Chapter 14

Image-based Identification of Chemical Compounds Capable of Trapping FOXO in the Cell Nucleus

Susana Machado, Catarina Raposo, Bibiana I. Ferreira, and Wolfgang Link

Abstract

Forkhead box O (FOXO) factors are tumor suppressor proteins commonly inactivated in human tumors. Furthermore, genetic variation within the FOXO3a gene is consistently associated with human longevity. FOXO proteins are usually inactivated by posttranslational modifications leading to cytoplasmic mislocalization. Therefore, the pharmacological activation by promoting nuclear localization of FOXOs is considered an attractive therapeutic approach to treat cancer and age-related diseases. We developed a cell-based imaging assay to screen for chemical agents capable of inhibiting the nuclear export and in turn trapping proteins that contain a nuclear export sequence including FOXO factors in the nucleus. The fluorescent signal of untreated assay cells localizes predominantly to the cytoplasm. Upon treatment with the nuclear export inhibitors the fluorescent-tagged reporter proteins appear as speckles in the nucleus. In a personalized medicine context, drugs capable of reactivating FOXO factors might be of enormous clinical value in human tumors in which these proteins are inactivated. Here, we describe the procedures for monitoring nuclear export which is suitable for high-throughput screening of compound collections.

Key words FOXO, Nuclear export, High-content screening, CMR1

1 Introduction

The subcellular localization of proteins is fundamental because it provides the physiological context for their function [1]. Cancer cells utilize aberrant localization of tumor suppressor proteins including Rb, APC, p53, BRAC1, INI1/hSNF5, galectin-3, Bok, nucleophosmin, RASSF2, Merlin, p21CIP, p27KIP1, N-WASP/FAK, estradiol receptor, Tob, and FOXO proteins as a mechanism for their inactivation and to evade anti-neoplastic mechanisms [2]. Studies have consistently shown FOXO transcription factors to be frequently inactivated in human cancer [3, 4]; and revealed them as important determinants in aging and longevity [5]. FOXO

Susana Machado and Catarina Raposo contributed equally to this chapter

factors exert their tumor suppressor function in the cell nucleus and contain a nuclear export sequence (NES) which mediates the nuclear export of non-DNA bound FOXOs through the export receptor CRM1. Contrary to proteins whose functions are blocked via genetic or epigenetic changes, the mode of inactivation via posttranslational modifications and subsequent mislocalization implies that aberrantly localized FOXO proteins are still reactivatable [6]. Hence, therapeutic interventions leading to nuclear localization of FOXO factors have been considered a very promising therapeutic strategy against cancer cells [7]. Mainly two strategies have been followed: Nuclear translocation and nuclear trapping of FOXO factors. We have developed the U2nesRELOC assay system to identify chemical compound capable of inducing nuclear localization via the inhibition of the nuclear export [8, 9]. U2nesRELOC is based on an osteosarcoma cell line stably expressing a fluorescent reporter protein fused to a NES allowing monitoring the CRM1-mediated nuclear export. U2nesRELOC has been established as a high-content screening assay suitable of testing chemical compounds at bench scale or chemical libraries in a high-throughput manner [10]. The experimental procedure that we describe here is cost effective and has shown to be a powerful tool for lead discovery.

2   Materials

2.1 Stable Expression of Reporter Construct in U2OS Cells

1. U2OS osteosarcoma cells (ATCC).

2. Standard cell culture reagents, plastic ware, and equipment.

3. Culture medium: Dulbecco's modified Eagle's Medium (DMEM) with 25 mM glucose (high glucose), 4 mM Ultra-glutamine I, with 10% heat inactivated fetal bovine serum (FBS).

4. Freezing medium: 10% DMSO in FBS.

5. Transfection reagent (such as FuGENE).

6. Expression construct: pRevMAPKKnesGFP that carries the NES from the MAPK kinase MEK cloned between the BamHI and AgeI sites of pRev(1.4)-GFP, sandwiched between the Rev. and the green fluorescent protein (GFP) coding sequences. It also carries G418 resistance [8].

