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Wolfgang Link *Editor*

FOXO Transcription Factors

Methods and Protocols

 Humana Press

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FOXO Transcription Factors

Methods and Protocols

Edited by

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 **Humana Press**

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Preface

FOXO transcription factors belong to the large family of forkhead proteins which is characterized by the presence of a ~100-residue forkhead (FKH) DNA-binding domain and bind as monomers to their consensus DNA-binding sites. FOXO proteins function as transcriptional regulators in the cell nucleus and activate the transcription of genes that are involved in numerous biologically relevant processes, such as metabolism, differentiation, proliferation, longevity, and apoptosis. FOXOs are key components of an evolutionary conserved pathway downstream of insulin and insulin-like growth factor receptors. FOXO proteins have been established as tumor suppressors and are known to determine longevity in a wide range of metazoans including humans. Recently, FOXO factors are receiving a great deal of attention as their therapeutic manipulation might be a useful strategy against cancer and age-related diseases. The past two decades have witnessed a broad variety of approaches to shed light on different aspects of FOXO biology. This volume in the *Methods in Molecular Biology* series gives an overview over experimental procedures and the underlying conceptual framework, and it is meant to serve as a compendium for state-of-the-art methods to investigate FOXOs. The current volume includes 23 chapters divided into 5 parts. Part I includes biochemical and molecular methods used in analyzing FOXO expression, regulation by posttranslational modifications, as well as transcriptional specificity and activity. Part II focuses on imaging approaches to monitor the subcellular localization of FOXO factors. Part III highlights the omics and bioinformatics approaches. Part IV provides insight into the different animal models used in FOXO research, and Part V focuses on human studies to investigate the role of FOXO factors in human disease and longevity.

Madrid, Spain

Wolfgang Link

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Chapter 1

Introduction to FOXO Biology

Wolfgang Link

Abstract

Forkhead box O (FOXO) proteins are a family of transcription factors with four members in mammals, namely FOXO1, FOXO3a, FOXO4, and FOXO6. FOXO factors, originally identified as downstream regulators of the insulin pathway, are known to bind to the promoters of a broad variety of target genes and control several processes of key importance for cellular homeostasis including cellular energy production, oxidative stress resistance, and cell viability and proliferation. Accordingly, deregulation of FOXO proteins has been shown to play an essential role in metabolic disorders, human longevity, and the suppression of tumors. As the activity of these transcription factors is controlled by posttranslational modifications, inactivation of FOXOs occurs mostly due to the overactivation of their upstream modifying enzymes providing a wealth of possibilities for restoring FOXO activity pharmaceutically.

Key words FOXO1, FOXO3a, FOXO4, FOXO6, Daf-16, PI3K/AKT pathway, Cancer, Longevity

1 The FOXO Story: a Historic Perspective

FOXO genes were identified in the 1990s in studies of chromosomal translocations found in human tumors [1]. FOXO1, FOXO3a, and FOXO4 lie at sites of chromosomal translocations generating fusion proteins in which the contribution of FOXO to carcinogenesis is still controversial. Shortly after, activity of DAF-16, the nematode ortholog of mammalian FOXO proteins, was shown to be suppressed through activation of the PI3K/AKT pathway by insulin or insulin-like growth factor 1 (IGF-1) in *Caenorhabditis elegans*. This regulation was found to be mediated by phosphorylation of DAF-16 at three consensus AKT sites which are conserved in all the members of the mammalian family of FOXO proteins. FOXO3a was shown to be phosphorylated by AKT resulting in its nuclear exclusion in 1999 [2]. DNA-binding studies revealed a consensus-binding element (DAF-16 family protein-binding or DBE: 5'-TTGTTTAC-3') and high-resolution structures of FOXO proteins bound to DNA have been characterized enabling the understanding of the structural basis for DNA

recognition by FOXO proteins and its regulation [3]. All FOXO proteins bind as monomers via the DNA-binding domain to the same DBE consensus-binding site within the promoter of their target genes. Single full body knock-out mice of FOXO1, 3a, and 4 revealed isoform specific and redundant functions of FOXO isoforms [4]. A major breakthrough in unveiling the role of FOXOs in cancer and stem cell biology has been made by generating conditional triple knock-out mice lacking FOXO1, 3a, and 4 [5]. Analysis of these animals formally established FOXO factors as bona fide tumor suppressors [5] and revealed their importance for the maintenance and integrity of stem cell compartments [6].

2 FOXO Genetics

FOXOs belong to the forkhead box gene family of transcription factors characterized by the presence of the forkhead domain, which is highly conserved across all members of the Fox family [7]. This protein family which comprises over 100 members is named after the typical phenotype caused by mutations in the *Drosophila* gene fork head which result in head structures that look like a fork. There is only one FOXO gene in invertebrates, daf-16 in *C elegans* and dFOXO in drosophila. In contrast mammals have four FOXO genes, FOXO1, FOXO3a, FOXO4, and FOXO6 located on different human chromosomes (Table 1). FOXO2 and FOXO3a are identical and FOXO5 is the ortholog of FOXO3 in fish. FOXO3b has been regarded as a pseudogene although a recent study shows that the FOXO3b locus encodes a protein without DNA-binding activity constitutively localized in the cytosol [8]. Genetic alterations of FOXOs are rare in human cancers where these tumor suppressors are mainly inactivated through posttranslational modifications [1].

3 FOXO Proteins

The human FOXO genes encode for proteins with similar length and a predicted molecular weight between 54 and 72 kDa (Table 1). FOXO proteins consist of four domains, a highly conserved forkhead DNA-binding domain that comprises 100 amino acid residues, a nuclear localization signal (NLS) located just downstream of the DNA-binding domain, a nuclear export sequence forkhead domain at the N terminus, the rest of FOXO proteins is predicted to be highly disordered. The isoforms of the FOXO family FOXO1, 3, and 4 have overlapping as well as isoform-specific upstream regulatory mechanism and display differential preferences for target genes [9]. It is increasingly recognized that different FOXO isoforms play nonredundant roles in different tissues

Table 1

Properties of mammalian FOXO proteins: location on human chromosomes, Number of amino acids, predicted molecular weight in kDa, expression in human tissues, predominant subcellular localization and phenotype of FoxO knock-out mice, embryonic lethal or viable

	FOXO1	FOXO3a	FOXO4	FOXO6
Chromosome	13	6	X	1
Amino acids	655	673	505	492
Mol. Weight	70	72	54	61
Expression	Ubiquitous	Ubiquitous	Ubiquitous	Nervous system
Localization	Nuc/Cyt	Nuc/Cyt	Nuc/Cyt	Nuc
Knock-out	Lethal	Viable	Viable	Viable

[10]. FOXO1, 3, and 4 are expressed in almost all tissues. FOXO6 expression is restricted to the central nervous system and its subcellular localization is not significantly changed by PI3K/AKT signaling [11].

4 Regulation of FOXO Activity

The FOXOs are regulated by synthesis, phosphorylation, acetylation, and ubiquitination at three different levels: subcellular localization, stability, and transcriptional activity [12]. The specific combinations of [posttranslational modifications](#) of FOXO proteins generate a molecular code to sense external stimuli and determine the transcriptional programs mediated by these transcription factors [13]. The main regulatory input to FOXOs activity comes from growth factor-dependent activation of the *PI3K/AKT* pathway and cellular stress signaling. Under stress conditions or in the absence of growth or survival factors, FOXO proteins translocate to the cell nucleus, where their transcriptional functions can be executed. Conversely, in the presence of growth factors or in cancer cells where the *PI3K/AKT* pathway is constitutively activated, AKT phosphorylates FOXO transcription factors in the nucleus, creating a docking site for 14-3-3 proteins that translocate FOXO proteins to the cytoplasm and inactivate them. Conversely, subcellular localization of FOXO6 is not significantly changed by *PI3K/AKT* signaling [11].

5 FOXO Targets

FOXO factors bind to a large set of target gene promoters and have been shown to regulate the transcription of genes even in the absence of direct DNA binding [14]. The recruitment of histone

Table 2

List of selected target genes of mammalian FOXO proteins. A more comprehensive list and the corresponding references can be found in [17]

Target gene	Cellular process
Bim	Apoptosis
FasI	Apoptosis
TRAIL	Apoptosis
PINK	Apoptosis
p27	Cell cycle
Cyclin G2	Cell cycle
Plk	Cell cycle
MnSOD	Stress resistance
Catalase	Stress resistance
Gadd45	DNA repair
LC3	Autophagy
BNIP3	Autophagy
CITED 2	Angiogenesis
InsR	Signaling
PI3K α	Signaling
SESN3	Signaling
RICTOR	Signaling
PEPCK	Metabolism
G6PC	Metabolism
IL-1 β	Inflammation
TLR4	Inflammation
C/EBP β	Differentiation
KLF2	Differentiation
OCT4	Pluripotency
SOX2	Pluripotency
MDR1	Drug resistance
RAG1	Immune response
RAG2	Immune response
FOXP3	Immune response

acetyltransferases (HATs) and histone deacetylases (HDACs) has been suggested as a mechanism for FOXO-mediated transcriptional regulation [15]. The list of FOXO target genes (Table 2) includes genes involved in cell cycle, apoptosis, autophagy, stem cell maintenance and terminal differentiation, DNA repair, glucose and lipid metabolism, stress resistance, pluripotency, immune response, and other cellular processes [16].

6 FOXO and Human Diseases

Given their central role in a broad variety of cellular processes, it is not surprising that FOXO proteins have been implicated in several human diseases including cancer, diabetes, infertility, neurodegeneration, and immune system dysfunction. There is also emerging evidence that deregulation of FOXO factors might account for the association between insulin resistance-related metabolic disorders and cancer. FOXO proteins are inactivated in a broad range of human tumors via posttranslational modifications and miRNA-mediated inhibition [18]. Many cancer cells show an increase in intrinsic level of reactive oxygen species activating FOXO factors promoting stress resistance in quiescent cells, but inhibiting proliferation and inducing apoptosis in the presence of growth factors [17]. The inactivation of FOXO proteins in cancer cells results in the suppression of transcriptional programs that control cell proliferation and cellular defence mechanisms. Accordingly, triple knock-out mice that conditionally ablate FOXO1, 3a, and 4 engendered a progressive cancer-prone condition [5]. FOXO proteins factors are final effectors of the insulin signaling pathway and respond in general to decreased nutrients by inducing gluconeogenesis in the liver, inhibiting adipocyte and myocyte differentiation, or shifting fuel utilization in muscle from glucose to lipids [17]. Accordingly, dysregulation of FOXO proteins is associated with pathogenesis of insulin resistance, dietary obesity, and type 2 diabetes.

7 FOXO and Longevity

Extreme longevity appears to be a result of genetic factors more than environmental factors when compared to an average lifespan and long-lived individuals have been thought to carry protective genes that may work in general cellular defense mechanisms [19]. Genetic FOXO3a variants are associated with longevity in worms, flies, and mammals [20] and protective alleles of FOXO3a are the second most-replicated genetic factors found so far to be associated with extended human lifespan [21]. In humans, FOXO3a contains about 40 noncoding single-nucleotide polymorphisms (SNPs) that have been consistently associated with

longevity. In addition, SNPs of FOXO1A were found to be associated with longevity in women [22]. Recent studies shed light on how these single-nucleotide polymorphisms might affect FOXO functions. Grossi et al. showed that a specific genetic FOXO3a variant allows for the creation of a novel transcription factor-binding site inducing *FOXO3a* transcript expression in response to diverse stress stimuli [23]. Conversely, it has been suggested that genetic FOXO3a variants may function at the genomic level to help regulate neighboring genes by virtue of its central location in chromatin conformation via topologically associated domains [24].

8 FOXO as a Therapeutic Target

Activation of FOXO based on its tumor suppressor and cell protecting properties is considered an attractive therapeutic approach to treat several human diseases including cancer and in the quest for compounds that could slow down the aging process. Contrary to other tumor suppressors like p53 or PTEN whose functions are abrogated via genetic or epigenetic changes, inactivation of FOXOs occurs mostly due to the overactivation of their inhibitory inputs [1]. That offers a wide range of possibilities for restoring FOXO activity with small molecule inhibitors. Although, no screening campaigns have been conducted in search for drugs that directly target FOXO factors with the aim to slow aging, a significant effort has been undertaken targeting FOXOs to identify anti-cancer molecules (Table 3). However, due to the extreme diversity of the

Table 3
List of selected compounds found to activate FOXO proteins

Agent	Effect	Action on FOXOs	Reference
Epigallocatechin gallate	DYRK1a inhibition	Nuclear accumulation/DNA binding	[28]
Aspirin	AMPK activation	Phosphorylation and activation	[29]
Resveratrol	SIRT1 activation	Nuclear translocation	[30]
Rifampicin	JNK/IIS pathways	Nuclear translocation	[31]
ETP-45658	PI3K inhibitor	Nuclear translocation	[32]
MDN-0105	Nuclear export inhibitor	Nuclear translocation	[33]
Ratjadone A	Nuclear export inhibitor	Nuclear translocation	[34]
Calmidazolium	CaM antagonist	Nuclear translocation	[35]
LOM612	N.D.	Nuclear translocation	[36]
GSK690693	14-3-3 inhibitor	Nuclear translocation	[37]
Cyc Pt(II)-ferrocene	N.D.	Nuclear translocation	[38]

cellular roles of the FOXO proteins, the benefit of FOXO activation is context and isoform-dependent [10]. For example, coincidence of FOXO3 with beta-catenin in the nucleus of colon cancer cells promotes metastasis [25], disease-induced catabolic states are characterized by increased FOXO3a expression levels [26], and FOXO4 has been shown to sustain viability of senescent cells impairing tissue homeostasis [27].

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Part I

Biochemical and Molecular Methods



Chapter 2

Characterization of MicroRNAs Regulating FOXO Expression

Doug Hanniford and Eva Hernando

Abstract

MicroRNAs are critical post-transcriptional regulators of a majority of genes, of which the FOXO family of transcription factors is no exception. Here, we describe generalizable methods, including 3' UTR reporter assays and western blotting after microRNA manipulation, to test if a candidate miRNA (*miR-182*) directly targets a candidate (FOXO3) gene product. We also provide guidance on candidate miRNA selection and unbiased miRNA-target identification methods.

Key words MicroRNA, FOXO, 3'UTR reporter assay, MicroRNA target identification

1 Introduction

FOXO proteins are members of the Forkhead box (FOX) family of transcription factors, which are defined by a winged helix DNA-binding motif [1]. The FOXO family comprises FOXO1, FOXO3, and FOXO4, which are widely expressed in human tissues, and FOXO6, which is restricted predominantly to CNS, muscle, and liver [2]. FOXOs are critical regulators of cell metabolism, apoptosis, and stress response during development and tissue homeostasis [3]. They are thought to coordinate cellular decision-making in response to upstream stimuli, particularly environmental stresses, to maintain cellular homeostasis [4]. FOXO proteins are heavily regulated by posttranslational modifications and nuclear-cytoplasmic shuttling, and also post-transcriptionally by microRNAs [5].

MicroRNAs are single-stranded 20–22 nucleotide noncoding RNA molecules that guide AGO2 and associated RNAi-induced silencing complex (RISC) components to target mRNA or other noncoding RNA (Fig. 1a) [6]. When bound to a target mRNA, the microRNA-RISC complex mediates mRNA degradation and/or represses mRNA translation.

Regulation of FOXO factors by microRNAs is now well established. As extensively catalogued [5], FOXO factors have been

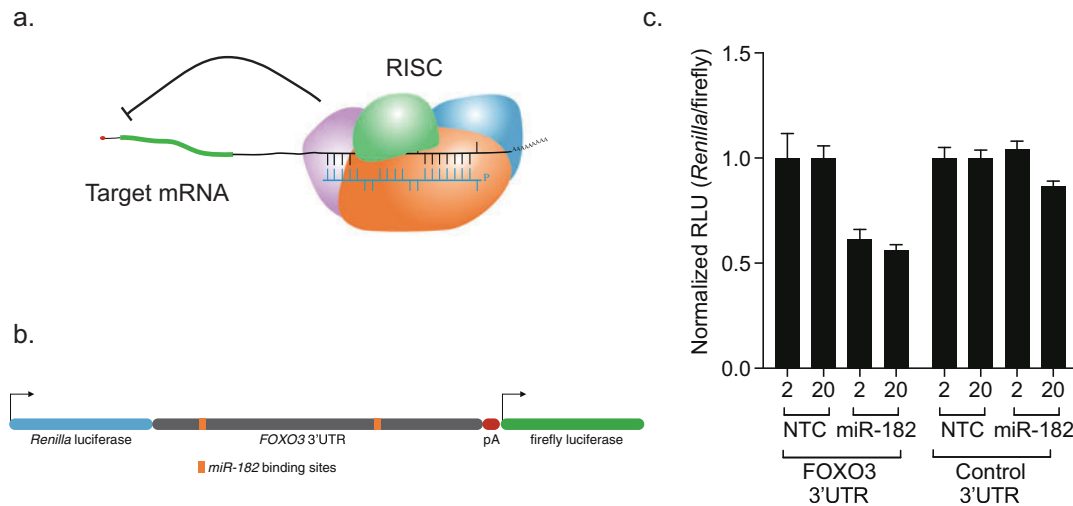


Fig. 1 Testing effects of microRNA mediated target repression by 3'UTR luciferase reporter assay. (a) Graphical depiction of a microRNA-containing RISC bound to the 3'UTR of a target mRNA to mediate translational repression. (b) Graphical depiction of the FOXO3 3'UTR reporter construct generated to test targeting by *miR-182*. (c) Normalized relative light unit (RLU) ratios (*Renilla*/firefly) of 293T cells co-transfected with the indicated reporter construct and microRNA control (NTC) or *miR-182* mimic at 2 or 20 nm

reported as direct targets of more than 30 microRNAs. Of relevance to this method, numerous studies have documented direct targeting of FOXO1 and/or 3 by *miR-96*, *miR-182*, and/or *miR-183* [7–16], a cluster of co-transcribed microRNA on Chromosome 7.

Here, we describe generalizable methods to test direct interaction between a 3'UTR of a target mRNA (*FOXO3*) with a candidate microRNA (*miR-182*) (Fig. 1b,c), and to measure effects of candidate microRNA (*miR-182*) manipulation on target (*FOXO3*) protein abundance (Fig. 2).

