adenovirus Transduction and Endogenous Protein Knockdown by siRNA Transfection in HeLa Cells Using a Cationic Lipid Transfection Reagent

NOTE: Here we present a protocol that was adapted for experiments that do not involve cell synchronization and/or when siRNA transfection cannot be performed by the CaPi method, for instance to avoid undesirable toxic effects in some cell lines. This protocol also includes a cell replating step after adenofection in order to work at a suitable cell density. We have only tested the cationic lipid transfection reagent.

Caution! Working with viruses requires special precautions and a proper disposal of all material that has been in contact with the virus.

1. Plate 1.75×10^5 cells per 35 mm dish in 2.5 ml α MEM^{10%} and incubate at 37 °C, 5% CO₂. Plate one 35 mm plate additional to the number of conditions in order to count the cell number prior to virus transduction.
2. Grow cells until the next day to achieve a cell density of at least 50%. A cell density of less than 50% results in a significant decrease of virus transduction efficiency, increased variations of protein expression per cell, and increased cell toxicity.
3. One day after cell plating, count the number of cells from the additional plated dish. Aspirate the medium and rinse once with 1 ml 0.05% Trypsin/EDTA. Add again 1 ml of 0.05% Trypsin/EDTA and incubate at 37 °C, 5% CO₂ until all cells have detached. Separate cells well using a 1 ml pipette and count the number of cells per ml.
4. Determine the quantity of virus needed to transduce a multiplicity of infection (MOI) of 20-40 plaque-forming units (PFU) of the protein of interest (POI) per cell and 0-20 PFU per cell of an empty vector (e.g., LacZ) to have a total of 40 PFU per cell.
5. Prepare in a sterile hood a 1.5 ml plastic tube for each condition and add 400 μ l of warm α MEM^{10%}.
6. Thaw an aliquot of the viruses slowly on ice and, if necessary, dilute the virus stock in order to pipet a volume greater than 1 μ l to minimize pipetting errors.
7. Add the calculated virus quantity per condition to each 1.5 ml plastic tube containing 400 μ l α MEM^{10%} and mix gently by pipetting. Place the virus stock immediately back at -80 °C to keep its activity.
8. Aspirate the medium from the cells and gently pipette the virus mix drop-wise. Incubate the cells at 37 °C, 5% CO₂ and agitate the plates carefully under the sterile hood each 15 min for 1 hr to well cover the cells with the virus dilution. Using a small amount of medium facilitates the contact of the virus with the cells.
9. Meanwhile, prepare the siRNA transfection mix following the transfection method. If no siRNA is necessary, replace the amount of siRNA by medium without serum to perform an *empty transfection*.
 1. The following example is given for a final siRNA concentration of 50 nM and has to be adapted for each protein of interest. For each adenofection, prepare one 1.5 ml plastic tube containing 416 nM siRNA in 150 μ l medium without serum (e.g., 6.25 μ l 20 μ M siRNA + 144 μ l medium without serum) and one 1.5 ml plastic tube containing 6.25 μ l cationic lipid transfection reagent + 144 μ l medium without serum.
 2. Mix the content of each plastic tube by pipetting up and down several times with a 200 μ l pipette. Combine the content of both tubes and mix by pipetting up and down several times with a 200 μ l pipette. Incubate for 5 min at RT.
10. After incubation with the virus, gently pipette 1.8 ml α MEM^{10%} to each plate to obtain a total volume of 2.2 ml and immediately add slowly drop-wise the siRNA transfection mix to each plate and agitate cross-wise. Transfer the plates at 37 °C, 5% CO₂ for 24 hr.
11. The next day, re-plate the cells from each 35 mm plate into four new 35mm plates in 2.5 ml α MEM^{10%} each and incubate for an additional 24 hr at 37 °C, 5% CO₂.
12. The next day, 48 hr after siRNA transfection and virus infection, harvest cells from one plate for each condition for preparation of protein extracts and Western blot analysis to determine the efficiency of knockdown and the expression of the endogenous protein¹⁵.

NOTE: With the remaining plates, proceed with cell fixation, using the protocol of choice and subject the samples to immunofluorescence analysis with the antibodies of interest¹⁴.