7. Antibiotic for selection of stable clones: G418 (Geneticin).

2.2 High-Content Nuclear Export Assay

1. Assay plates: 96-well plates with clear optical plastic bottom and black walls (Greiner Bio-One #655087 or similar).

2. Control compounds: Dimethylsulfoxid (DMSO), Leptomycin B (LMB).

3. Fixation: 6% paraformaldehyde in PBS at a pH of 7.2–7.4.

4. Nuclei staining: 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI).

2.3 Equipment

1. Inverted Fluorescence Microscope.

2. High-content screening platform (e.g., Opera LX, BD Pathway Bioimager).

2.4 Software

1. Image acquisition: AttoVision software (BD Biosciences), Acapela v2.0 (Perkin Elmer).

2. Image Analysis: AttoVision software (BD Biosciences), Acapela v2.0 (Perkin Elmer), or Cell Profiler.

3. Statistical analysis: BD Image Data, Microsoft Excel, SPSS, or similar.

3   Methods

3.1   Generation of the Assay Cell Line

3.1.1   Transfection of U2OS Cells

For transfection use FuGENE or a similar transfection reagent (*see* **Note 1**) at a ratio DNA/FuGENE of 1:6. Transfer DMEM to 12 μl FuGENE, and 2 μg of pRevMAPKKnesGFP (*see* **Note 2**) to a final volume of 200 μl in a 1.5 ml Eppendorf tube and incubate for 20 min at room temperature (RT). Harvest U2OS cells at 70–80% confluency (*see* **Note 3**) using trypsin and count the cells (*see* **Note 4**) after resuspending them in DMEM. Transfer 8 ml of these cells at a concentration of 80,000/ml to 2 plates/wells (60 mm plates, or 6-well plate), 4 ml each. To one of these plates/wells add the DNA/FuGENE complexes. Incubate for 48 h at 37 °C and confirm the presence of green fluorescent cells.

3.1.2   Selection of Stable Cell Clones

Add G418 at a final concentration of 1 mg/ml to one of the plates/wells (*see* **Note 5**). Maintain the concentration of the antibiotic for about 1–2 weeks, changing the medium of both plates/wells every other day and avoiding confluency (*see* **Note 6**). Cells in the control plate/well (untransfected cells) and transfected cells which have not integrated the resistance gene die during the first week of selection. Harvest the G418-resistant cell clones (*see* **Note 7**) using small sterile filter paper discs wet with trypsin (*see* **Note 8**), put on the colony to be cloned and transfer the discs to a new well (e.g., 24-well plate) with fresh DMEM supplemented with 100 μg/ml G418.

3.1.3   Selection of the Assay Cell Line

After expansion of the clones, analyze the expression level and cytoplasmic localization of GFP according to fluorescent signal observed in a fluorescent microscope. Select the most suitable cell clone, expand and freeze down aliquots (*see* **Note 9**). Confirm the capability of the assay cells to monitor the CRM-1-dependent
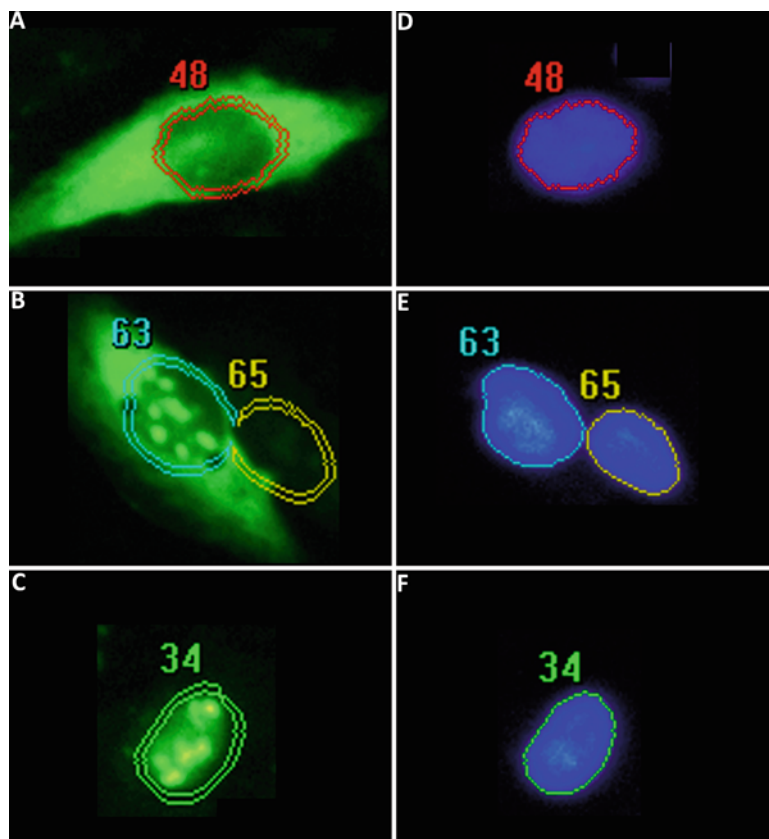
Fig. 1 Assay cells are capable of monitoring CRM-1-mediated nuclear export. (**a–c**) Images of cells taken in the GFP channel. (**a**) Well treated with DMSO, (**b**) Well treated with hit compound, (**c**) Well treated with 4 nM LMB, (**d–f**) Images of cellular nuclei taken in the DAPI channel

nuclear export, treating them with the nuclear export inhibitor Leptomycin B (LMB). Seed cells in a small plate/well, grow them to 80% confluency, and add 4 nM LMB. After incubation of 1 h, confirm the presence of intense green fluorescent speckles in the cell nucleus using a fluorescent microscope (Fig. 1). Maintain assay cell line with DMEM/100 µg/ml G418.