2 Materials

2.1 Cloning of FOXO3 3'UTR Luciferase Reporter

1. psiCHECK2 Dual Luciferase cloning vector (Clontech) or other luciferase or GFP reporter vector containing a cloning site(s) downstream of the reporter transgene. psiCHECK2 contains a multi-cloning site containing SgfI, XhoI, PmeI, and NotI downstream of *Renilla* luciferase.
2. Primers to amplify desired 3'UTR or 3'UTR fragment, containing restriction sites for cloning into psiCHECK2 or similar vector. See **Note 1** for 3'UTR fragment selection and **Note 2** for primer design. Primers to amplify a 1.4Kb fragment of the FOXO3 3'UTR:

F: CTCGAGTCACTGAGGAAGGGGAAGTG

R: GCGGCCGCTGTGCTAATCAGAGCATCGTT

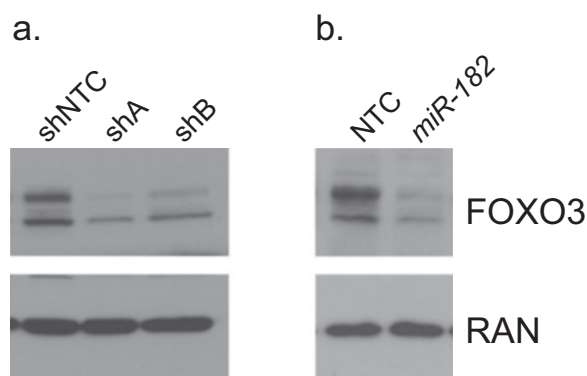


Fig. 2 Testing effects of microRNA-mediated target repression by immunoblotting. **(a)** Representative immunoblot of FOXO3 from protein extracts of A375 melanoma cells expressing control (shNTC) or *FOXO3*-targeting shRNA (shA/B). **(b)** Representative immunoblot of FOXO3 from protein extracts of A375 melanoma cells transfected with control (NTC) or *miR-182* microRNA oligo mimics

3. High fidelity PCR reagents, such as EXPAND high fidelity PCR System.
4. dNTPs (10 mM each).
5. cDNA or DNA derived from appropriate human cells (*see Note 3*).
6. PCR tubes.
7. Thermal cycler.
8. Agarose.
9. 50× Tris-acetate, Ethylenediaminetetraacetic acid (EDTA) (TAE): For 1 L, add 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL of 0.5 M EDTA (pH 8.0), QC to 1 L with H₂O. Dilute TAE in H₂O for 1× working solution (40 mM Tris-acetate, 1 mM EDTA).
10. Ethidium Bromide or other DNA binding dye.
11. 5× PCR loading dye (or equivalent).
12. DNA ladder.
13. Horizontal gel electrophoresis unit and power supply, gel casting trays, and combs.
14. UV imaging dock.
15. PCR cloning kit, such as TOPO TA.
16. Microfuge tubes.
17. Restriction enzymes, XhoI and NotI.
18. Gel extraction DNA recovery kit.

19. Scalpel or razor blade.
20. Alkaline phosphatase.
21. T4 DNA ligase and buffer.
22. Chemically competent DH5alpha or equivalent.
23. S.O.C. recovery media or equivalent.
24. Bacterial growth media and plates (LB) containing ampicillin.
25. Plasmid mini prep and maxi prep kits.

2.2 Dual Luciferase Assay to Measure Effect of miR-182 Overexpression on FOXO3 3' UTR Reporter

1. Synthetic *miR-182* and control mimics.
2. Lipofection reagent, such as Lipofectamine 2000.
3. Serum-free medium, such as Opti-MEM.
4. 5 mL round-bottom sterile tubes.
5. 293T cells or similar.
6. Dulbecco's Modified Eagle Media (DMEM)..
7. Phosphate-buffered Saline (PBS).
8. Fetal bovine serum (FBS).
9. Trypsin-EDTA.
10. 96-well, clear-bottomed, white-walled tissue culture plates.
11. Plate reader capable of measuring luminescence from 96-well plates.
12. Dual luciferase assay reagents, such as Dual-Glo Luciferase Assay System.
13. PBS.
14. 8- or 12-well multichannel (20–200 µL).
15. 200 µL pipette tips, multichannel compatible.
16. 8-channel aspirator.

2.3 Immunoblot to Measure FOXO3 Abundance in Melanoma Cells After miR-182 Overexpression

1. Synthetic *miR-182* and control mimics.
2. Lipofection reagent, such as Lipofectamine 2000.
3. Serum-free medium, such as Opti-MEM.
4. 5 mL round-bottom sterile tubes.
5. A375 melanoma cells (ATCC).
6. Dulbecco's Modified Eagle Media (DMEM)..
7. Phosphate-buffered Saline (PBS).
8. Fetal bovine serum (FBS).
9. Trypsin-EDTA.
10. 10 cm tissue culture dishes.
11. RIPA buffer.
12. Protease inhibitors.

13. Detergent compatible protein assay and bovine serum albumin standards.
14. Precast 8-well 4–20% Tris-Glycine polyacrylamide mini-gels.
15. 10× Tris-Glycine SDS (sodium dodecyl sulfate) PAGE running buffer.
16. 5× sample buffer: 0.25 M Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 0.5 M DTT (dithiothreitol), and 0.25% bromophenol blue.
17. Pre-stained protein ladder.
18. Mini gel tank and blot module, and power supply.
19. Immunoblot transfer sponges.
20. Whatman filter paper.
21. 10× Tris-Glycine transfer buffer: 250 mM Tris, 1.92 M glycine.
22. Nitrocellulose membranes.
23. 20× Tris-buffered saline (TBS): 3 M NaCl, 400 mM Tris, pH 7.6.
24. 25% v/v Tween 20 diluted in H₂O. Autoclave and store at 4 °C.
25. Antibodies.
 - (a) FOXO3.
 - (b) RAN or other loading control.
 - (c) HRP-conjugated secondary antibody.
26. Nonfat dry milk.
27. Plastic containers.
28. Chemiluminescence detection reagents.
29. Autoradiography film.

3 Methods

3.1 Cloning of FOXO3 3' UTR Luciferase Reporter

1. Dissolve lyophilized primers in nuclease-free H₂O to 100 μM.
2. Dilute primers to 10 μM working stocks in nuclease-free H₂O.
3. Prepare 50 μL PCR reactions using Expand High Fidelity PCR System or equivalent (*see Note 4*) on ice. Dilute all components in nuclease-free H₂O to 50 μL final volume. Add 5 μL provided 10× buffer with 15 mM MgCl₂. Add 1 μL each of 10 μM forward and reverse primer stocks (200 nM final). Add 1 μL dNTPs stock (200 μM final). Add template cDNA or DNA (1–200 ng).
4. Run reaction in thermal cycler using the following parameters:

- (a) 95 °C for 2 min.
 - (b) Denature at 95 °C for 15 s.
 - (c) Anneal at 55 °C for 20 s.
 - (d) Elongation at 72 °C for 90 s.
 - (e) Repeat **steps 2–4** 18×, 25×, or 30×.
 - (f) Final elongation at 72 °C for 7 min.
 - (g) Hold at 4 °C.
5. While PCR reaction is cycling, prepare 1% agarose gel. Weigh 2 g agarose. Dilute agarose in 200 mL of 1× TAE in a 500 mL glass Erlenmeyer flask. Heat mixture by microwave to dissolve agarose in TAE. Allow to cool briefly until steam is no longer visible. Add EtBr to 0.5 µg/mL and mix briefly. Pour hot gel into casting tray and add appropriate comb.
 6. Allow gel to solidify at room temperature for at least 30 min. Remove gel casting tray end caps and comb, and place gel into horizontal gel electrophoresis unit containing 1× TAE.
 7. Remove PCR reactions and place on ice. Dilute 40 µL of each PCR reaction with 10 µL 5× loading dye; retain at least 2 µL for TOPO cloning.
 8. Pipette 10–25 µL of diluted PCR reactions and DNA ladder into wells of agarose gel. Electrophorese gel at 100 V for 30 min. Expose gel using UV imager to determine if PCR amplification yielded an appropriately sized product. Assuming the expected product was amplified, proceed to TOPO cloning.
 9. Mix 1–2 µL of retained PCR product (*see Note 5*) with 1 µL TOPO TA vector, 1 µL salt solution, and H₂O to 6 µL. Incubate for 5 min at room temperature (22–23 °C). Positive and negative reactions may also be performed. Place reaction(s) on ice and briefly thaw DH5alpha competent bacteria on ice. Add 1–2 µL cloning reaction to a 50 µL aliquot of DH5alpha and mix gently. Incubate on ice for 10 min. Heat-shock bacteria for 30 s in a 42 °C water bath. Transfer to ice for 2 min. Add 250 µL S.O.C. media. Cap transformations and shake tube (200RPM) for 60 min at 37 °C. Spread 10–50 µL transformation on pre-warmed LB Amp plates and incubate overnight at 37 °C.
 10. Using a pipette tip, pick 4–5 isolated colonies, streak onto LB Amp plate, then transfer tip to 4 mL liquid LB in a 14 mL culture tube. Grow liquid cultures in a bacterial shaker (200 RPM) overnight at 37 °C for use in plasmid mini preps. Isolated colonies streaked on LB Amp plates should be incubated overnight at 37 °C, sealed tightly with Parafilm, and stored at 4 °C for up to 2 months.

11. Transfer 1 mL of each liquid culture to a 1.5 mL microfuge tube. Centrifuge at $10,000 \times g$ for 1 min to pellet bacteria. Perform mini prep following the manufacturer's instructions to isolate plasmid DNA. Perform Sanger sequencing to confirm isolation of TOPO clones containing the FOXO3 3'UTR and verify accuracy of the DNA sequence. A sequence-verified clone should be used for subsequent subcloning into psiCHECK2.
12. Digest 4 μ g each of psiCHECK2 reporter vector and FOXO3 3'UTR-containing TOPO clone with XhoI and NotI. Briefly, mix DNA in water with appropriate buffer and 10 U each of XhoI and NotI. Incubate 3–4 h at 37 °C. Prepare 1% agarose gel as before. Load digested products, DNA ladder, and controls (undigested vector(s), single-cut vector(s)) and run gel at 100 V for 30–45 min, as before (*see Note 6*). Place gel on foil on UV imager, position to reveal only a portion of digested vector(s), and cut gel slices containing appropriately sized bands (FOXO3 3'UTR: 1.5 Kb, psiCHECK2 vector: 3.5 Kb). Extract DNA from gel slices using Zymoclean gel extraction kit following the manufacturer's directions.
13. Dephosphorylate digested vector (10 $\times$ dephosphorylation buffer and 1 μ L Alkaline phosphatase) for 15 min at 37 °C followed by inactivation at 65 °C for 15 min. Quantify digested vector or FOXO3 3'UTR DNA by Nanodrop or Qubit. Set up overnight ligation reaction with 1:3 vector to insert molar ratio:

10 $\times$ Ligation Buffer	2 μ L
Dephosphorylated digested psiCHECK2	60 ng
Digested FOXO3 3'UTR	45 ng
T4 DNA Ligase	1 μ L
H ₂ O	to 20 μ L

Incubate ligation reactions overnight at 16 °C. As before, transform into chemically competent DH5alpha or equivalent and plate transformation on LB Amp plates. As before, pick 4–5 colonies, streak on LB Amp plates, and expand in liquid culture. Isolate plasmid DNA by mini prep following the manufacturer's instructions, and perform Sanger sequencing to confirm successful subcloning of FOXO3 3'UTR in psiCHECK2.

14. Expand a sequence-verified clone(s) in a larger liquid culture (300 mL LB) and use in maxi prep plasmid purification following the manufacturer's instructions. Assess concentration and quality of isolated DNA by Nanodrop.

**3.2 Dual Luciferase
Assay to Measure
Effect of miR-182
Overexpression on
FOXO3 3' UTR Reporter**

1. Harvest 293T cells by trypsinization, count, and wash $1 \times$ with PBS. Resuspend cells to 400,000 cells/mL in growth media (DMEM + 10% FBS), and plate 100 μ L per well (40,000 cells) in 96-well clear-bottomed, white-walled tissue culture plates. Incubate overnight in a humidified cell culture incubator at 37 °C/5% CO₂.
2. Dilute empty or FOXO3 3'UTR-containing psiCHECK2 maxiprep DNA in sterile, nuclease-free H₂O to generate 200 ng/ μ L stocks. Resuspend lyophilized *miR-182* and non-targeting control mimics to 20 μ M in sterile, nuclease-free H₂O. Keep microRNA mimics on ice and store in aliquots at –20 °C.
3. Design experiment and generate plate map to determine number of replicate wells for each transfection condition (*see Note 7*).
4. Prepare separately liposomal cocktails for psiCHECK2 constructs and microRNA mimics as follows:
 - (a) Plasmid cocktails—dilute 0.5 μ L/well of 200 ng/ μ L plasmid stock (100 ng) in 24.5 μ L/well Opti-MEM and 0.3 μ L/well Lipofectamine 2000 in 24.7 μ L/well Opti-MEM, separately in 5 mL round-bottom tubes. Scale accordingly with 5–10% excess for the required number of replicate wells. Gently mix and incubate for 5 min at room temperature. Mix diluted Lipofectamine 2000 with diluted DNA. Incubate for 20 min at room temperature.
 - (b) microRNA mimic cocktails—dilute 0.15 μ L/well (20 nM final) of 20 μ M stock mimics with 25 μ L/well Opti-MEM and 0.15 μ L/well Lipofectamine 2000 with 24.75 μ L/well Opti-MEM, separately in 5 mL round-bottom tubes. Scale accordingly with 5–10% excess for the desired number of technical replicate wells (3–8 recommended). Gently mix and incubate for 5 min at room temperature. Mix diluted Lipofectamine 2000 with diluted miRNA mimic. Incubate for 20 min at room temperature.
5. Aspirate media from wells of 293T-seeded 96-well plate using 8-channel vacuum manifold. Dispense 50 μ L per well each of plasmid DNA- and microRNA mimic-Lipofectamine cocktails to appropriate wells of 96-well plate. Add 50 μ L per well DMEM + 10% FBS for a final volume of 150 μ L with ~3.33% FBS. Incubate for 16–24 h in a humidified cell culture incubator at 37 °C/5% CO₂.
6. Prepare Dual-Glo luciferase assay reagents per the manufacturer's instructions. Store prepared Dual-Glo luciferase reagent in aliquots at –80 °C (stable for at least 1 year). Diluted Dual-Glo Stop & Glo substrate (1:100 in Dual-Glo Stop & Glo buffer) should be made freshly and not stored. Equilibrate reagents to room temperature prior to use.

7. Remove 96-well transfection plate, aspirate media using 8-channel vacuum manifold, and dispense 50 μ L/well of room temperature PBS.
8. Add 50 μ L/well Dual-Glo luciferase reagent. Incubate for 10 min at room temperature to allow cell lysis to complete. Measure luminescence in plate reader with the following parameters: premix for 5 s, 1000 ms integration time.
9. Add 50 μ L/well diluted Dual-Glo Stop & Glo reagent. Incubate for 10 min at room temperature. Measure luminescence in plate reader with the following parameters: premix for 5 s, 1000 ms integration time.
10. Calculate ratio of *Renilla* luciferase (experimental) output to firefly luciferase (control) output for each well. Technical replicates should be averaged and standard deviation calculated for plotting results. Standard statistical testing (two-sided Student's T test) should be performed on average ratio values compiled from at least three independent experiments.

3.3 Immunoblot to Measure FOXO3 Abundance in Melanoma Cells After miR-182 Overexpression

1. Culture A375 melanoma cells in DMEM + 10% FBS. Passage A375 twice weekly to new flasks by a 1:10 splitting ratio. 16–24 h prior to transfection with microRNA mimic oligos, harvest A375 cells by trypsinization, count, and wash $1 \times$ with PBS. Seed 1×10^6 cells per dish in 10 cm dishes and incubate overnight at 37%/5% CO₂.
2. Prepare liposomal cocktails for microRNA mimics (*see Note 8*) as follows: dilute 20 μ L (50 nM final) of 20 μ M stock mimics with 780 μ L Opti-MEM and 15 μ L Lipofectamine 2000 with 785 μ L/well Opti-MEM, separately in 5 mL round-bottom tubes. Gently mix and incubate for 5 min at room temperature. Add diluted Lipofectamine 2000 to diluted miRNA mimic. Incubate for 20 min at room temperature.
3. Aspirate growth media from 10 cm dishes seeded with A375 cells. Add 6.5 mL fresh DMEM + 10% FBS to each dish. Add dropwise ~ 1.6 mL of *miR-182* or non-targeting control mimic liposomal complex to 1×10 cm dish each. Incubate cells for 48 h at 37%/5% CO₂.
4. Prepare RIPA buffer by supplementing with protease inhibitors following the manufacturer's recommendations.
5. Remove cells from incubator. Do the following steps on ice. Wash A375 cells $2 \times$ with ice-cold PBS. Add 2 mL ice-cold PBS and harvest by scraping. Transfer detached cells to 15 mL conical tubes. Use an additional 2–5 mL ice-cold PBS per dish to collect residual cells. Pellet cells by centrifugation ($216 \times g$ for 5 min at 4 °C) and proceed to cell lysis and protein isolation. Alternatively, aspirate supernatant and snap-freeze on dry ice, or resuspend in 1 mL ice-cold PBS, transfer

to 1.5 mL microfuge tubes, pellet by centrifugation ($600 \times g$ for 5 min at 4°C), discard the supernatant, and snap-freeze pellets on dry ice. Store frozen pellets at -80°C for future use. Briefly thaw on ice immediately prior to cell lysis.

6. Resuspend cell pellets with 200 μL RIPA + protease inhibitors and incubate on ice for 30 min (*see Note 9*). Pellet insoluble material by centrifugation ($17,000 \times g$ for 10 min at 4°C), and transfer supernatant to fresh 1.5 mL microfuge tubes and store at -80°C . Prior to use in immunoblotting, quantify protein concentrations using a detergent compatible protein assay following standard methods.
7. Prepare $1\times$ SDS-PAGE running buffer by diluting $10\times$ buffer with picoPure H_2O .
8. Prepare 4–20% gel. Remove gel from package, remove white tape, and extract 8-well comb. Briefly rinse gel wells with picopure H_2O , then flick excess out. Place gel in mini gel tank apparatus and fill central chamber with $1\times$ SDS-PAGE running buffer to just above gel wells. Ensure no leaking occurs to outer chamber, then fill outer chamber approximately $2/3$ full with $1\times$ SDS-PAGE running buffer.
9. Dilute 25 μg protein per sample in H_2O to 20 μL , then add 5 μL of $5\times$ sample buffer. Heat samples in a 95°C heat block for 5 min. Transfer to ice briefly to cool, then briefly centrifuge to collect all liquid at the bottom of the tube. Load 10 μL Benchmark prestained protein ladder and 20 μL of each sample (20 μg) into one well each of prepared gel(s). Run gel at 125 V for 90 min or until dye-front reaches the gel bottom.
10. Prepare 1 L of $1\times$ Tris-Glycine transfer buffer by diluting 100 mL of $10\times$ Tris-Glycine transfer buffer in 700 mL H_2O and 200 mL Methanol and cool on ice. Prepare $1\times$ Tris-buffered saline-tween (TBS-T) by diluting 100 mL of $10\times$ TBS to 2 L with H_2O , then adding 8 mL of 25% Tween 20.
11. Remove gel by using a spatula to pry open the plastic casing and transfer gel to a plastic container filled with $1\times$ transfer buffer or picopure H_2O . Prewet 2 sponges, 2 Whatman filter papers, and 1 nitrocellulose membrane in $1\times$ transfer buffer. Prepare transfer by stacking components in order: sponge, whatman filterpaper, gel, nitrocellulose membrane, whatman filter paper, and sponge. Ensure that no air bubbles are present between stack components (*see Note 10*). Sandwich stack in transfer cassette and place in mini cell transfer apparatus. Fill apparatus with ice-cold $1\times$ Tris-Glycine transfer buffer and transfer blot at 100 V for 1 h (*see Note 11*).
12. Prepare blocking buffer: 50 mL of 5% nonfat dry milk by diluting 2.5 g nonfat dry milk in $1\times$ TBS-T. Vortex vigorously until solubilized.