5. Live Cell Imaging of Mitotic Cells and Data Analysis

1. Perform short-term live cell imaging experiments on mitotic cells with an inverted microscope equipped with a humidified/5%CO₂/thermo-regulated chamber.

NOTE: In this study, a spinning disk confocal microscope (40X, 0.75 NA) was used, equipped with an EMCCD cooled charge-coupled camera at -50 °C.
2. Prior to acquisition, verify that the chamber has reached the appropriate temperature of 37 °C.

NOTE: This may take several hours depending on the microscopic system.
3. Place the culture dishes in the microscope chamber 1 hr before acquisition to enable proper equilibrium of the medium and avoid focus drifting due to temperature changes. Monitor the mitotic status of the cells. At this point, 10-15% of the cells should be in the early stages of mitosis (prophase-prometaphase).
4. During the equilibration time, set up the acquisition parameters as determined in prior experiments. Typically, an exposure time for both channels (488 and 594) of 2-3 sec with a laser intensity of 100% and a sensitivity of 121-130 are suitable parameters in our hands.

NOTE: First tests should be made to determine the minimum laser intensity and acquisition time/interval that result in an appropriate resolution with minimum photobleaching that causes cell damages and perturbs mitotic progression.
5. Choose several fields per condition to obtain a significant number of cells to analyze without exceeding the acquisition interval. Using a spinning disk confocal system, a typical setup will include four different conditions, 7 fields per plate and 2 color channels (488 and 594) with a 1.5-2 min interval over a 75 min-period.
6. Choose cells that are at mitotic entry, re-set the focus once all fields have been chosen and start the acquisition as fast as possible.
7. Monitor the stability of the system for at least three time points and re-set the focus if necessary.
8. After the first short-term live cell imaging of 75 min, new fields of mitotic cells may be chosen to acquire a second set of movies to increase the number of cells being analyzed.

9. Determine well-defined criteria to analyze the mitotic phenotypes of cells, which will depend on the fluorescent markers being used. Defects in mitosis may include prolonged time spent in mitosis (from nuclear breakdown until anaphase), chromosome misalignment, spindle rocking, and cortex blebbing¹⁴.

6. LifeAct-TagGFP2 Adenovirus Transduction in Differentiating C2C12 Mouse Myoblasts

NOTE: The adenofection protocol is also applicable to hard-to-infect mouse C2C12 myoblasts undergoing differentiation.

1. Plate 2×10^5 C2C12 cells in 35 mm culture dishes in growth medium on plastic or on a substrate of choice.
NOTE: Cell differentiation is improved on gelatine- or matrigel-coated dishes. Plan one additional plate to determine the cell number prior to virus transduction.
2. The following day, cells should have reached 80% of confluency. Induce myoblast differentiation by washing the cells twice with warm PBS and adding 2 ml of differentiation medium (DM).
3. The next day, labeled as day 1 (D1) of differentiation, transduce myocytes with adenovirus LifeAct-TagGFP2 to visualize the actin cytoskeleton in live cells. Count the cell number from the additional plate as described under 3.2. Calculate 5 PFU/cell of LifeAct-TagGFP2 adenovirus and 45 PFU/cell AdLacZ following the example described in **Table 1**. The total virus quantity is 50 PFU/cell. Viruses were used as described¹⁴.
4. Prepare in a sterile hood a 1.5 ml plastic tube for each condition and add 400 μ l of warm DM.
5. Thaw an aliquot of the viruses slowly on ice and, if necessary, dilute the virus stock in order to pipet a volume greater than 1 μ l to minimize pipetting errors.
6. Add the appropriate amount of virus particles per condition to each 1.5 ml plastic tube containing 400 μ l DM and mix gently by pipetting. Place the virus stock immediately back at -80 °C to keep its activity.
7. Aspirate the medium from the dishes and gently pipette the virus mix drop-wise. Incubate the cells at 37 °C, 5% CO₂ and agitate the plates carefully under the sterile hood each 15 min for a total of 1 hr to well cover the cells with the virus.
8. After incubation, gently pipette 1.6 ml DM onto each plate and continue the incubation for an additional 2 hr at 37 °C, 5% CO₂.
9. Meanwhile, prepare an *empty transfection* mix following the CaPi transfection method. Calculate 200 μ l mix for each condition. Use the following example for a total volume of 400 μ l mix.
 1. In a 1.5 ml plastic tube, pipette 50 μ l 1 M CaCl₂ into 150 μ l sterile H₂O and mix by vortexing. After a quick spin, add carefully drop-wise 200 μ l HBS2x (280 mM NaCl, 50 mM HEPES, 1.5 mM Na₂HPO₄, pH 7.01-7.05). Gently mix by air injection three times using a 200 μ l pipette.
10. Incubate the mix for 30 min at RT. Add slowly drop-wise 200 μ l of the transfection mix to each plate and agitate cross-wise. Transfer the plates at 37 °C, 5% CO₂ for 16 hr.
11. The next day, rinse the cells twice with 2 ml warm HEPES (6.7 mM KCl, 150 mM NaCl, 10 mM HEPES, pH 7.3) and add 2 ml DM. Visualize the infection efficiency under an inverted fluorescent microscope at a magnification of 20-40X (air) and acquire three representative images per condition in both fluorescent and transmission channels for documentation.
12. Depending on the desired experiment setup, follow differentiation into myotubes for several days. Cells can be fixed and subsequently subjected to immunofluorescence analysis or live cell imaging studies can be performed.