3.2 Establishing the High-Content Screening Assay

3.2.1 Scaling Up the Assay to a 96-Well Format

Grow assay cells to 80–90% confluency in 100 mm plates in DMEM. Use standard trypsinization protocol and pellet cells by centrifugation in a 15 ml tube. Resuspend cells in DMEM and count them. Seed 10,000 cells per well in a blackwall clear-bottom 96-well microplate at a final volume of 200 µl and incubate at 37 °C for 24 h (*see* **Note 10**). Then add to 8 wells 2 µl of positive control compound (4 nM LMB) each and 2 µl DMSO as a negative control to another 8 wells each (*see* **Note 11**) and leave the remaining 8 wells untreated.

3.2.2 Fixation of the Cells		After incubation of the multi-well plate for 1 h at 37 °C remove DMEM, wash twice with 1× PBS, and fix cells with 100 μl 6% paraformaldehyde for 30 min at RT. Then aspirate the paraformaldehyde and wash twice with 1× PBS.

3.2.3 Nuclear Staining

Stain the nucleus adding 20 μl DAPI to a final concentration of 1 μg/ml, incubate for 20 min at RT. Wash twice with 1× PBS and store the plate at 4 °C before analysis.

3.2.4 Image Acquisition

Use a Bioimager equipped with a 488/10 nm excitation filter (GFP), a 380/10 nm excitation filter (DAPI), a 515LP nm emission filter (GFP), and a 435LP nm emission filter (DAPI). Acquire images in the DAPI and GFP channels for each well using a 10× dry objective (*see* **Note 12**).

3.2.5 Image Analysis

Start image analysis with the segmentation of the cell nucleus. As nuclei have been stained with DAPI, local thresholding can define their boundaries and generate a nuclear mask. The cytoplasm can be represented by an outer ring surrounding the nucleus. Based on this segmentation, the intensity of the fluorescent signal can be measured for the nucleus and the cytoplasm, separately. Quantify the level of nuclear and cytoplasmic GFP fluorescence based on the definition of cell compartments by calculating the pixels within the nuclear mask and within the circumferential ring surrounding the nuclear ring mask.

3.2.6 Data Analysis

Based on this quantification, the ratio of GFP intensity between nucleus and cytoplasm for each cell can be calculated by dividing the fluorescence intensity of the nucleus by the cytoplasmic fluorescence intensity. A threshold ratio greater than 1.8 can be used to define nuclear accumulation of fluorescent signal for each cell.

3.2.7 Determination of the Z'score

In order to determine the robustness of the assay calculate the Z score according to the following formula:

$$Z' = 1 - (3SD_{LMB} + 3SD_{DMSO})/|Mean_{LMB} - Mean_{DMSO}|$$

The assay is robust enough to screen complex compound libraries if the Z factor is above 0.5 [11].

3.3 High-Content Screening to Identify Nuclear Export Inhibitors

3.3.1 Preparation of Mother Plates

The following procedure has been designed to screen compounds at a single concentration of 10 μM in a 96-well format (*see* **Note 13**). Prepare the compounds of interest at a stock concentration of 1 mM in DMSO in a standard 96-well plate. Leave the first and the last row empty.

3.3.2 Treatment of the Assay Cells	Transfer 2 μl of each compound to the corresponding well in the assay plate that has been prepared as in Subheading 3.2, **step 1** (*see* **Note 14**). Add 2 μl DMSO to wells A1 to D1 in the first row and to wells A12 to D12 in the last row and 2 μl of 400 nM LMB to wells E1 to H1 in the first row and to wells E12 to H12 in the last row (*see* **Note 15**). The following steps have been described above (*see* Subheading 3.2, **steps 2–5**).
3.3.3 Hit Identification	Compounds that induce a nuclear accumulation of the GFP signal above 60% of that obtained from wells treated with LMB can be considered hits.
3.3.4 Hit Validation Procedure	Compounds that displayed nuclear export inhibitory activity in the primary screening and were considered hit compounds should undergo a stringent follow-up analysis. First, the hit compounds should be cherry picked and tested in an independent experiment with identical design. Then, dose response experiments should be carried out testing several different concentrations of the hit compound to obtain EC50 values. Before starting the hit to lead stage in drug discovery, hit compounds should be ranked according to their potency and drug likeness [12].