13. Remove membrane from transfer sandwich. Assess prestained protein ladder to ensure successful transfer, then place membrane in a plastic container and briefly rinse with picopure H₂O. Replace H₂O with blocking buffer and shake gently at room temperature for 60 min.
14. Prepare antibody dilutions in 1× TBS-T.
 - (a) FOXO3—dilute 1:1000 (Antibody validation performed using shRNA Fig. 2a).
 - (b) RAN—dilute 1:5000.
15. Remove blocking buffer and store at 4 °C for future use. Briefly rinse blocked membrane with 1× TBS-T, then cut membrane in half horizontally into two strips, one for FOXO3 (~95Kd) and one for RAN (~25Kd). Place strips into separate containers and rinse briefly with TBS-T. Replace TBS-T with primary antibody dilutions. Close containers and rock gently on a shaker overnight at 4 °C.
16. Prepare HRP-conjugated secondary antibodies by diluting 1:25,000 in 2.5% nonfat dry milk in TBS-T (dilute retained blocking buffer 1:1 with TBS-T).
17. Remove primary antibody solutions and wash membranes with TBS-T at least 4× for 5–10 min each.
18. Add diluted HRP-conjugated secondary antibodies. Incubate with gentle shaking at room temperature for 45 min.
19. Remove secondary antibody solutions and wash membranes with TBS-T at least 4× for 5–10 min each.
20. Prepare chemiluminescence HRP detection reagent. Place membrane protein-side up on a piece of Parafilm with folded up corners. Add HRP detection reagent and rock solution gently over the membrane for 1–3 min. Drain excess liquid from membrane, sandwich between two plastic transparencies, and place inside a film exposure cassette. Expose films in a darkroom for 10 s, 20 s, and 2 min, or as needed. Compare FOXO3 between conditions, ensuring equal loading (RAN) (Fig. 2b).

4 Notes

1. 3'UTR fragment selection is an important consideration when designing microRNA reporter experiments testing a microRNA-target interaction. Ideally, full-length 3'UTRs would be employed to most closely mimic the native transcript; however, frequently 3'UTRs are several kilobases or larger, making such experiments technically difficult. In our estimation, fragments of at least 1Kb should be employed for large

3'UTRs. For large 3'UTRs with 2 or more predicted target sites for a microRNA or interest, multiple 3'UTR fragments may need to be tested independently to determine the effect of the microRNA on the target 3'UTR and elucidate active sites.

2. Forward primer should be designed to amplify close to the translation stop codon if possible to mimic native transcript when cloned after *Renilla* luciferase. Forward primer should contain a restriction site compatible with the cloning vector on the 5' end (XhoI for psiCHECK2). Reverse primer should be designed to amplify as much 3'UTR as technically feasible and should contain a restriction site compatible with the cloning vector on the 5' end (NotI for psiCHECK2). Ensure that the amplified sequence does not contain the restriction sites added to the primers for subcloning. Alternatively, primers can be designed for Gibson cloning (20–40 bp homology to vector at insert site) or 3'UTR fragment can be produced synthetically (e.g., IDT gBlock) and subsequently cloned. Regardless of the chosen cloning method, use of TOPO or other shuttle vectors typically increases cloning (and subcloning) efficiency (particularly for PCR amplified products), simplifies Sanger sequencing from both ends to examine sequence accuracy, and provides additional flanking restriction sites to easily transfer to alternative vectors as needed.
3. To ensure sequence accuracy of the 3'UTR of interest, DNA/cDNA from non-malignant human cells, such as immortalized fibroblasts, should be used, unless mutations within a specific cell model are of interest.
4. Ensure that chosen polymerase is high fidelity but also compatible with TA cloning (many high fidelity polymerases do not leave adenine overhangs). Alternatively, taq polymerase can be used to add adenine overhangs to purified PCR products produced from high fidelity polymerases.
5. For TOPO cloning, use product from the least number of amplification cycles tested, even if no product is apparent on gel imaging (assuming successful amplification of the correctly sized product with additional cycling). Reducing the number of cycles performed limits propagation of mutations introduced during cycling, resulting in cloned products that are more likely to contain accurate sequences.
6. Combs with wide lanes are especially useful for running large amounts of DNA, but such combs typically lack small lanes for running DNA ladder, controls, etc. To create “wide” lanes in standard combs, tape two or more combs together with standard lab tape to customize lane sizes. This also allows running a portion of the digested vector(s) (5 μ L) next to the bulk of digested vector. By doing so, gel can be placed on foil when

imaging by UV and positioned to only reveal the small lane of digested vector, which can be used as a guide for cutting out the correct band while not directly exposing it to UV.

7. Design of 3'UTR luciferase assay is a critical step for interpretation of results. Technical replicate wells ($n = 3-8$ recommended) for each condition should be performed. At minimum, the candidate 3'UTR reporter (FOXO3) should be co-transfected with a non-targeting microRNA and a candidate microRNA (*miR-182*). As the candidate miRNA may directly target transcripts from the luciferase vector (and not the cloned 3'UTR), empty psiCHECK2 reporter vector should be used as a non-targeted negative control. Alternatively, a 3'UTR or fragment of similar length and lacking predicted binding sites to the candidate miRNA may also be used. More elegantly, site-directed mutagenesis can be performed to mutate predicted binding site(s) in the candidate 3'UTR reporter. Using standard oligo cloning methods described elsewhere, a positive control for the microRNA candidate can also be generated by cloning a single, perfectly complementary binding site in psiCHECK2. In addition to these controls, microRNA mimic controls can be used, including mock transfection, a second non-targeting mimic, or a microRNA not predicted to bind the candidate 3'UTR reporter as negative controls, or a microRNA known to bind and regulate the candidate 3'UTR as a positive control. Finally, replicate experiments should be performed independently to examine reproducibility of results.
8. These protocols (3'UTR luciferase assay and Immunoblot) describe overexpression of microRNA oligo mimics to test if a microRNA can directly target the 3'UTR and alter protein abundance of a candidate target gene of interest; however, they do not address if this interaction occurs under physiological conditions. There are many alternative approaches to provide additional evidence confirming such interactions and to test physiological binding. Oligo-based microRNA inhibitors can be used in both described protocols to further address this question by interrogating protein abundance and 3'UTR luciferase activity after microRNA inhibition. Alternatively, short, target-site blockers can be used to disrupt an interaction of a microRNA with a validated or predicted binding site on a target transcript [17], followed by interrogation of protein abundance by immunoblot. More elegantly, CRISPR-Cas9-based gene editing can be employed to genetically alter described microRNA binding sites to examine the impact of a given microRNA/gene interaction. Luciferase-based 3'UTR reporter assays are also amenable to unbiased arrayed screening with microRNA oligo mimics or inhibitors to identify all

directly targeting microRNAs. To examine interactions of all microRNA with all other RNA transcripts, several similar methods involving cross-linking of RNA and proteins, immunoprecipitation of AGO2-containing complexes, followed by next-generation sequencing of isolated RNA (AGO2 Clip-seq) have been developed [18–21]. As a limitation, such methods are intensive and require extensive training to conduct robustly. Moreover, all such methods, except CLASH/chimaera analysis [22, 23], employ predictive algorithms to infer bound microRNA at microRNA/mRNA interaction sites, thus individual microRNA/gene interactions require additional validation.

9. Ideally, protein extraction should be performed with minimal manipulation (e.g., –trypsinization) of cells prior to extraction. Limited manipulation is absolutely critical for rapidly changing proteins and many posttranslational modifications, and trypsinization should not be employed in the analysis of membrane proteins. Harvesting cells for protein extraction by scraping cells from plates directly in lysis buffer after 2 quick washes with ice-cold PBS is an excellent way to minimize cell manipulation. As a con to this approach, residual PBS on plates can easily double the intended lysis volume, altering the composition of lysis buffer, which may result in incomplete lysis, reduce yield, etc. For many proteins, it may be preferable to harvest cells in PBS, allowing pelleting and near complete PBS removal, prior to lysis. To further improve protein extraction, samples can be subjected to multiple rounds on freeze-thaw (dry ice to 37 °C) or sonication prior to removal of insoluble material by centrifugation.
10. To reduce the possibility of air bubbles during transfer, form stack while submerged in 1× transfer buffer, such as in a medium-sized, low-walled glass baking dish. In addition, rolling out air bubbles with a small roller or half a 25 mL pipette further reduces air bubbles trapped between layers. Alternatively, other methods of transfer, such as semi-dry blotting, can be performed.
11. Transfer performance is dramatically improved when transfer buffer is kept cold. Small, reusable ice blocks can be placed directly in the transfer tank, tank can be placed in a bucket of ice, or transfer can be performed in a cold room.

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Chapter 3

Identification of Transcription Factor-Binding Sites in the Mouse FOXO1 Promoter

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Abstract

One critical determinant of levels of gene expression is binding of transcription factors to cognate DNA sequences in promoter and enhancer regions of target genes. Transcription factors are DNA-binding proteins to which transcriptional co-regulators are bound, ultimately resulting in histone modifications that change chromatin structure to regulate transcription. Examples of transcription factors include hormone-activated transcription factors such as the glucocorticoid receptor, transcription factors regulated by cell surface receptors such as FOXO1 and Smad2/Smad3, and many others. Promoter regions typically contain multiple, diverse transcription factor-binding sites. Binding sites for cell-type-specific transcription factors involved in cell fate determination such as Runx2, MyoD, or myogenin are frequently observed. Promoter regions are located within ~2 kb upstream of the transcriptional start site, whereas enhancers may be located at some distance from promoter sequences and exert long-range effects. Here, we will discuss classical and emerging technologies by which one can understand the role of binding of specific transcription factors in regulation of transcription of FOXO genes.

Key words Promoter, Enhancer, Transcription factor, Transcriptional effector, Glucocorticoids, Glucocorticoid receptor, Chromatin, Epigenetics, Histones

1 Introduction

The final effector of many hormones, growth factors, and homeostatic mechanisms is regulation of gene transcription. Genes affected may include those for growth, cell cycle, mitochondrial biogenesis, fission and fusion, anabolism, protein catabolism, glucose and fat uptake, storage and breakdown, and cell type specific genes. FOXO1 is now known to be regulated by several transcription factors including the glucocorticoid receptor, a ligand-activated transcription factor that binds DNA [1, 2]. Given the crucial roles of FOXOs in cell homeostasis and metabolism, and clear links between their expression and cell physiology, understanding of the molecular mechanisms regulating the expression levels of FOXOs is of great importance. Here, we will discuss

classical and emerging omics-based approaches for mapping and understanding biological relevance of transcription factor-binding sites within FOXO genes. Classical approaches are discussed using experience in my lab over the past 15 years to illustrate strategies by which they may be employed. Omics approaches are discussed more broadly with the intent of demonstrating their tremendous power and illustrating how one manages the scale of the data generated at the conceptual level.

When discussing transcriptional regulation it is important to recognize that transcription factors may regulate transcription through directly binding to DNA or indirectly, through binding other, DNA-bound transcription factors in an interaction called tethering. An additional mechanism of transcriptional regulation is through the action of other proteins (transcriptional effectors) that bind transcription factors to displace other transcriptional regulators which are often transcriptional repressors; β -catenin and Notch intracellular domain are well-characterized examples of transcriptional effectors [3–6]. In addition, a growing list of proteins that normally have membrane or cytosolic localizations have been found in the nucleus bound to chromatin and are believed to affect transcription [7]. The discussion below will summarize work in our laboratory that characterized transcriptional regulation through these methods of action as examples of how one might study the transcriptional regulation of FOXO expression.

2 Materials

Materials required for classical methods of analysis of promoter-binding sites include the usual tools of molecular biology. With the widespread availability of kits for library preparation, cloning, sub-cloning, ligation of DNA inserts into reporter genes, and gel shift assays, a detailed discussion of materials is less relevant. Materials required include a high-quality Tac polymerase with a low rate of errors, agarose, ethidium bromide or other RNA/DNA detection method, suitable restriction enzymes and buffers for determining if ligation of the insert into a plasmid was successful, and electrophoresis buffers that can be commercially obtained in most cases. Where gel shift assays are employed it is recommended that commercially prepared solutions of Bis-acrylamide be purchased to reduce risk of exposure to this neurotoxin. Commercially produced gel-shift assays are available and offer nonradioactive detection methods that should reduce the overhead associated with managing P^{32} in the lab.

3 Methods

The advent of inexpensive sequencing of cDNA clones or inserts and development of commercially produced kits and reagents which are provided with detailed instructions has rendered has reduced the need for description of methods for many standard techniques involved in cloning promoter regions, verifying their sequences, subcloning them into sequencing vectors or reporter genes, and mutating them. Procedures such as gel shift assays, while not conducted in our work on transcription factor-binding sites in FOXO promoter, have been described in great detail elsewhere.

4 Notes

1. Classical Methods.

Prior to the advent of next-generation sequencing and the subsequent development of this technology to provide affordable sequencing of large numbers of RNAs or DNA fragments, analysis of promoter regions was performed through traditional approaches of molecular biology. These included a combination of computational analysis, chromatin immunoprecipitation (ChIP), gel shift analysis, DNase I footprinting, and examination of activity of reporter genes in which transcription is controlled by cloned regions of genes of interest. Promoter sequences can be downloaded through <https://genome.ucsc.edu/FAQ/FAQdownloads.html>. Regions of interest can be searched for putative transcription factor-binding sites using online tools such as Patch (<http://gene-regulation.com/pub/programs.html>).

As an example of this approach in the context of FOXO1, we recently asked whether the upregulation of FOXO1 observed in muscle from glucocorticoid-treated animals was attributable to glucocorticoid response elements (GRE, glucocorticoid receptor DNA-binding sites) in the FOXO1 promoter [8]. Specifically, we examined the possibility that upregulation of FOXO1 resulted from transcriptional activation of FOXO1 promoter activity as a result of binding of glucocorticoid receptors (GR) to GREs within the proximal promoter. mRNA stability studies demonstrated that glucocorticoids increased FOXO1 mRNA levels but did not alter FOXO1 mRNA turnover [8]. In these studies, mRNA degradation was measured by the decrease over time of levels of FOXO1 mRNA after the addition of actinomycin which inhibits transcription. These studies were performed in cultured C2C12 myoblasts and indicated that the rise in FOXO1 mRNA levels induced by glucocorticoids was attributable to increased transcription. Computational analysis of the mouse FOXO1 promoter revealed several

clusters of GREs. Several DNA sequences were cloned by PCR (each containing a different cluster of GREs) and ligated into luciferase reporter genes. Luciferase activity was then measured in the presence or absence of the synthetic glucocorticoid dexamethasone. Some but not all GRE clusters conferred glucocorticoid responsiveness on the reporter genes, suggesting that these sequences contained authentic GREs. To prove that predicted glucocorticoid receptor-binding sites were indeed capable of binding this receptor, gel shift studies were performed. Commercially prepared recombinant glucocorticoid receptor was incubated with synthetic oligonucleotides with sequences corresponding to specific putative-binding sites. This analysis confirmed that some of these sequences bound the recombinant glucocorticoid receptor.

Other strategies we have found informative involve sequential removal of specific regions of the entire upstream promoter. This approach is most useful when there is no computationally predicted binding site, a situation that most commonly arises when tethering is involved. In the past, such truncations have been done using either restriction enzyme digests, as we have done for analysis of androgen receptor-binding sites in the MAFbx promoter [9], or by cloning regions of interest using PCR, as was done in our studies of the FOXO1 promoter described above and our work toward identifying the region of the human IGF-1 promoter containing androgen response elements [10]. The advent of CRISPR provides an expedient alternative technology for manipulating sequences.

A tethering-based mechanism should be suspected when either no obvious DNA-binding site is observed by computational analysis or when sequences identified during ChIP-seq do not contain obvious DNA-binding sites for the transcription factor of interest. We encountered this phenomenon when attempting to understand why androgens downregulate expression of MAFbx [9]. We found that removal of a small, 200 bp region of the first exon of the human MAFbx promoter blocked repression of human MAFbx reporter genes by androgens. DNase I sensitivity analysis was used to understand which transcription factors bound this DNA sequence.

For these studies, a 400 bp fragment of the human MAFbx gene was removed from the reporter by restriction enzyme digestion. This fragment was incubated with nuclear extracts from C2C12 mouse myoblasts. DNase I sensitivity of this protein-coated DNA fragment was then determined. Two of the chromatin regions protected from DNase I had sequence similarities to the consensus-binding site for Oct1. Chromatin immunoprecipitation confirmed that androgen receptor and Oct1 each pulled down this sequence in cells expressing wild-type androgen receptor or androgen receptor with a mutant DNA-binding sequence that prevents effective DNA binding. These findings indicated that binding of the androgen receptor to DNA was not required for it to associate

with the 200 bp region responsible for MAFbx repression. Recruitment of androgen receptor to this DNA region was blocked by Oct1 gene knockdown. Ability of Oct1 to bind the putative Oct1-binding sites was confirmed in gel shift assays. Deletion of the two Oct1-binding sites identified by this analysis from reporter genes blocked suppression by androgens of the MAFbx reporter gene. Collectively, these data demonstrated that androgen receptor repressed MAFbx expression through association with DNA-bound Oct1. This general approach can be used to study any situation in which one hypothesizes that tethering is responsible for transcriptional regulation of a target gene by transcription factors or other proteins.

In much of our work, the cell culture studies were conducted using cells that do not express sufficient levels of the transcription factor being studied. This can be both an advantage and a hindrance. The advantage is that one can understand basal activity of the reporter gene(s) being studied. The disadvantage is that overexpression of the transcription factor of interest raises the specter of quenching of other transcriptional regulators by the overexpressed transcription factor, as discussed further below. When one needs to express the transcription factor being studied, traditional methods such as transfection or viral transduction using retrovirus, lentivirus, or adenovirus can be employed. The availability of commercially prepared replication-incompetent adenoviral or lentiviral vectors obviates the time-consuming task of preparing expression plasmids or viral vectors in the lab, thus improving safety and greatly accelerating progress of the research. The high expression levels achieved by these techniques assure a robust response. Data interpretation requires some caution because of concerns of quenching, whereby there is so much of the overexpressed transcription factor that it binds all available transcriptional co-regulators thus biasing results. This is nevertheless a valuable approach.

Where the gene for the transcription factor of interest is expressed at biologically relevant levels, an alternative approach is gene knockdown with the aim of showing that the transcriptional regulation in question is blocked by the removal of the transcription factor under study. This approach is complimentary to overexpression. As noted above, we found this strategy to be very valuable in demonstrating that Oct1 was necessary for association of androgen receptor with DNA sequences within the first exon of the human MAFbx promoter [9] and in identifying androgen response elements in the human IGF-1 promoter [10]. Of note, one can take advantage of species differences in RNA sequence to do gene knockdown using siRNA against the mRNA of interest in the species for the cell being studied and then rescue using plasmids or viruses expressing the same gene but from another species. For example, in many cases, at the level of DNA binding and basic

biological function, human genes introduced into mouse cells recapitulate function quite well.

Another strategy that is valuable in proving involvement of a transcription factor in binding to a specific sequence in a particular promoter is to introduce mutant versions of the transcription factor with constitutive activity or containing mutations that block nuclear entry, DNA binding, or association of transcriptional co-regulators. One can then test how such mutations alter chromatin-binding (ChIP assays) and biological activities (reporter gene activities). These types of studies are most effective when performed using cells that do not express the transcription factor of interest.