Representative Results

Transfection of BAG3-GFP plasmid DNA using cationic lipids was associated with heterogeneous expression in HeLa cells, some cells showing barely detectable levels of the protein and others bearing very high BAG3 levels (**Figure 2A**). In these cells, loss of protein homeostasis was evidenced by accumulation of BAG3-GFP into perinuclear aggregates (**Figure 2A**, arrows). In contrast, cell transduction with adenoviruses carrying BAG3-GFP exhibited more homogenous low expression and accurate localization of BAG3-GFP (**Figure 2B**, infection alone). Remarkably, addition of cationic lipids during adenovirus transduction (*i.e.*, transfection of adenovirus particles) significantly increased BAG3-GFP expression per cell at similar MOI, while it allowed keeping homogenous expression in the majority of cells (**Figure 2B**, Adenofection).

Adenofection allowed efficient depletion of endogenous BAG3 and reintroduction of BAG3-GFP proteins at near endogenous levels, whether using CaPi precipitates or liposome-based compounds to increase the transduction-transfection efficiency in HeLa cells. **Figure 3** shows a typical experiment with wild type BAG3 (WT)-GFP or a BAG3 (IPV)-GFP variant bearing mutations that abolish binding to one of its chaperone partners HSPB8 (**Figure 3A, 3B**). Consistent with a role for BAG3 in stabilization of HSPB8⁷, silencing of BAG3 led to a ~50% decrease in the levels of HSPB8, which was restored to normal levels upon reintroduction of BAG3 WT, but not by expression of similar levels of the mutant of BAG3 (IPV)-GFP or of GFP alone. Under these conditions, BAG3-GFP proteins were appropriately localized within ~75-90% of the cells, being enriched at the perinuclear-centrosomal regions (**Figure 3C, 3D**). This suggested that adenofection preserved the dynamics of BAG3 and of the BAG3-HSPB8 complex in cells.

HSPB8 and BAG3 are upregulated by various proteotoxic stresses that also perturb cytoskeletal proteostasis¹⁰. Hence overexpression of the chaperones may potentially disturb the assembly-disassembly of macromolecular structures controlling cellular morphodynamics. In order to assess the role of BAG3-HSPB8 under non-stressed conditions, it was important to verify that protein homeostasis was minimally perturbed by the adenofection procedure. To that end, monitoring variations in the levels of chaperones of the heat shock protein family is a good indication of the status of protein homeostasis. As shown in **Figure 4**, adenofection by the CaPi or liposome-based methods did not significantly increase the levels of endogenous HSPB8 and BAG3, or that of the major HSP70/HSPA1 chaperone system. In contrast, typical proteotoxic treatments like heat shock or MG132, a proteasome inhibitor, increased the levels of the chaperone-cochaperone proteins in HeLa cells.