4   Notes

1. As U2OS cells are readily transfectable, several different transfection agents might be used.

2. DMEM refers to medium supplemented with 10% FBS as the protocol does not require removal of serum. However, alternative protocols might require removal of serum.

3. Confluency is important because transfection is most successful when cells are near 50–80% confluent and actively growing. Too few cells result in lack of cell-cell contact, to many cells in contact inhibition.

4. For example by using a hemocytometer or an automated cell counter.

5. The concentration of stock G418 can vary considerably from batch to batch. The selection condition might be established experimentally.

6. Expand if necessary as too many cells might compromise viability.

7. Confirm the expression of green fluorescence. You can label the position of the colony with a small circle on the bottom of the plate.

8. Use standard procedure for trypsinization: Wash with PBS, aspirate, put trypsin discs on top of the selected cell colony. Incubate at RT for 5 min.

9. Trypsinize cells, resuspend cells in media, centrifuge, remove supernatant, resuspend pellet in freezing media, and transfer 1 ml freezing media plus cells to each vial. Freeze aliquots at a slow cooling rate by using a freezing foam device or similar in a −80 °C freezer. Transfer vials to a −150 freezer or liquid N2 tank for long-term storage.

10. Seeding of cells can be done manually by using a multichannel pipettor or an automated reagent dispenser.

11. For example, add DMSO to wells A6–H6 of row 6, LMB to wells A7–H7 of row 7 and leave wells A8–H8 of 8 untreated. Note also that the maximum DMSO concentration is 1%. Higher concentrations are cytotoxic. Even 1% of DMSO can impact the viability of cells. In the present assay, however, the toxic effect is limited due to the short exposure time.

12. We expose plates for 0.066 ms (Gain 0) to acquire DAPI images and 0.85 ms (Gain 30) for GFP images on PD Pathway Bioimager.

13. Alternative approaches such as 384-well format or testing several different concentrations are possible.

14. Liquid handling can be performed by using multichannel pipettors or robotic work stations.

15. As 2 μl of 1 mM compound of interest is being transferred to 200 μl the final concentration will be 10 μM and as 2 μl of 400 nM LMB is being transferred to the same volume of cells, final volume of LMB is 4 nM.

Acknowledgments

References

1. Hung MC, Link W (2011) Protein localization in disease and therapy. J Cell Sci 124 (Pt 20):3381–3392. https://doi.org/10.1242/jcs.089110124/20/3381 [pii]

2. Hill R, Cautain B, de Pedro N, Link W (2014) Targeting nucleocytoplasmic transport in cancer therapy. Oncotarget 5(1):11–28. https://doi.org/10.18632/oncotarget.1457 1457 [pii]

3. Zanella F, Link W, Carnero A (2010) Understanding FOXO, new views on old transcription factors. Curr Cancer Drug Targets 10 (2):135–146 doi:EPub-Abstract-CCDT-02 [pii]

4. Link W, Fernandez-Marcos PJ (2017) FOXO transcription factors at the interface of metabolism and cancer. Int J Cancer 141 (12):2379–2391. https://doi.org/10.1002/ijc.30840

5. Martins R, Lithgow GJ, Link W (2016) Long live FOXO: unraveling the role of FOXO proteins in aging and longevity. Aging Cell 15 (2):196–207. https://doi.org/10.1111/acel.12427

6. Zanella F, Dos Santos NR, Link W (2013) Moving to the core: spatiotemporal analysis of Forkhead box O (FOXO) and nuclear factor-kappaB (NF-kappaB) nuclear translocation. Traffic 14(3):247–258. https://doi.org/10.1111/tra.12034

7. Kau TR, Way JC, Silver PA (2004) Nuclear transport and cancer: from mechanism to intervention. Nat Rev Cancer 4(2):106–117. https://doi.org/10.1038/nrc1274

8. Zanella F, Rosado A, Blanco F, Henderson BR, Carnero A, Link W (2007) An HTS approach to screen for antagonists of the nuclear export machinery using high content cell-based assays. Assay Drug Dev Technol 5(3):333–341. https://doi.org/10.1089/adt.2007.058

9. Zanella F, Rosado A, Garcia B, Carnero A, Link W (2009) Using multiplexed regulation of luciferase activity and GFP translocation to screen for FOXO modulators. BMC Cell Biol 10(14):1471–2121

10. Kauselmann G, Dopazo A, Link W (2012) Identification of disease-relevant genes for molecularly-targeted drug discovery. Curr Cancer Drug Targets 12(1):1–13 doi:BSP/CCDT/E-Pub/00174 [pii]

11. Zhang JH, Chung TD, Oldenburg KR (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 4(2):67–73. https://doi.org/10.1177/108705719900400206

12. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 46 (1–3):3–26 doi:S0169-409X(00)00129-0 [pii]