Gel shift studies continue to offer certain specific opportunities to understand binding interactions between proteins and DNA sequences. In the past, P^{32} -labeled oligonucleotides were required for these studies. However, nonradioactive alternatives using chemiluminescence and other detection technologies are available and offer a very attractive way to avoid the need for P^{32} . The most challenging part of these assays is obtaining recombinant proteins which may be purchased from commercial sources, purified from bacterial expression systems using GST or other affinity purification methods, or expressed in rabbit reticulocyte lysates that are ideal for labile proteins or proteins that require mammalian systems for proper protein folding. We have used commercially produced recombinant glucocorticoid receptor for gel-shift studies to understand the role of putative glucocorticoid response elements in the FOXO1 promoter [8]. In another study, a GST-tagged androgen receptor was used to isolate overexpressed androgen receptor for gel shift studies examining the location of androgen response elements in the IGF-1 promoter [11]. Using gel shift methods one can test effects of mutations in the nucleotide sequence to understand the roles of various nucleotides in the binding interaction when the binding site is not well characterized or when noncanonical binding sites are identified. A summary of the concepts and decision points in utilizing this classical approach is shown in Fig. 1.

While the focus of this chapter is on identification of DNA-binding sites of transcription factors, a growing number of proteins bind to and modulate DNA-bound transcription factors through tethering as noted above. Tethering is increasingly recognized for its potent effects on gene expression. Classical, well-studied examples include β -catenin, which binds TCF/LEF family members and Notch Intracellular domain which interacts with RBPj_K/CBF-1. Evidence that Ankrd1 binds chromatin-bound androgen receptor has also been presented [12]; interactions between Numb and the myogenic differentiation factor MyoD have also been described [13]. Approaches for identifying and characterizing these interactions use the same toolkit employed for defining transcription factor-binding sites above as was

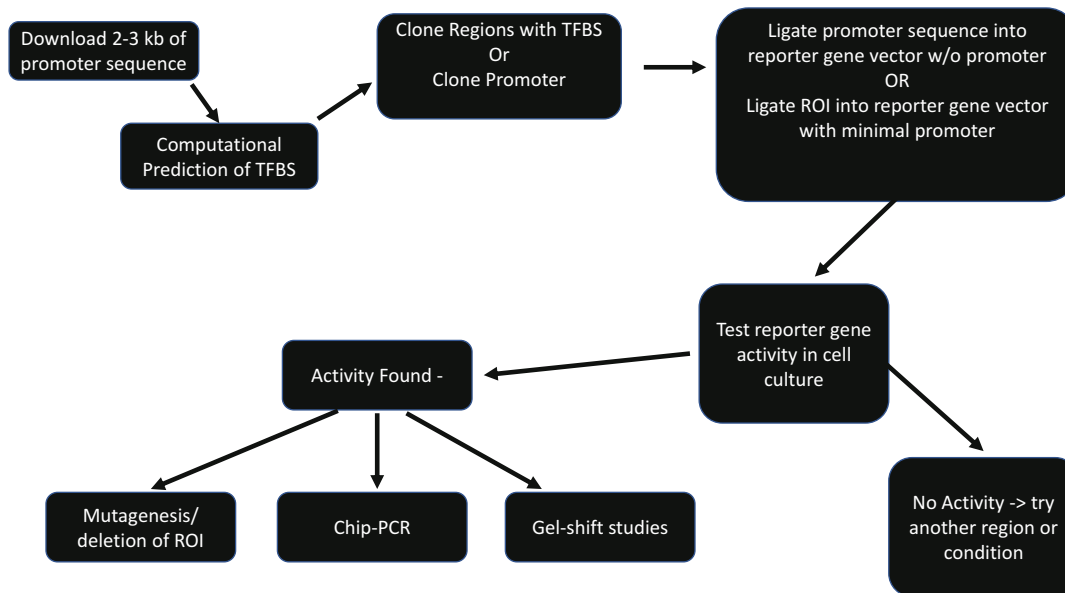


Fig. 1 The sequence of classical procedures used to identify transcription-binding sites within a defined region of interest, usually a proximal promoter, is shown

described for binding of androgen receptor to Oct1 in the first exon of the MAFbx promoter.

Tethering of transcriptional effectors such as β -catenin to transcription factors is more difficult to predict computationally. We have had some success in this regard nonetheless by a reverse engineering approach. We recently sought to understand how canonical Wnt signaling upregulated expression of the Numb gene [14]. We hypothesized that there were TCF/LEF-binding sites in the proximal promoter of the Numb gene. We reasoned that if this was the case then we would see genomic DNA sequences from the Numb promoter containing TCF/LEF-binding sites when we did ChIP with anti- β -catenin antibodies. ChIP studies confirmed that anti-Numb antibodies pulled down the region of the Numb promoter containing one cluster of these sites. A reporter gene containing this promoter sequence was activated by Wnt 3a, a ligand for canonical Wnt signaling. Mutation of the TCF/LEF sites in this reporter blocked upregulation of its activity by Wnt 3a.

A similar though less detailed approach was taken in our examination of the biological relevance of interactions between Numb and MyoD [12]. We hypothesized that if this interaction, identified by co-immunoprecipitation studies, was significant then we should observe recruitment of Numb to MyoD bound to DNA at its target genes. We tested this possibility by determining if Numb was recruited to a published MyoD-binding site in the slow myosin heavy chain promoter and confirmed that this was the case.

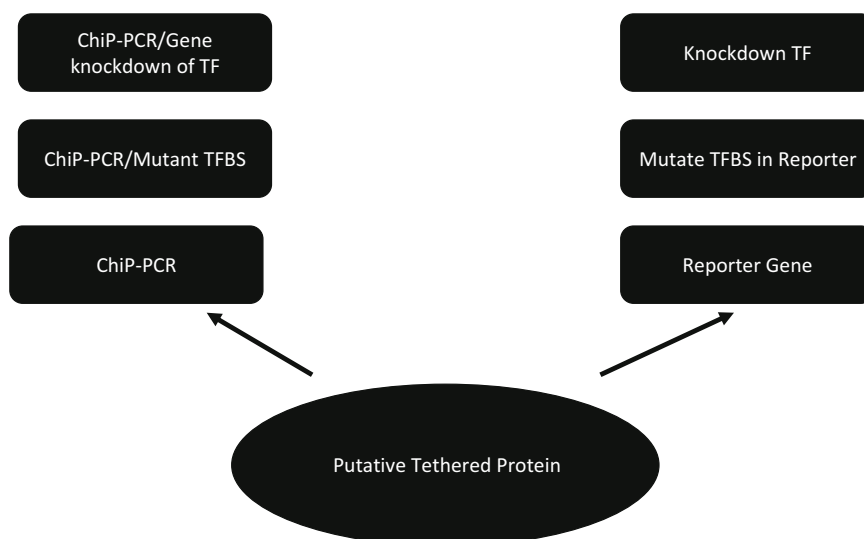


Fig. 2 A strategy of parallel and complimentary procedures that may be used to confirm tethering at specific transcription factors or regions of DNA as a mechanism responsible for regulating transcription

The above approaches provide a means to examine in more detail the biological relevance of such binding interactions. The growing number of growth factor receptors found in the nucleus where they bind chromatin adds greatly to the scope and scale of the potential binding interactions that must be studied. The conceptual framework for our approach to identifying sites of tethering is shown in Fig. 2.

In closing, classical methods are excellent for studying DNA-protein interactions in detail in a single gene for one or a few sites. However, they are impractical for larger numbers of sites and do not provide an integrative view of the interactions between the many regulatory elements present in a single gene. Alternative, omics approaches must be considered when larger numbers of sites are involved.

2. Omics-based approaches.

The major weakness of the classical approach is that because of the scale of the genome, and indeed any particular gene, it is difficult to identify transcription factor-binding sites outside proximal promotor regions. Furthermore, it is now clear that much of the regulation of transcription is the result of enhancers located up or downstream of the promoter region [15] which would not be detected by this strategy.

This limitation has been addressed through development over the last decade of a variety of omics-based strategies made possible by the rapid advances in chromatin immunoprecipitation and next-generation sequencing which make possible combinatorial

approaches to map probable chromatin-binding sites throughout the genome as well as within specific genes and to define how specific signals or hormones influence transcriptional regulation. By use of chromatin immunoprecipitation, one can now identify at high resolution those chromatin regions bound by transcription factors and map how such patterns of chromatin binding change under various biological conditions, such as addition of glucocorticoids, cellular stress, high or low glucose levels in the extracellular space, addition or removal of growth factors, and many others. This strategy will identify both sites at which the transcription factor binds DNA directly and sites at which the transcription factor is tethered to other DNA-bound transcription factors. It is able to identify binding sites in introns, 3' untranslated regions, and distant sequences. By comparing regions pulled down using ChIP with sequences of known transcription factor-binding sites one can infer at which chromatin-binding sites the transcription factor is tethered or bound directly to DNA. An overview of the conceptual basis of these approaches to identify transcription factor-binding sites on DNA and through tethering is shown in Fig. 3.

Several parallel omics techniques provide different perspectives regarding biological relevance of specific transcription factor-binding sites. These include RNA-seq, genomic finger printing, and analysis of histone modifications. The ready availability of RNA seq provides a powerful tool for understanding genome-

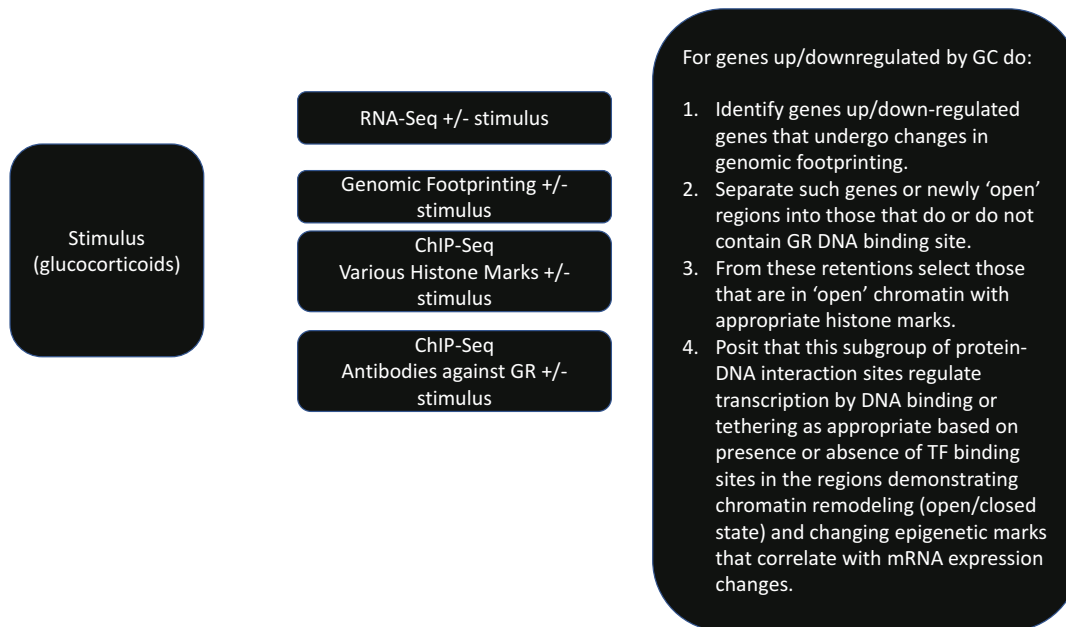


Fig. 3 The overarching logic by which omics approaches may be coupled with RNA-seq data to understand in an unbiased manner how transcription factor binding to promoters and enhancers regulates expression of target genes

wide effects of hormones, cell stresses, and other signals on gene expression. Interpreting changes in mRNA levels detected by RNA-seq makes an assumption that any changes observed are due to altered rates of transcription rather than changes in mRNA stability, which can be confirmed independently in cell culture using quantitative real-time RT-PCR to follow mRNA levels in cells treated with actinomycin to arrest transcription.

Histones undergo a variety of posttranslational modifications that directly affect chromatin folding and include histone methylation and acetylation. These post-transcriptional histone modifications direct folding and unfolding of chromatin into an open (transcriptionally active) or closed state. High-quality antibodies recognizing common histone modifications are available for chromatin immunoprecipitation of covalently modified histones and the DNA bound to them. The general procedure involves sonication to shear genomic DNA, chromatin immunoprecipitation to pull down DNA sequences bound to covalently modified histones, next-generation sequencing to identify these DNA sequences and computer-assisted mapping of the sequences identified. The map then provides the locations within the genome at which corresponding histone modifications occurred. One can overlay the map of transcription factor-binding sites with that of histone modification and thereby identify those transcription factor-binding sites that are associated nearby with epigenetic changes in response to biological cues (e.g., binding of glucocorticoid receptors to DNA). Binding sites that are associated with histone modifications in response to biological signals are presumed to be active in regulating gene transcription. Use of ChIP-seq to study histone modifications is a well-developed field and there are excellent recent reviews on the topic [16–19]. Much progress has been made toward understanding how specific histone modifications influence chromatin structure and transcription. Recent reviews on the topic are included [20–22].

Analysis of hypersensitivity of genomic DNA to cleavage by endonucleases is increasingly used to identify regions of open, transcriptionally active DNA. Hypersensitive regions are considered to be open (unfolded) and transcriptionally active. This approach is sometimes referred to as genome footprinting, alluding to its similarities to the classical DNA footprinting approach we used in our studies of transcription factors bound to the first exon of the MAFbx gene [9]. In these assays, chromatin is digested with DNase I and then sequenced. Sequences are assembled to generate genome-wide maps of endonuclease sensitivity from which chromatin structure is inferred. When maps of open and closed sequences are overlaid with RNA-seq data and maps for histone modifications and transcription factor-binding sites, one obtains a complete understanding of which binding sites are biologically relevant and how they affect gene expression. Methods and

bioinformatics used for DNase I sensitivity analysis are continually evolving. Several recent reviews on the topic include [23–25].

Acknowledgments

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Chapter 4

Investigating Mechanisms that Control Ubiquitin-Mediated DAF-16/FOXO Protein Turnover

Thomas Heimbucher and Coleen T. Murphy

Abstract

Protein turnover of FOXO family transcription factors is regulated by the ubiquitin-proteasome system. A complex interplay of factors that covalently attach certain types of ubiquitin chains (E3-ubiquitin ligases), and enzymes that are able to remove ubiquitin conjugates (deubiquitylases), regulate the degradation of FOXO proteins by the proteasome. Here, we describe methods to characterize candidate E3-ubiquitin ligases and deubiquitylases as regulators of the FOXO ubiquitylation status. Our protocol can be utilized to purify and enrich a ubiquitylated FOXO pool from cultured cells under denaturing conditions, which inactivates cellular deubiquitylases and thereby protects ubiquitin conjugates on FOXO proteins. In addition, our method describes how ubiquitylated FOXO proteins can be renatured in a stepwise fashion to serve as substrates for in vitro deubiquitylation (DUB) assays.

Key words FOXO, DAF-16, Protein stability, Ubiquitylation assay, Deubiquitylation (DUB) assay, Ubiquitin, Proteasome

1 Introduction

The stability of FOXO proteins is regulated through ubiquitylation via ubiquitin pathways and degradation by the proteasome [1]. Attachment of ubiquitin tags to FOXO proteins requires the concerted function of an enzymatic cascade including ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) [2]. A variety of E3-ligases have been identified for FOXO transcription factors in diverse physiological contexts [1]. Posttranslational modifications on FOXO proteins, predominantly phosphorylation, mediate E3-ligase binding to FOXO family members. Upstream kinases, including AKT, ERK, and I κ B, phosphorylate FOXO proteins, and thereby promote their binding to E3-ligases, catalyzing covalent attachment of ubiquitin to FOXO proteins [3–6]. Deubiquitylases (also known as deubiquitylating enzymes or DUBs) act as antagonists to E3-ligases and can reverse ubiquitylation on FOXO proteins [7–9]. Depending on

the type of ubiquitylation, removal of mono-ubiquitin or poly-ubiquitin chains can affect FOXO transcriptional activity or stability, respectively [7, 8].

Here, we describe methods that are instrumental for the study of candidate E3-ligases and deubiquitylating enzymes as FOXO regulators. Using these techniques, novel E3-ligases and DUBs isolated by purification of FOXO-binding partners or obtained through genetic screens in model organisms can be tested for their ability to alter the ubiquitylation status of FOXO proteins.

Our methods focus on the characterization of candidate deubiquitylases for FOXO proteins in vitro. To generate a ubiquitylated pool of FOXO proteins for in vitro DUB assays, we coexpress the FOXO transcription factor DAF-16 with the RING-finger E3-ubiquitin ligase RLE-1 [10] in cultured cells (Fig. 1a). This approach can also be utilized to test novel E3-ligase activities as FOXO/DAF-16 regulators. In order to study deubiquitylation on FOXO proteins by a candidate DUB, we isolate and enrich ubiquitylated DAF-16 along with ubiquitylated proteins from cultured cells under denaturing conditions (Fig. 1b). Denaturing lysis conditions remove non-covalently interacting proteins from FOXOs and inactivate cellular DUBs. Following purification of poly-ubiquitylated DAF-16, we stepwise renature DAF-16 proteins by dialysis (along with ubiquitylated cellular proteins) to utilize it as a substrate for in vitro-based DUB assays (Fig. 1c). We have derived this method from a protocol that was originally established to analyze the function of the DUB USP7/HAUSP on the tumor suppressor p53 [11]. We have optimized this protocol for the FOXO transcription factor DAF-16 and the DUB MATH-33, which are the *C. elegans* FOXO and USP-7/HAUSP orthologs, respectively [12]. Our assays can be easily adapted to FOXO proteins of other species, as they are cell culture and in vitro based.

2 Materials

2.1 Expression and Purification of Ubiquitylated DAF-16/FOXO from Cultured Cells

1. HEK293T cells (ATCC) and standard cell culture equipment.
2. Transfection reagent, e.g., polyethylenimine (PEI).
3. Expression constructs: pHA-MEX-*daf-16* [8], pCMV-FLAG-*rle-1* [10], pCMV-8 × (His₆-ubiquitin) [13].
4. Cell scraper.
5. 1 × Phosphate-Buffered Saline (PBS).
6. Guanidine hydrochloride lysis buffer: 6 M guanidine hydrochloride (GuHCl), 25 mM HEPES pH 7.5, 5 mM imidazole, 0.2% NP40, and 10% glycerol.
7. Syringe needles (18 gauge, 26 gauge) and syringes.