We then sought to determine if the adenofection procedure combined with baculoviruses driving expression of actin and tubulin fluorescent probes (BacMam-RFP-actin and GFP- α tubulin) was suitable for tracking mitotic cell dynamics. As shown in **Figure 5**, adenofection of HeLa cells with a control siRNA (siCTL) did not disturb mitotic spindle dynamics (green) or the average time spent in mitosis (**Figure 5A**, representative confocal time-lapse sequences). The proportion of these cells showing abnormal mitotic events was in line with the levels of mitotic defects generally observed in cancer cell lines (~30%-40%, **Figure 5B**). In contrast, cells adenofected with BAG3-specific siRNA alone (siBAG3) exhibited a ~2-fold increase in the level of mitotic phenotypic defects, which was restored to near the level in control cells by BAG3-GFP (WT), but not by GFP alone. This validated the suitability of adenofection for functional analysis of the impact of BAG3 and its associated chaperones on cytoskeletal dynamics that regulate proper progression of cells in and out of mitosis, as shown by *Fuchs et al*¹⁴.

To verify the versatility of the adenofection method, we then adapted the protocol to enable visualization of F-actin during differentiation of mouse C2C12 myocytes, using the commercially available adenovirus-LifeAct-GFP to label F-actin¹⁶. C2C12 cells were induced to differentiate for one day before Adenofection. Using the CaPi-based adenofection protocol, remarkably low amounts of Adenovirus-LifeAct-GFP sufficed to express the probe at a level that was easily detected by fluorescence microscopy in a significant proportion of differentiating myocytes (~3-5 PFU/cell; **Figure 6**). This was in marked contrast to the extremely high multiplicity of infection reported in the literature to transduce mouse C2C12 cells (in the order of 250-400 MOI)¹⁷⁻¹⁹. Furthermore, by monitoring the differentiation process for 7 days, we established that myotube formation was not significantly impaired by the procedure. This suggested that myocyte fusion, a process that relies on finely tuned actin dynamics²⁰, was not perturbed by expression of low levels of LifeAct-GFP (**Figure 6**, Day 6 and Day 7). Since the fluorescent marker can still be detected many days after adenofection of C2C12 cells, we believe that this method will be suitable for functional analyses of the impact of chaperones on actin dynamics at different stages of C2C12 myogenesis.

<i>Virus Stock:</i>	AdPOI #1	1x10 ⁵ / μ l
	AdPOI #2	4x10 ⁵ / μ l
	AdLacZ	8x10 ⁵ / μ l
	Baculovirus	1x10 ⁵ / μ l
<i>Cells counted:</i>	3x10 ⁵ / ml	
<i>Virus per plate:</i>	2PFU:	2 x 3x10 ⁵ = 6x10 ⁵
	4PFU:	4 x 3x10 ⁵ = 12x10 ⁵
	18PFU:	18 x 3x10 ⁵ = 54x10 ⁵

Condition	AdPOI	AdLacZ	Baculovirus	α MEM-minus ^{10%}
2PFU AdPOI #1 + 18PFU AdLacZ +4PFU Baculovirus	6 μ l	6.75 μ l	12 μ l	400 μ l
2PFU AdPOI #2 + 18PFU AdLacZ +4PFU Baculovirus	1.5 μ l	6.75 μ l	12 μ l	400 μ l

Table 1. Calculation of adenovirus MOI. Shown is an example on how to calculate the number of adenovirus plaque-forming units (PFU) necessary for infection of a defined number of cells considering different virus titers.

Figure 1. Planning of a typical adenofection protocol. Successive steps of a typical experiment are shown for the protocol using CaPi precipitates (underlined in grey) or the protocol using a cationic lipid transfection reagent (highlighted in yellow), including cell plating, transduction of adeno- and baculoviruses, transfection of siRNA duplexes, and cell synchronization with a double thymidine block. Time lines in hours for both protocols are shown on the right side of the figure. [Please click here to view a larger version of this figure.](#)

Figure 2. Homogenous and efficient expression of BAG3-GFP using Adenofection. (A) Representative epifluorescence images of HeLa cells that had been transfected with BAG3-GFP plasmid DNA, showing perinuclear aggregates of the protein at high expression levels (designated by arrows) and heterogeneous expression within the cell population. Western blots show higher expression of BAG3-GFP relative to endogenous BAG3 levels in the overall cell population, indicating that the protein is largely overexpressed in some cells. (B) Representative epifluorescence images of HeLa cells that had been transduced only or adenofected using increasing amounts of Ad-BAG3-GFP virus particles. Images were acquired using identical parameters and were equally processed for background subtraction and intensity; Bar: 50 μ m. [Please click here to view a larger version of this figure.](#)