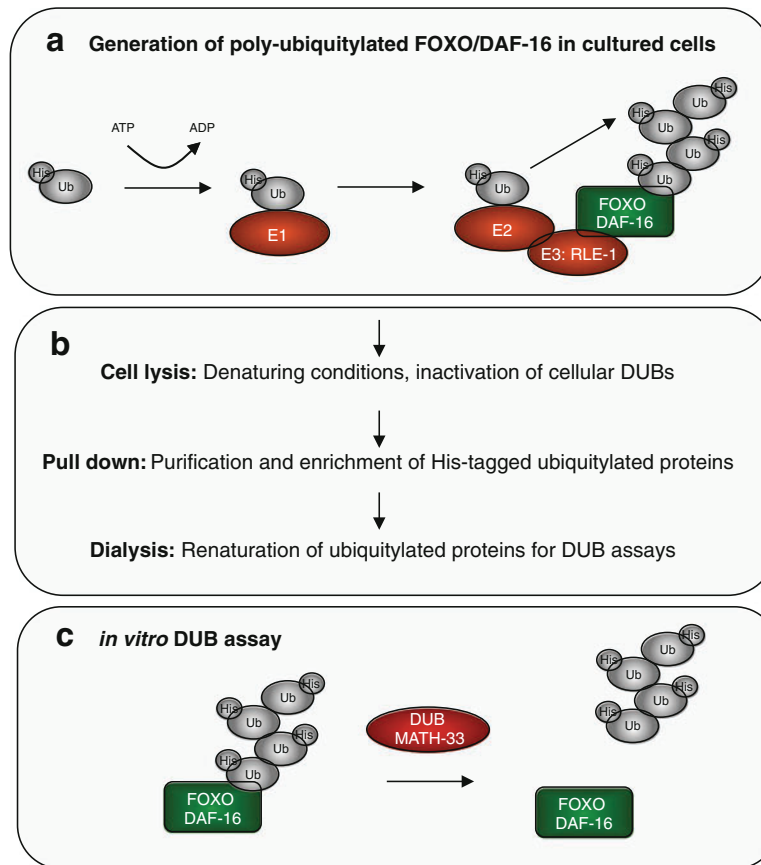


Fig. 1 Generation and purification of poly-ubiquitylated FOXO/DAF-16 for in vitro DUB assays. **(a)** Coexpression of the FOXO transcription factor DAF-16 with the RING-finger E3-ubiquitin ligase RLE-1 and His-tagged ubiquitin results in poly-ubiquitylation of DAF-16. Cellular E1-activating and E2-conjugating enzymes act upstream of RLE-1 to activate and conjugate ubiquitin. **(b)** Flow chart for cell lysis, purification, and renaturation of His-tagged ubiquitylated proteins. **(c)** In vitro DUB assay with recombinant MATH-33 (USP7 ortholog in *C. elegans*) results in deubiquitylation of renatured, poly-ubiquitylated DAF-16

8. 2× SDS-Laemmli sample buffer: 100 mM Tris-HCl pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 200 mM DTT, 0.02% bromophenol blue.
9. TALON Metal Affinity Resin.
10. DAF-16 specific antibody [14].
11. Proteasome inhibitor (MG132).

2.2 Renaturation of Ubiquitylated DAF-16/ FOXO Proteins

1. Slide-A-Lyzer mini dialysis devices, 20 K MWCO.
2. 6 M urea buffer: 6 M urea, 25 mM HEPES pH 7.5, 20 mM imidazole, 0.1% NP40, 10% glycerol.

3. 4 M urea buffer: 4 M urea, 25 mM HEPES pH 7.5, 0.02% NP40, 10% glycerol.
4. 1 M urea buffer: 1 M urea, 25 mM HEPES pH 7.5, 150 mM NaCl₂, 0.02% NP40, 10% glycerol.
5. HEPES buffer: 25 mM HEPES pH 7.5, 150 mM NaCl₂, 12.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, protease inhibitors (0.1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin), 10% glycerol.
6. Elution buffer: 25 mM HEPES pH 7.5, 300 mM imidazole, 150 mM NaCl₂, 12.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, protease inhibitors (0.1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin), 10% glycerol.

2.3 In Vitro DUB Assay for Ubiquitylated DAF-16/FOXO Proteins

1. Bacterially expressed and purified His-USP2-cc (Boston Biochem).
2. Insect cell derived and purified glutathione-S-Transferase (GST)-3×Flag-MATH-33 and GST-3×Flag-MATH-33 C202S (catalytically inactive version of MATH-33) [8].
3. Assay buffer: 50 mM HEPES pH 7.6, 150 mM NaCl₂, 4 mM DTT, 20 mM EDTA, and 5% glycerol.
4. MATH-33 specific antibody [15].

3 Methods

3.1 Expression and Purification of Ubiquitylated DAF-16/FOXO from Cultured Cells

This method can be used to test whether a candidate E3-ligase mediates ubiquitylation on FOXO proteins in cultured cells. Here, we coexpress the FOXO transcription factor DAF-16 with the RING-finger E3-ubiquitin ligase RLE-1 [10] and His-tagged ubiquitin to generate a pool of poly-ubiquitylated DAF-16. His-tagged ubiquitylated DAF-16 proteins are subsequently purified and enriched (along with cellular ubiquitylated proteins) using metal affinity chromatography.

1. Perform a standard transfection of cultured cells. An example is shown below for the transfection of HEK293T cells in a 60 mm plate using polyethylenimine (PEI): 1.5 µg pHA-MEX-*daf-16*, 6 µg pCMV-FLAG-*rle-1* (see Note 1), 3 µg pCMV-(His₆-ubiquitin)₈ (see Note 2).
2. 44–48 h after transfection add the proteasome inhibitor MG132 (final concentration: 20 µM) and incubate cells for 2.5 h. MG132 will result in an accumulation of ubiquitylated DAF-16/FOXO proteins.
3. Aspirate off the cell culture medium, rinse the cells with ice-cold phosphate-buffered saline (PBS). Collect cells by scraping them off the plate in ice-cold PBS with a cell scraper.

4. Set aside an aliquot of the cell suspension (~5%) and boil its cell pellet (95 °C) in SDS-Laemmli sample buffer 10 min for immunoblotting (expression analysis).
5. Pellet cells of the main sample. Continue with cell lysis (*see below*) or shock freeze cells in liquid nitrogen and store them at -80 °C.
6. Lyse cell pellets in guanidine hydrochloride (GuHCl) lysis buffer (*see Note 3*). For a pellet volume of ~200–300 µL use 2–3 mL of GuHCl lysis buffer. Pass the lysate ~30 times through an 18-gauge syringe needle, then ~10 times through a 26-gauge syringe needle to reduce viscosity.
7. Clear the lysate by centrifugation at $16,000 \times g$ for 15 min. Transfer the supernatant to a new tube. Set aside a small aliquot of the cleared lysate (~5%) and boil it in SDS-Laemmli sample buffer 10 min for immunoblotting (this serves as input for pull down assay).
8. Equilibrate 40 µL TALON Metal Affinity Resin (80 µL 50% slurry per pull-down condition) in guanidine hydrochloride lysis buffer.
9. Add the equilibrated Metal Affinity Resin to 1 mL cleared lysate and incubate ~16–18 h on a rotator at 4 °C (*see Notes 4 and 5*).
10. Set aside an aliquot of the resin (~10%) and boil it in SDS-Laemmli sample buffer for immunoblotting. Poly-ubiquitylated DAF-16 is detected with a DAF-16-specific antibody [14] and displays an increase in the “ubiquitin ladder” above the DAF-16 band when the RING-finger E3-ligase RLE-1 is cotransfected [8, 10] (*see Note 6*).

3.2 Renaturation of Ubiquitylated DAF-16/ FOXO Proteins

To analyze whether a candidate DUB is able to deubiquitylate FOXO/DAF-16, the denatured, purified, and enriched pool of His-tagged ubiquitylated proteins including FOXO/DAF-16 needs to be renatured by dialysis. Only renatured FOXO/DAF-16 proteins can be efficiently bound and deubiquitylated by a DUB.

1. Wash the Metal Affinity Resin containing poly-ubiquitylated DAF-16 (*see step 10* in Subheading 3.1.) two times with 6 M urea buffer. Urea solutions need to be prepared fresh (*see Note 7*).
2. Transfer the resin to a 0.5 mL mini dialysis device (e.g., Slide-A-Lyzer mini dialysis device) in a small volume (~100–200 µL) of the 6 M Urea buffer. Fix the mini dialysis device on a polyfoam floating rack. Dialyze against a large volume (200 mL) of 4 M urea buffer in a beaker with stirring for 2 h at 4 °C (*see Note 8*).

3. Dialyze against a large volume (200 mL) of 1 M urea buffer in a beaker with stirring for 2 h at 4 °C.
4. Dialyze against a large volume (200 mL) of HEPES buffer in a beaker with stirring for 4 h (or overnight) at 4 °C.
5. Transfer the Metal Affinity Resin in ~50 µL HEPES buffer to a PCR tube. Set aside an aliquot of the resin (~10%) and boil it in SDS-Laemmli sample buffer for immunoblotting (for detection of bound poly-ubiquitylated DAF-16 proteins before elution).
6. For elution of ubiquitylated proteins from the Metal Affinity Resin add imidazole (final concentration: 300 mM). Seal the lid of the PCR tube with a parafilm strip and rotate the tube for 4 h at 4 °C.
7. Remove supernatant containing eluted poly-ubiquitylated DAF-16 proteins and store on ice. Perform a second elution with 40 µL Elution buffer. Seal the lid of the PCR tube with a parafilm strip and rotate the tube for 4 h (or overnight) at 4 °C.
8. Pool the eluate from the second elution with the eluate from the first elution (results in ~80 µL total eluate). Shock freeze aliquots of eluate in liquid nitrogen and store them at –80 °C. Set aside an aliquot of the resin (~10%) and boil it in SDS-Laemmli sample buffer for immunoblotting (for detection of non-eluted ubiquitylated proteins on the resin, *see Note 9*). Poly-ubiquitylated DAF-16 is detected with a DAF-16-specific antibody in immunoblots [8] (*see Note 10*).

3.3 In Vitro DUB Assay for Ubiquitylated DAF-16/FOXO Proteins

Renatured poly-ubiquitylated DAF-16 (which is in a pool with additional renatured, ubiquitylated cellular proteins) can be utilized in in vitro deubiquitylation assays to detect whether a candidate DUB is able to deubiquitylate DAF-16. The DUB MATH-33 (HAUSP/USP-7 ortholog) derived from insect cells [8] was tested in the DUB assay described below. A catalytically inactive version of the DUB (MATH-33 C202S) served as a negative control. The stable DUB activity of bacterially purified USP2-cc, the highly active catalytic core of USP2, was used as a positive control [8]. Deubiquitylation on DAF-16 through a particular DUB is detected by immunoblotting using a DAF-16 specific antibody (*see Note 10*).

1. Activate the DUB by incubating in assay buffer at room temperature for 10 min (*see Note 11*).
2. Incubate 4 µL of the eluate from above (*see step 8* in Subheading 3.2) containing poly-ubiquitylated DAF-16 with 20–150 nM of the purified DUB in an assay buffer volume of 30 µL at 37 °C for 1 h. Titrate the amount of the DUB, e.g., from 20 to 150 nM.

3. To terminate the reactions add $2\times$ SDS-Laemmli sample buffer and boil at $95\text{ }^{\circ}\text{C}$ for 10 min. Deubiquitylation on DAF-16 is detected in immunoblots using a DAF-16-specific antibody [8], (*see Note 10*). MATH-33 is detected with a MATH-33-specific antibody. In addition, immunoblotting utilizing a His-tag-specific antibody (or a ubiquitin-specific antibody) reveals all cellular His-tagged ubiquitylated proteins and thus serves as a DUB specificity control.

4 Notes

1. A catalytically inactive E3-ligase derivative can be used as a negative control (e.g., pCMV-FLAG-*rle-1 C34S*), or the E3-ligase can be omitted in the control transfection.
2. Octameric ubiquitin is efficiently processed in vivo by cellular ubiquitin-C-terminal hydrolases and thus is more efficiently conjugated to substrates than monomeric ubiquitin [13].
3. A 6 M guanidine hydrochloride solution efficiently denatures proteins and disrupts non-covalent interactions in protein complexes. In addition, 6 M GuHCl denatures all cellular deubiquitylases (DUBs) to prevent deubiquitylation of FOXO/DAF-16 during lysis and purification. Alternatively, lysis may be performed under native conditions in the presence of the DUB inhibitor N-Ethylmaleimide (NEM, 20 mM), which is an irreversible inhibitor of cysteine peptidases. However, NEM could modify cysteine residues on FOXO family proteins as well, which could potentially interfere with the actual DUB assay described in the method Subheading 3.3. Modified cysteine residues on FOXO proteins might block the association of the DUB with FOXO proteins and their subsequent deubiquitylation.
4. We recommend using one-third to half of each lysate (1 mL–1.5 mL) for the pull-down experiment and store the rest of each lysate as a backup at $-80\text{ }^{\circ}\text{C}$.
5. Only a small fraction of DAF-16/FOXO proteins is usually ubiquitylated in cultured cells. The Metal Affinity Resin based purification specifically binds His-tagged ubiquitin and enriches the ubiquitylated pool of DAF-16/FOXO (along with ubiquitylated cellular proteins) for subsequent analysis.
6. Detection of ubiquitylation on DAF-16/FOXO mediated by an E3-ligase activity in cultured cells does not necessarily prove that a particular E3-ligase directly ubiquitylates DAF-16/FOXO. Additional factors acting between the E3-ligase and FOXO proteins as a substrate could be involved causing the ubiquitylation on FOXO/DAF-16. In vivo ubiquitylation

assays are required to test whether an E3-ligase or a subunit of an E3-ligase complex directly ubiquitylates FOXO proteins [16].

7. Urea containing buffers need to be prepared fresh to prevent carbamylation of proteins.
8. Previously established protocols suggest performing stepwise dialysis *after eluting* proteins from the Metal Affinity Resin [11]. However, we found that dialysis of ubiquitylated proteins bound to the resin seemed to greatly improve the renaturation of poly-ubiquitylated DAF-16. Stepwise dialysis of proteins on the affinity resin increased the subsequently eluted fraction of poly-ubiquitylated DAF-16. DAF-16 was more efficiently deubiquitylated in DUB assays when renatured on the resin.
9. Only a fraction of ubiquitylated proteins bound to the Metal Affinity Resin is usually eluted. The non-eluted fraction might largely contain proteins, which are not properly renatured and thus might “stick” to the affinity resin.
10. Poly-ubiquitylated DAF-16 has been purified and renatured along with ubiquitylated cellular proteins. As these additional ubiquitylated proteins are present in the DUB assay, deubiquitylation on DAF-16 through a particular DUB needs to be detected by immunoblotting using a DAF-16-specific antibody (or an antibody against a tagged version of DAF-16/FOXO). An antibody specific to ubiquitin (or specific to the His-tag on ubiquitin) detects all ubiquitylated proteins purified from the cell lysate.
11. The reducing agent dithiothreitol (DTT) is essential for DUB activation. In the DUB assay DTT prevents oxidation of the catalytic cysteine residue of MATH-33/USP7 to maintain the DUB activity.

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Chapter 5

Phosphorylation of FOXO Proteins as a Key Mechanism to Regulate Their Activity

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Abstract

Phosphorylation of FOXO transcription factors is one of the key mechanisms involved in the regulation of the activity, nucleo-cytosolic shuttling, and stability of this family of proteins. Here, we describe several experimental approaches allowing analysis of changes in the phosphorylation of these proteins upon exposure to different stimuli.

Key words FOXO phosphorylation, Western blot, Immunofluorescence, cell fractioning

1 Introduction

It has been widely reported that the FOXO family of transcription factors plays an important role in the regulation of multiple cellular processes via their ability to control the expression of different genes [1]. One of the mechanisms that regulates FOXO activity is phosphorylation. Thus, various stimuli have been shown to trigger FOXO transcription factors phosphorylation in different residues. This event can control multiple aspects of the function of this family of transcription factors, including their degradation [2], nucleo-cytosolic presence [3, 4], or transcriptional activity [5]. It is worth noting that phosphorylation of FOXO transcription factors can take place specifically in the cytosol [6], or in the nucleus [7]. In this chapter we describe different approaches that may be used to characterize FOXO phosphorylation. Namely, analysis of FOXO phosphorylation by Western blot using anti-phospho-FOXO-specific antibodies in whole cell lysates or in subcellular (cytosolic and nuclear) fractions; and analysis of FOXO phosphorylation using anti-phospho-FOXO-specific antibodies in fixed cells or tissues by

Alba Orea-Soufi and David Dávila contributed equally to this work.

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using immunofluorescence followed by microscopic analyses. To this aim, we will describe the protocols used to detect FOXO1 and FOXO3a phosphorylation upon different experimental conditions and cellular models [8].

2 Materials

Prepare all buffers using double distilled water. Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 M Ω -cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. We do not add sodium azide to reagents.

2.1 Preparation of the Lysate Buffers for Western-Blotting

50 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.1% Triton X-100, 1 mg/mL leupeptin, 1 mM EDTA, 1 mM EGTA, and 10 mM sodium β -glycerophosphate (*see Note 1*).

2.2 Preparation of Buffers Used in Nuclear and Cytosolic Protein Extraction

1. Pre-buffer: 20 mM HEPES, 20 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 1.6 mM KOH (*see Note 2*).
2. Buffer A (cytosolic buffer): 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.8 mM KOH, 2 mM dithiothreitol (DTT), protease inhibitor cocktail tablets from Roche (1/2 tablet per 5 mL buffer) (*see Note 3*).
3. Buffer B (nuclear buffer): 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.8 mM KOH, 2 mM DTT, 400 mM NaCl, 1% NP-40, protease inhibitor cocktail tablets from Roche (1/2 tablet per 5 mL buffer) (*see Notes 3 and 4*).

3 Methods

3.1 Analysis of FOXO Phosphorylation by Western Blot

1. Culture the cells with the appropriate medium and experimental conditions and incubate in the presence of the stimuli expected to promote changes in FOXO phosphorylation. For cultures of adherent cells typically this may require culturing cells in plates of 55 cm² at a density of 20,000–30,000 cells per cm² (in any case please note that these experimental conditions may vary significantly for each cell type). In the case of the example shown in Fig. 1, RasV12/E1A-transformed embryonic fibroblasts were cultured at a density of 30,000 cells per cm² (*see Note 5*).
2. For adherent cell cultures, add ice-cold lysis buffer (normally we add 80 μ L in 55 cm² plates) and keep the plates in ice.

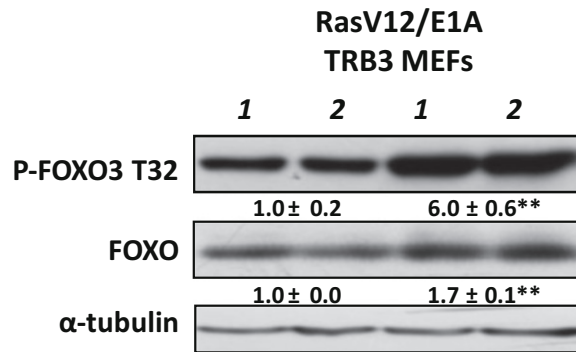


Fig. 1 Analysis of FOXO phosphorylation by Western blot. FOXO phosphorylation was evaluated by Western blot in two experimental conditions: lysates obtained from RasV12/E1A-transformed mouse embryonic fibroblasts wild type (lanes 1 and 2) and knock-out for the expression of the protein TRIB3 (lanes 3 and 4). TRIB3 genetic inhibition promotes AKT stimulation and the subsequent phosphorylation of FOXO. Please note that both FOXO phosphorylation and total FOXO levels were increased, although the increase in FOXO phosphorylation was stronger than that in total FOXO levels. Total FOXO and α -tubulin were included as loading controls. (experiment extracted from Salazar et al. *Cell Death Differ.* 22:131–44 (2015)). Values below the Western blots correspond to the densitometric analysis of the ratio Phospho-FOXO (P-FOXO)/tubulin and total FOXO (t-FOXO)/tubulin respectively and are expressed as the mean fold change $\pm$ s.d. relative to Trib3+/+ cells. ($n = 4$; $^{**} P < 0.01$ from Trib3+/+ cells)

Scratch the cells with a cell scraper until all cells have been removed from the substrate and transfer the cell lysate of each plate/well to an Eppendorf tube.

3. Spin the lysates in a microfuge at $12,000 \times g$ for 15 min at 4°C and keep the supernatant.
4. Determine protein concentration.
5. Load 25 μg of each sample in an 7.5% PAGE-SDS gel.
6. Transfer to PDVF (100 v, 2 h).
7. Block the membrane with 5% BSA during 45 min–1 h.
8. Incubate membranes with anti-PhosphoFOXo antibodies. [In the case of the example shown in Fig. 1) a 1:1000 dilution of the antibody was used (*see* **Note 6**).

3.2 Cytosolic and Nuclear Fractionation and Further Analysis of FOXO Phosphorylation in Cytosolic and Nuclear Fractions

The protocol proposed to obtain the nuclear and cytosolic fractions from cell extracts is based on a protocol previously described [9] with some modifications.

3.2.1 Cell Preparation

1. Plate between 7.5×10^5 and 1×10^6 adherent cells in a 55 cm² plate (or place the same number of cells in suspension in a F75 flask) (*see Note 7*).
2. After performing the corresponding treatment, wash the cells once with ice-cold PBS.
3. Resuspend cells in cold PBS for adherent cells use gentle scraping. If necessary trypsinize them to facilitate resuspension.
4. Centrifuge cells at $300 \times g$ for 5 min at 4 °C, then resuspend the pellet in 1 mL of ice-cold PBS, and transfer to an Eppendorf tube.

3.2.2 Cytosolic Protein Extraction

1. Prepare fresh buffer A and B (*see Note 8*) from the pre-buffer solution and keep them on ice, add 100–150 µL of buffer A to the tube and vortex the pellet for 15 min on ice.
2. To lyse the cells, add 1% (v/v) of NP40 detergent and mix the tube gently for 1 min (*see Note 4*).
3. Centrifuge cells at $13,000 \times g$ for 5 min at 4 °C to separate the supernatant containing the cytosolic protein extract from the pellet containing the nuclear extract.
4. Snap-freeze the supernatant (cytosolic extract) and store at –70 °C (*see Note 9*).

3.2.3 Nuclear Protein Extraction

1. Wash the pellet obtained from centrifugation in the above protocol (point 3) with 1 mL of buffer A, centrifuge at $13,000 \times g$ for 5 min at 4 °C, and discard the supernatant.
2. Resuspend the pellet containing the nuclear material in 50 mL of buffer B, and rock the tube gently for at least 20 min at 4 °C.
3. After incubation, centrifuge the tube at $13,000 \times g$ for 5 min at 4 °C. Collect the supernatant containing the nuclear protein extract and discard the pellet.
4. Snap-freeze the supernatant (nuclear extract) and store at –70 °C (*see Note 9*).
5. For SDS-PAGE Western-Blot analysis load at least 20–100 µg of nuclear or cytosolic extract (*see Note 10*). Use specific protein markers for each fraction (e.g., Iκβ-α for cytosolic fraction and Lamin A/C for nuclear fraction) to verify the correct development of the protocol and normalize the protein content.

3.3 Analysis of Foxo Phosphorylation by Immunofluorescence of Cells and Tumor Samples

1. Culture cells on 12 mm coverslips and treat with the corresponding stimuli.
2. Wash with PBS and fix with 4% paraformaldehyde (15 min at room temperature).
3. Wash with PBS (5 times).

Table 1
List of antibodies used in western blot analyses

Antigen	Host	Company	Application
Phospho-FoxO1 (Thr24)/FoxO3a (Thr32)	Rabbit	Cell Signaling	Western blot and immunofluorescence
Total FoxO3a	Rabbit	Cell Signaling	Western blot
Lamin A/C	Rabbit	Cell Signaling	Western blot
IKB α	Rabbit	Santa Cruz Biotechnology	Western blot
β -actin	Mouse	Sigma Aldrich	Western blot
α -tubulin	Mouse	Sigma Aldrich	Western blot

- Unmask the epitope by incubation with citric acid (10 mM pH = 6) 10 min at 55 °C.
- Permeabilize cells and block to avoid nonspecific binding with PBS + 0.25% Triton X-100 + 10% goat serum (15 min at room temperature) (*see* **Note 11**).
- Incubate the coverslips with the corresponding anti-phospho-FOXO antibody (a 1/300 dilution is recommended) (*see* Table 1 at the Subheading 3) (*see* **Note 12**).
- After wash with PBS (3 times), incubate the sample with Alexa-488 or Alexa-594-conjugated secondary antibodies (Invitrogen; Carlsbad, California, US) in the dark at room temperature for 1 h (*see* **Note 13**).
- Stain cell nuclei with Hoechst 33342 (Invitrogen).
- Mount coverslips in ProLong Gold antifade reagent (Invitrogen).
- Visualized in the microscope.

3.4 Analysis of Foxo Phosphorylation by Immunofluorescence in Tumor Samples

- Once the in vivo experiment has been performed, dissect tissue or tumor samples (in the example of Fig. 2 we show samples derived from subcutaneous tumor xenografts) embed in Tissue-Tek (Sakura; South Carolina, US).
- Before performing the staining procedures, fix samples (previously sliced in the cryostat) in acetone for 10 min at room temperature and freeze down (*see* **Note 14**).
- After fixation, permeabilize and block to avoid nonspecific binding with 10% goat antiserum and 0.25% Triton X-100 in PBS for 1.5 h (*see* **Note 11**).

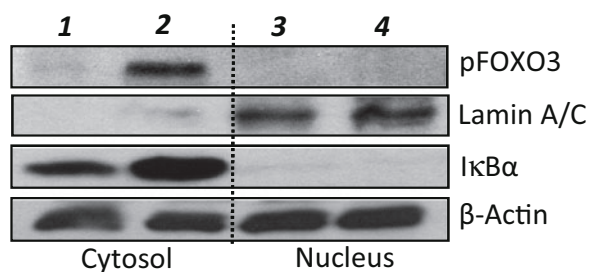


Fig. 2 Analysis of FOXO phosphorylation in nuclear and cytosolic fractions. FOXO phosphorylation was evaluated by Western blot in cytosolic and nuclear fractions obtained in two experimental conditions: lysates from RasV12/E1A-transformed mouse embryonic fibroblasts wild type (lanes 1 and 3) and knock-out for the expression of the protein TRIB3 (lanes 2 and 4). Note that Phosphorylated FOXO is detected only in the cytosolic fraction of *Trib3*^{-/-} cells. (experiment extracted from Salazar et al. Cell Death Differ. 22:131–44 (2015))

4. Incubate with the corresponding anti-phospho FOXO primary antibodies (a 1/200 v/v dilution of the anti-phospho FOXO antibody is recommended, *see* **Note 12**).
5. Wash with PBS (3 times).
6. Incubate with the corresponding Alexa-488 or Alexa-594-conjugated secondary antibodies (Invitrogen; 90 min, room temperature) (*see* **Note 13**).
7. Wash with PBS (3 times).
8. Stain nuclei with DAPI (Invitrogen; 10 min, room temperature).
9. Mount with Mowiol mounting medium (Merck, Darmstadt, Germany).
10. Acquire images in fluorescent microscope. In the case of the example shown in Fig. 3, Metamorph-Offline 6.2 software (Universal Imaging) and Zeiss Axioplan 2 Microscope were used.

4 Notes

1. This buffer must be prepared fresh for each experiment.
2. Pre-buffer can be stored at room temperature.
3. Buffers A and B are recommended to be prepared fresh for each experiment. Both can be prepared diluting pre-buffer 1:2 in ultrapure water and adding 2 mM DTT and protease inhibitors. Posteriorly, Buffer B must be completed with 400 mM NaCl and 1% NP-40.

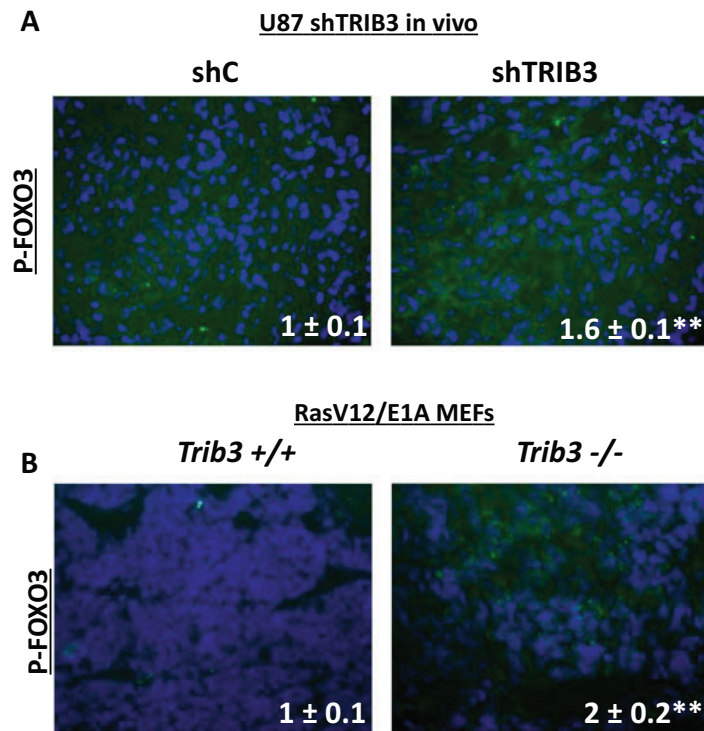


Fig. 3 Analysis of FOXO phosphorylation by immunofluorescence in tumor samples. FOXO phosphorylation was evaluated by immunostaining in tumor xenografts generated by subcutaneous injection of shC and sh*TRIB3* U87MG cells (a) and WT and *Trib3*^{-/-} RasV¹²/E1A-transformed MEFs (b) cells in nude mice. Values in the lower right corner of each panel correspond to the phospho-FOXO stained area normalized to the total number of nuclei in each section and are expressed as the mean fold change $\pm$ SD relative to shC U87MG tumors and WT tumors, respectively (18 sections for each of 3 dissected tumors for each condition were analyzed; $^{**}P < 0.01$ from tumors generated with shC U87MG cells). Note that genetic inhibition of TRIB3 enhances FOXO phosphorylation in these tumors. (experiment extracted from Salazar et al. Cell Death Differ. 22:131–44 (2015))

4. Use a cropped tip to facilitate pipette load and release of the viscous detergent solution NP40.
5. To obtain enough protein to be detected by Western blot we recommend loading at least 25 μ g of protein per well.
6. The incubation with anti phospho FOXO antibodies is overnight at 4 °C in a shaking device.
7. Please note that these experimental conditions may vary significantly for each cell type. In the case of adherent cells should be collected from around 70% confluent plates.

8. For instance, prepare at least 10 mL of buffer A and 1 mL of buffer B for an experiment with 8 samples.
9. In order to avoid repeated freeze/thaw cycles perform protein quantification before freezing the extracts.
10. When using anti-phospho FOXO antibodies with low sensitivity load the maximum possible amount of protein extract per well.
11. Permeabilization and blocking is performed in one step.
12. The incubation with anti-phospho FOXO antibodies is overnight at 4 °C in a wet chamber.
13. The incubation with secondary antibody is performed in a wet chamber.
14. The recommended width of the slices is 12 µm.

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Chapter 6

Methodological Approach for the Evaluation of FOXO as a Positive Regulator of Antioxidant Genes

María Monsalve, Ignacio Prieto, Andreza Fabro de Bem, and Yolanda Olmos

Abstract

All four FOXO isoforms have been shown to respond to changes in the cellular redox status of the cell, and regulate the expression of target genes that in turn can modulate the cellular oxidative status. However, the mechanisms involved are still controversial. It is clear though that redox regulation of FOXO factors occurs at different levels. The proteins themselves are redox-sensitive and their capacity to bind their target sites seems to be at least partially dependent on their oxidative status. Importantly, several of the cofactors that are known to regulate FOXO transcriptional activity are also sensitive to changes in the cellular redox status, in particular the deacetylase SirT1 is activated in response to reduced levels of reducing equivalents (increased $\text{NAD}^+/\text{NADH}^+$ ratio) and the coactivator PGC-1 α is induced in response to increased cellular oxidative stress. Furthermore, nuclear localization of FOXO factors is also regulated by proteins that, like AKT, are themselves regulated directly or indirectly by the cellular levels of reactive oxygen and nitrogen species. In this technical review, we aim to update the current status of our knowledge of how to handle redox-regulated FOXO factor research in order to better understand FOXO biology.

Key words FOXO, Vectors, Activity, Cell culture, Pro-survival, Metabolism, Stress response

1 Introduction

FOXO factors control critically how the cell responds to metabolic and environmental stressors and channels both pro-survival and pro-apoptotic responses. Therefore, when studying a particular type of FOXO activity is crucial to control adequately the experimental conditions and this is particularly true when analyzing FOXO regulation of oxidative stress resistance [1].

Just as an example, since FOXO activity is tightly regulated constitutively active mutants have been extensively used to analyze FOXO functions in a wide array of biological settings. However, under standard conditions, this will result in the induction of cell death, making it difficult the evaluation of FOXO protective, cell survival-related activities both in vitro and in vivo [2, 3].

Most actively-proliferating cells have very limited FOXO levels and activity. Therefore, in order to study FOXO functions the most widely used protocol involves a serum deprivation step to induce cell cycle arrest. However, in sub-confluent and serum deprivation conditions the activation of FOXO normally results in the strong induction of pro-apoptotic pathways and cell death [4]. This pro-apoptotic response can be enhanced by oxidative stress, regardless of whether the source of oxidants is exogenous or endogenous [5].

Therefore, in order to study FOXO pro-survival activities, such as antioxidant gene expression, it is advisable to work in confluent cultures, avoid standard serum deprivation conditions, and control tightly the level of FOXO activity.

2 Materials

Prepare all solutions using sterilized ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 M Ω -cm at 25 °C) and analytical grade reagents.

Handling of DNA and Viral stocks should be done in a tissue culture hood using sterile pipettes and pipette tips with filter. DNA and viral stocks should be kept at -80°C for long-term storage. DNA should be free of endotoxins and dissolved in ultrapure sterile water.

Viral stocks can be maintained in different solutions, but we generally use cell culture grade, sterile $1\times$ PBS. All viruses lose infectivity upon freezing and thawing. Keep handling time of viruses and DNA at a minimum and keep the stock vials on ice. For viruses, use of screw cap microcentrifuge tubes is recommended, preferably those that have a cap that screws over and not inside the vial, to avoid contamination. Before opening and after closing, a viral vial, spray it with 70% Ethanol. Work in a bio-safety level 2 tissue-culture hood and follow bio-safety and waste disposal regulations.

Test Cells. To generate adenovirus 293-A cells should be used, to prepare retroviruses and lentiviruses, Phenix cells are a good choice. These two cell types derive from HEK293 (ATCC[®] CRL1573[™]) human kidney carcinoma cells, and therefore have the same origin than 293T cells. 293T cells are easy to cultivate (DMEM, 10% FBS, antibiotics) and are probably the best choice to evaluate protein overexpression/suppression driven by adenoviral/retroviral/lentiviral infection, or plasmid transient transfection because they are easy to transfect/infect, so require low amounts of viruses or DNA. Using adenovirus a moi of 1 is enough to control changes in protein levels with most vectors, the recommended dose for FOXO vectors is 0.2 moi. Split cells $\frac{1}{4}$, 48 h later cells should be confluent. Mix gently the virus with fresh

media and add it to the confluent culture. Infection with most vectors is generally done overnight. For FOXO vectors 4–6 h is recommended in the presence of serum. To evaluate FOXO levels cells can be harvested as early as 12 h post infection (maximum 24 h).

Target cells of adenoviral infection. The most commonly used adenoviral vectors derive from human adenovirus serotype 5 (HAd5). Targeted cells can vary extensively as reported in the literature. Essentially all cell types of human, mouse, rat, and bovine origin can be infected. Lung epithelial cells are the natural target of adenovirus and hence particularly easy to infect. We have extensively used adenoviruses to infect human, mouse, and bovine endothelial cells (HUVEC, BAEC, MLEC, MAEC), for which the recommended moi is generally 25–50, but for FOXO vectors should be kept below 10. We have also used FAO (rat hepatoma cells) cells and mouse primary hepatocytes, they require 2–4 less adenoviral levels than endothelial cells. Fibroblast are generally hard to infect and for NIH3T3 cells it is almost impossible. Both confluent and subconfluent cells can be used for Ad infection, preferably in the presence of serum to reduce toxicity and cell death as indicated above. To enhance FOXO pro-cell survival activities after the infection the cells can be incubated in cell culture media without glucose and with 5 mM galactose in the absence of serum for 48–72 h or placed in an oxygen controlled cell culture incubator at 3–5% O₂.

Other common reagents that can be used to increase FOXO activity. The most common one is low serum, under 2% FBS serum this will induce cell death in most cell types, use as an alternative 2% Horse Serum (HS) is recommended. The second most common reagent used is hydrogen peroxide (H₂O₂), use of subtoxic doses is recommended. 50 µM for endothelial cells is enough to drive the activation of FOXO, the activated protein can be detected up to 9 h after the treatment (nuclear, dephosphorylated, deacetylated protein). Nitric oxide (NO), an important regulator of vascular function, can also be used to inhibit FOXO activity in endothelial cells. A variety of NO donors can be used to induce the phosphorylation of Akt and the inactivation of FOXO, each has an optimum time and dose, we generally used 62 µM DETA-NO for 12 h in serum deprivation conditions on confluent BAEC cultures.

Vectors and mutants. We used the plasmid vectors originally generated in the laboratory of Dr. K. Walsh and published in [6] to overexpress HA tagged wt FOXO3, and two mutant variants, subcloned into a shuttle vector pAdTrack-CMV, which contains green fluorescent protein (GFP) under the control of a separate cytomegalovirus (CMV). The FOXO3a-AAA triple mutant (TM-FOXO3a) is not phosphorylatable by AKT [7]. The dominant-negative form (dn-FOXO3a) was constructed by the deletion of the C terminus

transactivation domain. The shuttle vector was cotransformed into *Escherichia coli* with the adenoviral backbone plasmid pAdEasy-1 to generate recombinant adenoviral DNA and then transfected into the packaging cell line 293A to produce the recombinant adenoviral vectors. The control virus expressing only the GFP transgene (Ad-GFP) was prepared by the same system [8]. Silencing adenoviral vectors were generated using the pSilencer™ Adeno CMV Promoter System from Ambion. Silencing oligonucleotides were designed and tested by transfection, cloned in pSilencer and transfected into 293A cells to generate viral particles. Silencing oligonucleotides and controls were described in [9].

Antibodies. To monitor FOXO protein levels and activity is recommended to test the level of AKT phosphorylation that largely controls FOXO nuclear exclusion. There are a number of good antibodies in the market that are directed specifically against AKT phosphorylation sites. It is also relevant to test the levels of FOXO acetylation that is important in the control of the levels and activity of the protein. Due to the lack of specific antibodies, the protein has first to be immunoprecipitated and then the IP material tested by WB with a second antibody against FOXO and with an anti-Acetyl Lysine antibody. To efficiently IP the FOXO protein even in very stringent conditions like those required for CHIP assays (up to 2% SDS), the HA-tagged protein can be used since there are very good monoclonal antibodies in the market directed against the HA tag, such as the mouse monoclonal antibody 12CA5. It is also relevant to be able to visualize FOXO subcellular localization directly with specific antibodies that can be used in fixed cells and tissues. Finally, when using plasmids or viral vectors that also encode for a reporter protein, like GFP, evaluation of its levels by western blotting can be helpful to estimate the quality of our viral preparation in terms of levels of FOXO/GFP.

3 Methods

3.1 Vectors

To overexpress or knock down FOXO factors, in principle any kind of vector can be used; however, the nature of FOXO activities makes the use of many common strategies somehow difficult, particularly when overexpression of constitutively active mutants is involved.