Figure 3. Efficient knockdown of BAG3 and reintroduction of BAG3-GFP proteins at near endogenous levels. (A-B) HeLa cells expressing RFP-H2B (A) or parental HeLa cells (B) were adenofected with the indicated siRNAs and recombinant adenoviruses using the CaPi method (A) or liposome-based compounds as transfection reagent (B). Cells were synchronized by the double thymidine block method and total cell extracts were prepared during the second phase of release. Western blots show BAG3 depletion levels (BAG3 endogenous), the levels of adenofected BAG3-GFP proteins, and endogenous levels of HSPB8; GAPDH levels: loading control. Depletion was estimated at >75% by loading decreasing amounts of control extracts (adenofected with control siRNA-siCtl and BAG3-GFP WT, *i.e.*, 1/2, 1/4). Note that individual BAG3-GFP proteins were introduced at near the endogenous level of BAG3 and that wild type BAG3-GFP, but not BAG3 (IPV)-GFP or GFP alone, restored HSPB8 levels in BAG3-depleted cells. (C-D) Representative epifluorescence images of HeLa cells that had been adenofected with the indicated recombinant Ad-BAG3-GFP using CaPi or cationic lipid transfection reagent. Bars: 20 μ m. Representative results shown in (A) and (C) are modified from *Fuchs et al., PLoS Genet. 2015 Oct 23;11(10):e1005582*, doi:10.1371/journal.pgen.1005582¹⁴. [Please click here to view a larger version of this figure.](#)

Figure 4. Adenofection does not induce a stress response in HeLa cells. Western blots of total cell extracts prepared from control HeLa cells (NT: non treated) or HeLa cells transfected with control siRNA alone (siCtl, no adenovirus), or adenofected with siCtl and Ad-GFP using either CaPi or cationic lipid transfection reagent, or from HeLa cells submitted to typical proteotoxic treatments (HS: heat shock at 44 °C for 60 min followed by 16 hr recovery at 37 °C; MG132: proteasome inhibitor, 5 μ M for 16 hr), showing the levels of BAG3, HSPB8 and of other stress-inducible chaperones, namely HSP70/HSPA/ and HSP27/HSPB1; GAPDH: loading control. Note that while the levels of all proteins except GAPDH were increased upon proteotoxic stress treatments, they remained unchanged by adenofection. Protein level variations were assessed by loading varying amounts of HS cell extracts that bear typical increases in HSPs (HS:1, 1/2, 1/4, 1/8; for instance, HSP70 was induced by more than 8-fold in response to proteotoxic stress). [Please click here to view a larger version of this figure.](#)

Figure 5. Progression of HeLa cells through mitosis is not significantly perturbed by Adenofection. (A) Representative confocal time-lapse sequences from HeLa cells that had been adenofected with a control siRNA (siCtl) together with BacMam- GFP- α -tubulin and BacMam- RFP-actin and imaged by spinning disk confocal microscopy for 60 to 90 min at ~1.5 min intervals. White and yellow asterisks designate the position of spindle poles that remained relatively stable. Bar: 10 μ m. (B) Quantification of cells adenofected with siCtl or BAG3-specific siRNA, with or without the indicated GFP proteins (GFP alone or wild type BAG3-GFP: WT). The graph indicates the percentages of cells with abnormal mitosis defined as spindle rocking and stalled in mitosis +/- or chromosome misalignment. Shown are the means +/- SE. Representative results shown in (B) were taken from Fuchs *et al.*, *PLoS Genet.* 2015 Oct 23;11(10):e1005582, doi:10.1371/journal.pgen.1005582¹⁴. Please click here to view a larger version of this figure. Please click here to view the movie associated with panel (A).

Figure 6. Adenofection of C2C12 myocytes with Ad-LifeAct-GFP and myotube formation. Representative epifluorescence images of C2C12 cells that had been induced to differentiate and processed for adenofection 1 day later using 5 PFU/cell of LifeAct-GFP and 45 PFU/cell of LacZ. Images show expression of the GFP marker during the differentiation process (Day 2, Day 6 and Day 7). Bars: 20 μ m. Please click here to view a larger version of this figure.