3.1.1 Transient Transfection

Usual transfection protocols require that cells are transfected at 30–70% confluency in serum deprivation conditions, and after 6 h to overnight, cells are washed and incubated with regular serum-containing medium with serum prior to analysis 24–72 h post-transfection. Conventional constitutive vectors that drive the overexpression or knockdown of FOXO, specially the constitutively active form, will induce cell cycle arrest and/or apoptosis in these

conditions. To overcome this problem, it is advisable to use either an inducible vector or alternatively a very low copy number of a plasmid that codes for the wild-type construct or expresses it at relatively low levels (*see Note 1*). In this case, transfections work best when cells are transfected at a relatively high confluency, 70–90%. To be able to make the transfection work in these conditions the cells have to be split just before transfection. If the transfection reagent requires serum deprivation, transfection time should be reduced to a minimum (4–6 h) [9, 10].

3.1.2 Viral Vectors

FOXO activity in a virus-infected cell will induce cell death. This is especially true when using constitutive viral vectors but also the inducible ones will be unhappy bearing this gene. Therefore, when a viral stock is prepared that bears a FOXO active gene it can never be assumed that it will always have the same activity, independently of the number of active viral particles. Also, the use of previous viral stocks to generate a new one will result, in most cases, in an important reduction in the level of FOXO activity. Consequently, when working with viral vectors is advisable to limit viral expansion cycles and prepare viral stocks from the viral plasmids. The viral stocks have to be carefully evaluated both for the number of viral particles and the level of FOXO activity and both have to be controlled in the actual experimental setting.

Another consequence of FOXO activity is that its expression in the packaging cell line used to generate viruses will be detrimental both to the cell and to the virus. Therefore, high levels of transfection (high copy number/cell), low density of the packaging cell in culture, and lengthy transfection protocols should be avoided, as noted before in general for transfection protocols. Also, the number of viral particles produced cannot be expected to be very high. In our experience, the average number of particles with FOXO vectors is 10–100 times lower than with other genes.

3.1.3 Infection of Target Cells

To achieve an effective integration of the retroviral or lentiviral vector in the target cell genome, viral infection is carried out in subconfluent conditions, and cells are then incubated in media with serum so that they proliferate prior to the selection of positive clones.

Similarly, cell lines that bear retroviral and lentiviral FOXO genes will tend to silence these genes (more than other genes) since they reduce their proliferation rates. Consequently, FOXO activity should never be taken for granted even following 1–2 cell passages, and coexpressed gene expression (i.e., GFP, luc) will not guarantee FOXO activity. Accordingly, the use of inducible vectors would always be strongly recommended, since it will noticeably reduce (not cancel) the problem.

Use of Adenoviral vectors, in this context, offers an advantage over other gene delivery systems. Infections do not require to work in subconfluency or serum deprivation conditions. Nevertheless, the generation of the viruses themselves has the same limitations as with other viral particles. Similarly, low overexpression rates and use of wild-type over constitutively active FOXOs are highly recommended [9–11].

3.2 Standard Infectio Protocol

Let cells just reach 80–90% confluency. Reduce serum levels to 2%–5% (depending on cell type to reduce proliferation rate), infect the cells to result in a $2\times$ increase over endogenous FOXO levels for 4–6 h. Change media and let the cells recover o/n with media +10% FBS. On the morning cell culture should have just reached confluency. Test FOXO activity.

3.3 Additional General Considerations

Dosing. Taking into account that the precise control of FOXO activity can be difficult, we routinely tested 3 doses in each experimental test. The reference dose would be the one expected to yield the maximum activity, and along with it a 50% dose and a $2\times$ dose would be tested.

3.4 Generation of Stable Cell Lines

In general, overexpression of FOXO will result in the reduction of cell growth and as indicated, may result in the induction of cell death, particularly in subconfluent cell cultures in the presence of serum. Generally generation of stable cell lines requires the infection with retroviruses or lentiviruses in subconfluent cultures and the cells have to be able to grow and expand later. Therefore, the use of conditionally activable vectors is highly recommended or when it is not possible, an alternative could be the use of vectors with the wild-type protein, not the TM constitutive active version and under a relatively weak promoter (not like CMV), and avoid tags, that may stabilize the protein. In these conditions overexpression levels will never be very high, but changes in activity may still be significant (Note 2).

3.5 Activity Assays

FOXO proteins are highly regulated at a post-transcriptional level and as a consequence its overexpression or even its silencing does not guarantee a measurable change in activity. To overcome this problem both constitutively active and negative dominant mutant versions have been developed and widely used [6]. By far the most commonly used version has been the so called TM mutant, for FOXO1 and 3, that carry three point mutations that make the proteins insensitive to inactivation by AKT-dependent phosphorylation [12]. The handling of vectors that carry these mutants is particularly difficult since these constitutively active forms effectively drive apoptotic cell death in the cells that express them, including both the packaging cell for viral particles and the target cells. To tackle the problem there are two options. An obvious one

is to avoid these mutants, but if that is not possible. There are some considerations that can be helpful.

3.5.1 Dosage

If you intend to evaluate a FOXO activity that is different from cell death it is advisable to reduce the dosage of the protein to the minimum that will provide a readout of the intended determination and that means to work in conditions where overexpression is less than $2\times$ over the endogenous protein.

3.5.2 Timing

FOXO TM mutants do not only activate pro-apoptotic genes even if the obvious endpoint is cell death. Evaluation of the impact of FOXO on other target genes, like stress resistance genes with these mutants, is therefore possible before the apoptotic cascade is induced. In general, a fully regulated apoptosis process takes 24 h from the initial signal to the final cell death. If the stimuli is weak (i.e., low levels of active FOXO) it can take 48 h or more. That provides a window where other FOXO activities can be evaluated prior to the onset of the apoptotic activities of up to 24 h but that requires that evaluation quickly follows infection or transfection procedures (**Note 3**).

Whenever possible it is better to handle the wild-type protein to both increase and decrease its activity using wt vectors for its overexpression and sh vectors for its knockdown. Overexpression even of the wt protein should be moderate (about twofold) and the activity could be exogenously stimulated.

3.5.3 Cell Culture Conditions

1. *Confluency*. As noted above the cell death-promoting activities of FOXO proteins will be importantly reduced when working with confluent fully cell cycle arrested cultures. As a result, to evaluate FOXO protective roles, it is advisable to work with primary cells or cell lines that effectively respond to confluency and fully arrest cell growth. When that is not feasible it is advisable to work within the time frame in which cells are confluent but not over-confluent.
2. *Serum withdrawal*. To enhance FOXO protective activities and to control cell overgrowth it is common to reduce the serum concentration and/or not to use fetal serum to reduce the levels of growth factors. However, growth factor deprivation in standard culture conditions can also result in the activation of apoptotic cell death, in order to prevent it, oxidative metabolism should be activated [5]. This will increase the activity of several FOXO cofactors like PGC-1 α and SIRT1 [11] that cooperate with the FOXO protein to induce protective responses.
3. *Glucose/galactose*. The easiest way to activate oxidative metabolism is to use a cell media where glucose has been replaced by galactose, this change reduces the glycolytic flux and activates

oxidative metabolism. Reduction in the concentration of glucose in the media would have the same effect but can compromise cellular viability. Most non-tumor cells when they reach confluency can be maintained in media without serum in the presence of galactose for at least 48 h without compromising their viability [13].

4. *Oxygen*. Most cell incubators do not control oxygen tension, and as a result oxygen concentrations are higher than those encountered by the cells in the organism. Hyperoxia results in the inhibition of oxidative metabolism in order to prevent the resulting oxidative stress, since superoxide production in the mitochondrial ETC increases with supra-physiological oxygen concentrations. Even though each cell type has a different optimal oxygen concentration a good estimate is that incubation in 3–5% oxygen will result in the activation of mediators of oxidative metabolism [14, 15], conditions that result increase FOXO protective roles, preventing the induction of cell death pathways. Under about 2% oxygen, oxidative metabolism would be compromised.

3.6 Step by Step Protocols

3.6.1 Transfection

1. A standard protocol would be:

- (a) Split cells.
- (b) Transfect.
- (c) Add fresh media.
- (d) Harvest.
- (e) Analyze.

Example (Fig. 1).

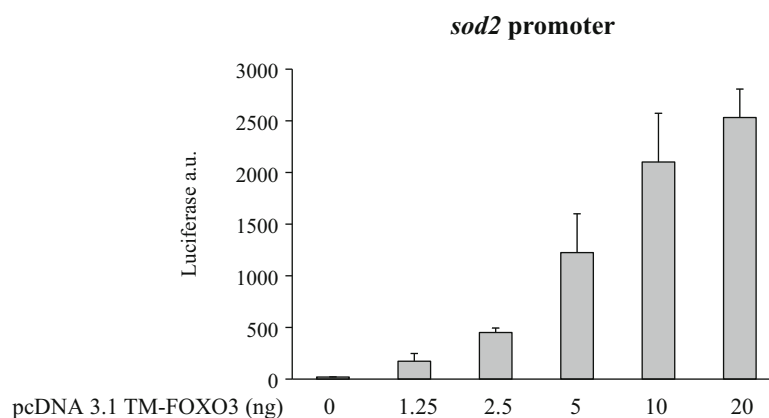


Fig. 1 Subconfluent BAEC cells seeded on 12-well plates were transfected with Lipofectamine 2000 for 6 h. The DNA mixture contained per well 250 ng of a luciferase reporter plasmid, containing 2 Kb of the promoter region of a FOXO3-regulated antioxidant gene (*sod2*) and 20 ng of a mixture of a TM-FOXO3 vector with its control. Cells were harvested 24 h post-transfection

1. Day 1, Split. BAEC cells passage 3–6, 90% confluent split 1/4 on 12-well plates. Split one 10 cm dish or a 75 cm² flask in 4 12-well plates.
2. Day 2, transfection with lipofectamine 2000
 - (a) Lipofectamine (Invitrogen) is diluted in culture media without serum or antibiotics (1 µL lipofectamine/70 µL media).
 - (b) The DNA pool is diluted 1 µg DNA/140 µL media. Vector concentration should be reduced at least 5× over standard. The DNA pool included per well 250 ng of the luciferase reporter vector (pGL3 in which 2 kb of the sod2 promoter had been cloned) and the indicated amounts of the TM-FOXO3 plasmid and the corresponding control vector, so that the total DNA amount per well is constant.
 - (c) The dilutions are mixed 1:1 and complexes were allowed to form for 20 min at RT.
 - (d) Remove the media from the plates.
 - (e) Mix the complexes with fresh cell media (0.5 mL) and then add them to the cells.
3. Transfection is allowed to proceed in standard media but in the absence of serum for 6 h. Fresh media with 10% FBS is then added.
4. Harvested 24 h later, the cell culture should be 100% confluent.
 - (a) Each well is washed 3× with cold PBS.
 - (b) Add 150 µL of lysis buffer to the cells and keep in a rocker on slow motion for 30 min at RT.
 - (c) Collect the lysate and transfer it to a 1.5 mL microcentrifuge tube. Keep in ice.
 - (d) Centrifuge to remove cell debris 1 min at 13,500 rpm at 4 °C.
 - (e) 30 µL of the lysate is allowed to react with 25 µL of luciferase substrate.
 - (f) Measure the emitted light in a luminometer, capturing the signal for 2 min.

3.6.2 Adenoviral Infection

1. Preparation of viral stocks.
 - (a) Transfect 293A cells with the adenoviral plasmid vector. Take 1 µg of DNA to transfect using Lipofectamine 2000 one well (90% confluent) in a 6-well plate dish for 4–6 h.
 - (b) Do not change the media unless it turns yellow. Harvest the cells by scraping when cytotoxicity becomes evident and before the cells detach from the dish. This should occur about 7–10 days post-transfection, if after 2 weeks

in culture the cells do not show signs of viral infection, discard and start again. Wash with cold PBS 2×, scrap the cells from the dish, resuspend in 300 µL PBS, lyse the cells using 3 cycles of fast freezing (in liquid N₂) and thawing (37 °C bath), centrifuge for 5 min at 5000 rpm (2500 × *g*) at 4 °C to remove cell debris. Take the supernatant, aliquot it in freezing vials, and store at −80 °C. Evaluation of GFP positive cells using a fluorescence inverted microscope can be used to evaluate transfection efficiency.

- (c) Use 1/3 of the total viral preparation to infect a 3 cm dish of confluent 293A cells. Gently mix the viral preparation with the cell media and then add it to the cells. Reduce the media volume to a minimum (1.5 mL). Change the media 6 h later. As in the previous cycle, harvest the cells by scraping when cytotoxicity becomes evident (loss of cell volume, cytosol shrinkage) and before the cells detach from the plate, now this should occur about 5–7 days post-infection. If the number of days that it takes to detect cytotoxicity is not significantly reduced in comparison to the previous amplification cycle, discard. Lyse in 1 mL.
- (d) Repeat the infection/harvesting cycle again using 1/3 of the total viral preparation but infecting one 10 cm cell plate, and then again in a 15 cm cell plate. At this point the cytotoxicity should be evident about 2 days post infection, if there are no signs after 3 days of infection, discard.
- (e) Use 1/3 of the viral preparation to infect 20 15 cm cell dishes, cytotoxicity should be evident about 2 days post infection, if there are no signs after 3 days of infection, discard. Prepare 5 mL of cell lysate.
- (f) Purify the preparation passing it through a commercially available purification column (i.e., Cell Biolabs) and following the manufacturer's instructions.
- (g) To evaluate the number of infective viral particles (pfu), prepare serial dilutions in PBS up to 10–15 and use them to infect 10 wells of a 96-well cell plate with confluent 293A cells (seeded directly to confluency and infected 6–12 h later). Count the number of wells with viral halos (clear circles) per dilution to estimate the pfu/mL. For FOXO viruses, the final viral yield is generally 10^{10–11} pfu/mL.
- (h) When you run out of the stock, DO NOT use it to re-amplify de virus. Start again from the previous cycle of viral preparation. Since FOXO viral vectors are highly unstable we avoided serial re-amplification of viral stocks and always used previous cycle preparations or prepared from plasmid vectors to generate new stocks.

- (i) Following the evaluation of the pfu using a standard serial dilution procedure it is highly recommended to test for the level of protein expression. Both primary cells (i.e., BAEC) and cell lines (i.e., EBNA) can be used for Adenoviral (Ad) load testing. As a rule, we tested first on a cell line that is easily infected, which requires 1–2 moi to get all the cells infected, and retested to confirm the optimal working conditions on the cell type that is under investigation (BAEC). Using FOXO viruses is highly recommended to use a vector that co-expresses a reporter gene, like GFP and, as indicated above, use the FOXO/GFP ratio as a quality control evaluator of the viral preparation.

Example (Figs. 2 and 3)

Infect confluent cultures in 10% FBS for 6 h with a control Ad-GFP virus, the Ad-TM-FOXO3 virus and carry on noninfected cells.

Change to fresh media and let cells recover o/n, change media again and 24–48 h later monitor infection in an inverted

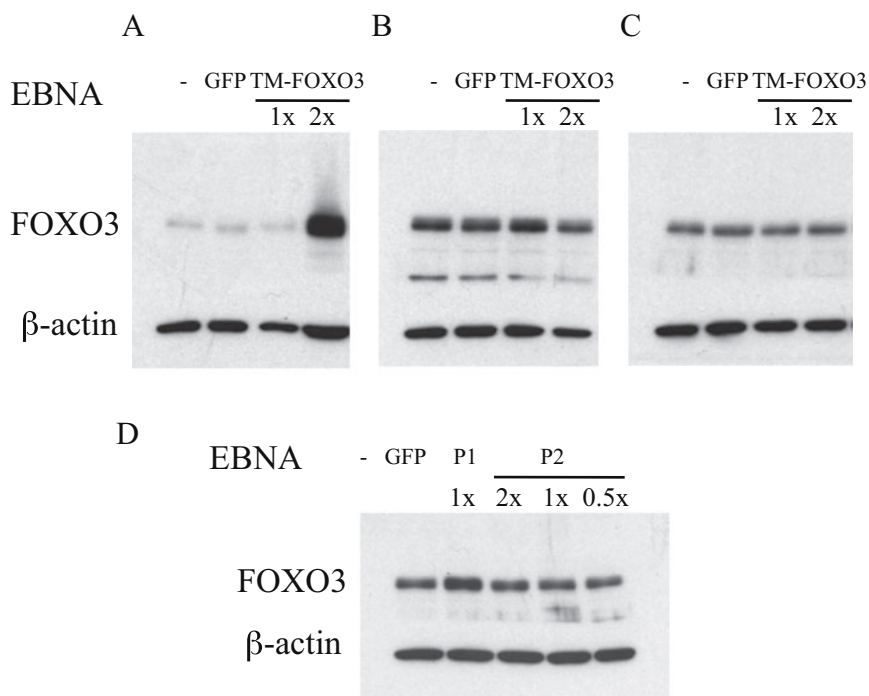


Fig. 2 Confluent EBNA cell cultures were noninfected (C), infected with a GFP control Ad vector (GFP) and with the indicated doses of the tested FOXO3 Ad vector. In the panels A-C three different viral preparations were tested. The preparation on panel A yielded a good level of FOXO3 expression, the preparation on panel B was marginally positive for the recombinant protein expression, and the preparation on panel C was negative for FOXO3 expression. On panel D two different preparations are compared: preparation 1 was positive and preparation 2 negative for FOXO3 levels

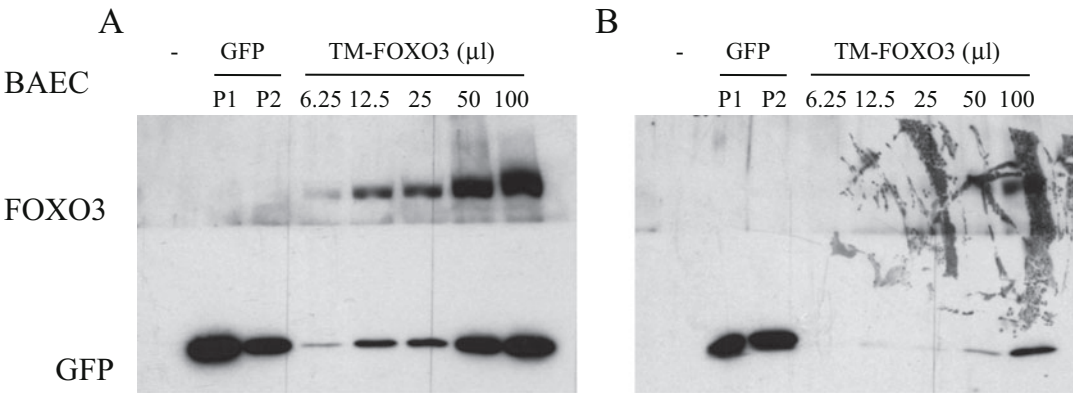


Fig. 3 Confluent BAEC cell cultures were noninfected (C), infected with a GFP control Ad vector (GFP) and with the indicated doses of the tested FOXO Ad vector. The viral concentration of the preparation in panel B (second preparation) was markedly lower than that of panel A (first preparation) but the FOXO/GFP ratios were found to be similar, so the two preparations were considered to be suitable for experiments

fluorescent microscope to evaluate GFP levels and harvest cells. Whole cells extracts were used for WB for GFP, FOXO3, and β -actin as loading control. GFP controls for viral load (**Note 4**).