Discussion

Here, we described a method enabling depletion-rescue experiments to be performed, which is applicable to functional analyses of cell biological processes that are particularly sensitive to overexpression of proteins affecting the stoichiometry and dynamics of protein complexes and macromolecular structures. Mitotic cell division is an extreme example of finely tuned cell morphodynamics that involves the most dramatic and spectacular changes in the overall structure of a cell. Using adenofection combined with commercially available BacMam reagents to introduce low but detectable amounts of actin and tubulin markers for cell imaging, the contribution of the chaperone complex BAG3-HSPB8 to proper mitotic cell remodeling could be clearly demonstrated. In a recent study by Fuchs *et al.*, we have shown that depletion of BAG3 causes defects in spindle orientation that are related to an inability to establish a rigid mitotic actin cortex and assemble actin-rich retraction fibers¹⁴. Proper spindle dynamics could be restored by reintroduction of wild type BAG3-GFP, which also corrected the decrease seen in HSPB8 levels upon BAG3

silencing. This implies that adenofection enables the recovery of a physiologically relevant chaperone complex that correlates with functional recovery of spindle dynamics.

Use of adenofection for depletion-rescue experiments provides an advantage over plasmid DNA transfection or nucleofection, which can result in a potent induction of a stress response in some cell types (*i.e.*, autophagy)²¹, making it virtually impossible to analyze the impact of a given chaperone and its physiological role. Indeed, in our hand, transfection of BAG3 plasmid DNA is associated with higher expression per cell, aggregate formation, and effects on cell apoptosis/survival in several cell types (**Figure 2**). BAG3 is a modular cochaperone with scaffold activity, which may play multiple roles depending on its partner proteins⁹. Hence, perturbations of complex stoichiometry upon overexpression of BAG3 may have undesirable dominant negative effects and induce toxicity. High capacity recombinant adenovirus is an ideal vehicle for the transient and safety delivery of large genes in both dividing and non-dividing cells in culture as it does not integrate into the host-cell genome, in contrast to lentivirus-based vectors for which some safety concerns still remain. Potential disadvantage of the use of adenoviruses for depletion-rescue experiments is that they require repeated preparation, which may be time-consuming. They also rely on careful titration of infectious particles for reproducible transduction efficacy.

Using adenofection to screen the contribution of known BAG3 functional domains, we obtained the first evidence, to our knowledge, for the existence of an HSPB8-dependent BAG3 function in the normal operation of dividing cells that does not require its interaction with the HSP70/HSPA1 chaperone system. Adenofection should be applicable to track F-actin during the process of myocyte differentiation into myotubes, as suggested by the data presented here. Thus our method provides a versatile and efficient protocol for siRNA-based depletion-rescue experiments with minimum impact on cell morphodynamics that should be useful in a wide range of projects where structure-function analysis of a gene of interest is being pursued.

Exploiting cationic compounds-lipids in order to achieve efficient transfection-transduction of cells with the lowest amount of virus particles is the key to this method. While it provides a larger window for controlling the levels of exogenous proteins per cell, we believe that it further allows minimizing potential side effects on signal transduction pathways that result from adenovirus cell binding-entry, which should mitigate the impact of a protein of interest on morphogenetic pathways.

It should be noted that different reagents to increase adenovirus transduction efficiency are commercially available, such as the CAR receptor booster. Such reagents are expensive, however, and are expected to promote virus binding-entry into cells in a way that requires the CAR receptor, which as stated above has been shown to activate signaling pathways associated with cell shape and adhesion. While CaPi is cheaper than cationic liposomes as a means to potentiate adenovirus entry via a CAR-independent pathway, it is also more toxic to some cell lines. We recommend prior testing of an empty adenofection to orient the choice between CaPi vs cationic lipid reagent, depending on the cell line used and the biological readout of interest.

Together with new biotechnological tools of genome editing, RNA interference-based knockdown-rescue approaches such as the one described here offer an array of powerful molecular tools to uncover gene function in cells, which can now be optimally chosen by investigators depending on specific applications³. We believe that adenofection provides a relatively fast and simple system to create hypomorphic knockdowns for structure-function analyses of the contribution of a protein of interest in multiple cellular backgrounds.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by the Canadian Institutes of Health Research (Grant no 7077), and by the Bellini Foundation and Roby Fondazione.

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