3.6.3 Evaluation of FOXO Stress Resistance Activity

As outlined above, FOXO needs to work in conditions when oxidative metabolism in quiescent confluent cultures to efficiently induce the pro-survival stress resistance programme, because in these conditions the activity of the cofactors that boost its antioxidant activity will be maximized. Therefore, the levels of oxidants will be kept under control, and will not be able to drive cell death. However, it has also to be noted that once a certain oxidant level is reached, even in these conditions (actually boosted by them), the cell death program will be activated in a FOXO-dependent manner. In other words, FOXOs control ROS more efficiently in conditions of nutrient limitation but also drive apoptosis in these conditions once a certain threshold level of oxidants is reached.

As a consequence, to induce antioxidant or stress resistance genes efficiently in a FOXO-dependent manner, several procedures can be implemented as indicated above. The simplest one of them is to work with constitutively active proteins in standard culture conditions, but using very short time spans, before the cell death program is activated. Obviously, this will not suffice to evaluate cell death resistance but will provide a robust and highly reproducible gene expression control setting (**Note 5**).

3.6.4 Working with a Constitutively Active Mutant. Example (Fig. 4)

1. Infect confluent HUVEC cells for 6 h with equivalent viral doses of Ad-GFP or Ad-TM-FOXO3 in triplicates.
2. Change the media and allow cells to recover o/n.
3. Harvest cells prior to WB analysis

Silencing sh-FOXO vectors provide a more physiological testing setting, however to induce FOXO activity and be able to detect

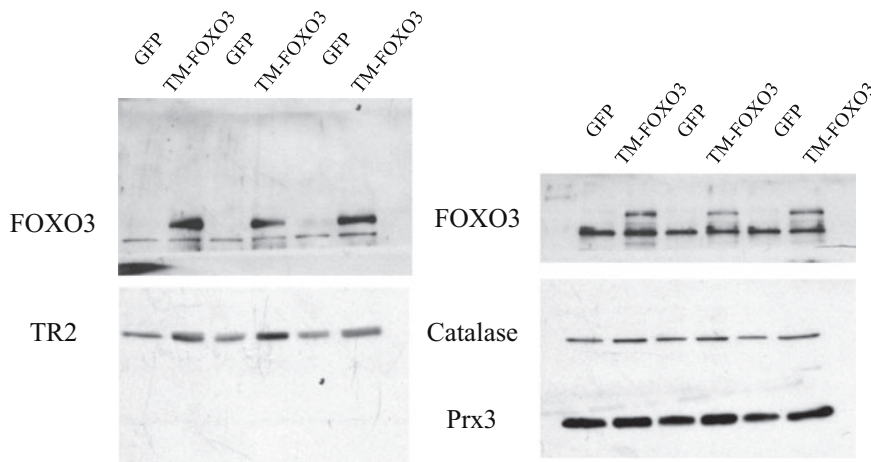


Fig. 4 6-well plates of confluent HUVEC cells (p6) were infected with equivalent viral doses of Ad-GFP or Ad-TM-FOXO3a in triplicates for 6 h, media was changed and cells allowed to recover o/n and cells were harvested prior to WB analysis

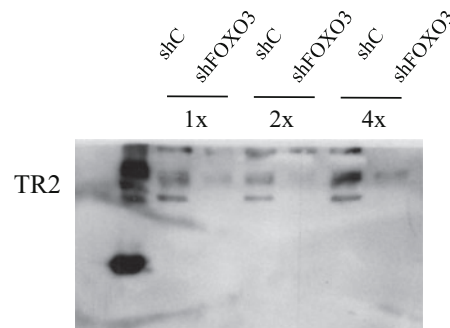


Fig. 5 6-well plates of confluent MEF cells (p2) were infected o/n with three equivalent doses of Ad-shControl or Ad-shFOXO3 in standard media with 10% FBS, cells were allowed to recover during the day in media supplemented with 10% FBS, and then the cells were serum deprived o/n (0.5% FBS) and harvested prior to WB analysis

differences in stress resistance gene expression, it has to be activated, the simplest procedure being serum deprivation. Since serum deprivation induces cell death, partial deprivation and short experimental time frames are the most convenient setting.

3.6.5 Working with a Silencing Vector. Example (Fig. 5)

1. Infect o/n confluent cultures of targeted cells in standard culture media with 10% FBS with Ad-shControl or Ad-shFOXO3.
2. In the morning add fresh media with 10% FBS.
3. The same evening change the media again, this time the media would be supplemented with just 0.5% FBS.
4. Harvest the cells the next morning.

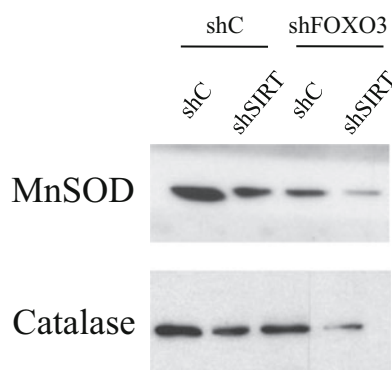


Fig. 6 6-well plates of confluent BAEC cells were infected with equivalent doses of Ad-shControl, ad-shSIRT1, and Ad-shFOXO3 as indicated. Cells were first infected o/n with ad-shControl and Ad-shSIRT1, allowed to recover for 8 h, and then re-infected o/n with Ad-shControl and ad-shFOXO3, serum deprived for 8 h (0.5% FBS) and harvested prior to WB analysis

3.6.6 Evaluating
the Effect of a Co-Factor.
Example Fig. 6

When evaluating the effect of a co-regulator on FOXO activity we may have to simultaneously modify the levels of the co-regulator and FOXO. Working with viral vectors or transfections means to work with relatively high doses of vectors (double what would be normally required). Since dealing with FOXO in standard culture conditions may result in enhanced cell death, it is highly recommended to establish two step protocols, where the vector for the cofactor is incorporated first.

Example.

1. Confluent BAEC cells (p3) in 6-well plates are infected o/n with Ad-Control or Ad-shSIRT1, aiming to silence the FOXO activator SIRT1 in standard culture media with 10% FBS.
2. The following morning fresh media is added supplemented with 10% FBS and the cells are allowed to recover during the day.
3. Then, the cells are infected with Ad-control or Ad-shFOXO3 o/n.
4. The following morning, free media is added supplemented with 0.5% FBS,
5. 8 h later the cells are harvested.

4 Notes

1. Transfection assays for reporter gene expression. When testing for transcription factor activity using reporter vectors encoding luciferase, it is generally of critical importance to test the dose and time of transfection. Even though this is generally true, we found that it is even more so for FOXO evaluation, to test a wide range of plasmid concentrations and time of response, and

it is generally advisable to work with as low levels of FOXO driving plasmid as possible unless it is an inducible vector. Working with low levels of FOXO will prevent cell cycle arrest and will facilitate the testing of FOXO activity in confluent cell cultures where its activity is more readily induced without the concomitant activation of programmed cell death.

2. FOXO antioxidant activity depends to a large extent on the coactivator PGC-1 α that is frequently downregulated or completely inactive in highly proliferative cells such as cancer cells. As a result, working with primary cells is easier to analyze FOXO antioxidant activities.
3. Oxidative stress induces the acetylation of FOXO and simultaneously activates the deacetylase SIRT1. The capacity of FOXO to induce antioxidant genes in response to increased ROS therefore largely depends on the activity of SIRT1. Since the acetylation of FOXO drives its degradation, to follow FOXO activity in response to oxidative stress it is necessary to identify the time window in which FOXO is acetylated by not yet degraded.
4. While working with virus, it is common to use a concentration that ensures that every cell in the dish is infected, as evaluated by a reporter like GFP. Viral infection *per se* is stressing your cells, and when you increase simultaneously FOXO activity, that may compromise viability and cell cycle progression. So, working with low levels of infection can be actually an advantage.
5. Every cell type has a different level of antioxidants that this will change the dose of pro-oxidant molecules or level of pro-oxidative insult required to activate FOXO. Testing of the minimal dose that mediates FOXO activation in your cell of choice is highly recommended since that will allow you to follow the capacity of FOXO to respond to the oxidative challenge as well as its functional consequences. When apoptosis is induced simultaneously the impact of the process on redox control is so huge that will prevent any meaningful determination of ROS levels.

Acknowledgments

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Chapter 7

Characterization of FOXO Acetylation

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Abstract

FOXO3 is a tumor suppressor that orchestrates the expression of genes that regulate cell cycle progression, apoptosis, metabolism, oxidative stress, and other important cellular processes. Its inactivation is closely associated with tumorigenesis and cancer progression. On the other hand, sirtuin proteins have been demonstrated to be able to deacetylate, thus causing FOXO3 inactivation at the posttranslational level. Therefore, targeting sirtuin proteins renders new avenues for breast cancer treatment. Here, we describe three procedures for studying FOXO3 posttranslational modifications controlled by sirtuin proteins in cancer cells.

Key words FOXO3, Acetylation, Deacetylation, Sirtuin, SIRT1, SIRT2

1 Introduction

Forkhead box (FOX) proteins belong to a superfamily of transcription factors that are responsible for the spatio-temporal regulation of a wide range of transcriptional programmes during normal development [1]. The FOXO is a FOX subfamily that belongs to the class O which contains a common conserved “wing-helix” of DNA-binding domain. In this subfamily, there are four members including FOXO1, FOXO3, FOXO4, and FOXO6. Functionally, FOXO3 acts as a tumor suppressor and inhibits cell growth by inducing many transcription of genes, such as p130 (RB2), Bim, FasL, or p27^{Kip1} which are essential for cell cycle arrest, cell death, and cell differentiation [2, 3]. Furthermore, the inactivation of FOXO3 has been shown to be related to oncogenic transformation. FOXO3 can be regulated by different posttranslational modifications including phosphorylation, acetylation, methylation, ubiquitination, and glycosylation. FOXO3 can be phosphorylated by different kinases, including protein kinase B (Akt), extracellular signal-regulated kinases (MAPK/ERK), IKB kinase (IKK), and serum and glucocorticoid-regulated kinase (SGK), facilitating the

cross-talk between different signaling cascades that are often deregulated in most of the cancers. Recent evidence has shown that anticancer drugs with cytostatic and/or cytotoxic effects such as paclitaxel, doxorubicin, lapatinib, gefitinib, cisplatin, or tamoxifen affect the FOXO3 activation by inactivation of the PI3K-Akt pathway in many cancers [4]. Interestingly, FOXO3 overexpression inhibits cell cycle progression and prevents the DNA damage induced by genotoxic agents and oxidative stress.

On the other hand, posttranslational modification of FOXO3 by acetylation also controls its transcriptional activity. The FOXO3 acetylation/deacetylation depends on the action of two types of proteins: histone deacetylases (HDACs) that include silent information regulator 1 (SIRT1) and SIRT2, and histone acetyl transferase (HATs), such as CBP/p300 [5]. SIRTs are class III HDACs and belong to the sirtuin family that are NAD-dependent deacetylases, which can deacetylate FOXO3. SIRT1, through FOXO3 deacetylation, can induce cell proliferation in response to oxidative stress and also control the FOXO induction of expression of apoptotic genes negatively. In addition, FOXO3 deacetylation by SIRT1 increases FOXO3 ubiquitination and subsequent proteosomal degradation. Furthermore, SIRT2 regulates FOXO3 activity inducing deacetylation of FOXO3 under oxidative stress increasing the binding of FOXO3 to the promoter region of p27^{Kip1}. In addition, CBP/p300 acetylates FOXO3 in three conserved lysine residues (Lys242, Lys245, and Lys262) and this acetylation inhibits DNA-binding activity, ubiquitination and promotes Akt-mediated phosphorylation [5].

Previous studies in our laboratory demonstrated that breast cancer cell lines treated with tyrosine kinase-inhibitors (e.g., Lapatinib) have different expression profiles of FOXO3. Interestingly, we also found that the levels of SIRT1 and SIRT2 are different in these cell lines suggesting that posttranslational modifications of FOXO3, such as acetylation/deacetylation, are associated with tyrosine kinase-inhibitor sensitivity in breast cancer cells [6, 7]. In this chapter we examine the methodology [i.e., co-immunoprecipitation (co-IP), western blot and proximity ligation assay (PLA)] for studying the reversible acetylation of FOXO3 in breast cancer cells.

2 Materials

2.1 Cell Culture

1. BT474 cell line, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).
2. Lapatinib (LC laboratories, Woburn, MA, USA), dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C (*see Note 1*).

3. Dulbecco's modified eagle's medium (DMEM), supplemented with 10% (v/v) fetal calf serum (FCS), 100 unit/mL penicillin/streptomycin, and 2 mM glutamine.
4. 25 g/L trypsin solution in 0.02% ethylenediaminetetraacetic acid (EDTA) to passage cells.
5. The cells, maintained at 37 °C in a humidified atmosphere containing 10% CO₂.

2.2 Western Blotting (WB)

1. SMARTpool siRNAs: siSIRT1 (L-003540-00), siSIRT2 (L-004826-00), and siSIRT6 (L-013306-00-0005) and non-specific siRNA (D-001210-01-05) (Dharmacon, Lafayette, CO, USA).
2. Oligofectamine (Invitrogen, Paisley, UK).
3. Serum-free DMEM medium or Optimem (Life Technologies Gibco/BRL, Paisley, UK).
4. NP40 lysis buffer: 1% (v/v) Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 5 mM EDTA, 1 mM DTT, 1 mM NaF, 2 mM PMSF, 1 mM sodium orthovanadate.
5. Protease inhibitor cocktail.
6. Pierce BCA Protein Assay kit (Thermo Scientific, Paisley, UK).
7. Tecan Sunrise Microplate Reader.
8. Gel preparation (Table 1).
9. Bio-Rad Mini-PROTEAN system-casting stand, appropriate casting frame, combs, and glass plates.
10. SDS-running buffer: 0.1% (w/v) SDS, 25 mM Tris, 192 mM glycine.
11. Prestained Protein Standard.
12. Electrophoresis chambers and power pack.
13. Ponceau.
14. Nitrocellulose membrane 0.45 µm.
15. Whatman 3 mm paper.
16. 100% Ethanol.
17. Transfer buffer: 25 mM Tris, 190 mM glycine, and 20% ethanol.
18. SDS-PAGE gel wet transfer apparatus.
19. Rabbit anti-acetylated FOXO3 (Lysine 242/245) antibody (Prepared in Eric Lam's Laboratory).
20. Antibodies: SIRT1 (Ab32441, Abcam, Cambridge, MA, USA), FOXO3 (07-702, EMD Millipore, Billerica, MA, USA); EP300 (sc-585) and beta-tubulin (H-235, Santa Cruz Biotechnology, Santa Cruz, MA, USA) and FOXO3 (D7D3Y) (#99199), FOXO3 (75D8) (#2497), acetylated-Lysine (#9441) and negative control rabbit IgG (#2729) (Cell Signaling Technology, Leiden, The Netherlands).

Table 1
Composition of resolving and stacking gel (see Note 2)

	Resolving gel				Stacking gel 5%
	7%	10%	12%	14%	
dH ₂ O-mL	5.02	4.02	3.35	2.68	3.67
1.5 M Tris pH 8.8	2.5	2.5	2.5	2.5	–
1.5 M Tris pH 6.8	–	–	–	–	0.42
30% Acrylamide	2.33	3.33	4.00	4.67	0.83
10% SDS- μ L	100	100	100	100	50
25% APS- μ L	40	40	40	40	20
TEMED- μ L	10	10	10	10	10
Total-mL	10	10	10	10	5

21. Anti-rabbit horseradish peroxidase-conjugated secondary antibody (Dako, Ely, UK).
22. 5% (w/v) bovine serum albumin (BSA).
23. TBS-T: 20 mM Tris pH 7.6, 136 mM NaCl, 0.01% (v/v) tween.
24. Enhanced Chemiluminescence (ECL) substrate kit.

2.3 Chemicals, Reagents and Equipment Used for Immuno-precipitation (IP)

1. Lysis Buffer: (50 mM Tris-HCl pH 8.8, 150 mM NaCl, 5 mM EDTA, 1% NP-40, mM DT, completed before use with 2 mM NaF, 2 mM Na₃VO₄ and protease inhibitor cocktail tablet.
2. Pierce BCA Protein Assay Kit.
3. Dynabeads Protein A/G (Invitrogen).
4. Loading buffer 2 $\times$ (2% SDS, 25% glycerol, 62.5 mM Tris-Cl pH 6.8, 350 mM dithiothreitol (DTT), and bromophenol blue (~0.05 mg/mL)).
5. DynaMag-2, Magnetic Particle Concentrator.
6. Tecan Sunrise Microplate Reader.
7. Sirtinol (Sigma-Aldrich, Poole, UK) dissolved in DMSO: SIRT1 (IC₅₀ = 131 μ M) and SIRT2 (IC₅₀ = 0.58 μ M) inhibition.

2.4 Chemicals, Reagents, and Equipment Used for Duolink In Situ Proximity Ligation Assay (PLA)

1. PLA probe MINUS.
2. PLA probe PLUS.
3. Detection reagents.
4. Wash buffer A and B (provided by Sigma-Aldrich, composition unknown).

5. $1 \times$ PBS.
6. 4% Paraformaldehyde/PBS, pH 7.4.
7. 0.1% Triton X-100/PBS.
8. High purity water, e.g., Milli-Q water.
9. Falcon 8-well chambered cell culture slides.
10. Cover slips.
11. Incubator at 37 °C.
12. 4,6-diamidino-2-phenyl indole (DAPI).
13. Fluorescence confocal microscope.
14. Microscope imaging software.

3 Methods

3.1 *siRNA Transfection and Drug Treatment*

The role of sirtuins in regulating the acetylation of non-histone proteins such as FOXO3 and p53 renders them attractive therapeutic targets for anti-cancer drug development [8]. Sirtuin inhibitors have been previously shown to enhance chemosensitivity and promote the expression of apoptotic genes through enhancing FOXO3 acetylation [9, 10]. Moreover, knockdown of sirtuin has been shown to induce apoptosis and senescence and enhances chemosensitivity in cancer cells [11]. Therefore, dissecting the role of protein acetylation can be attempted by the use of siRNA-mediated knockdown of sirtuin as well as by sirtuin inhibition. Most commonly used inhibitors are sirtinol (SIRT1 and SIRT2 inhibitors) and EX527 (SIRT1 inhibitor) [12]. Some other commonly used sirtuin inhibitors are nicotinamide and its derivatives, cambinol and AGK2 [13].

3.1.1 *siRNA Transfection*

1. Seed 2×10^5 cells per well in a 6-well plate (70%–80% confluent is ready for transfection).
2. Mix 70 μ L of Optimem and 5 μ L Oligofectamine for each reaction.
3. Incubate at room temperature for 10 min.
4. Prepare 250 μ L of Optimem and 7.5 μ L of $1 \times$ universal siRNA buffer (used as the siRNA vector) containing no siRNA as a control.
5. Prepare 250 μ L of Optimem and 7.5 μ L (50 nM final concentration) siRNA oligos specific for each gene.
6. Prepare 250 μ L of Optimem and 7.5 μ L of a nonspecific siRNA which does not target any known proteins.
7. Mix Optimem and Oligofectamine from **step 2** and Optimem and siRNA from **steps 4** and **5** or **6**.

8. Incubate for 25 min at room temperature.
9. Add 167.5 μL Optimem to the mixture, reaching a final volume of 500 μL for each reaction.
10. Remove the medium from 6-well plates and wash with pre-warmed PBS.
11. Add the total 500 μL mixture to the well.
12. Incubate for 4–5 h in an incubator at 37 °C and 10% CO_2 .
13. Add 2 mL of prepared medium to each well.
14. Incubate in the incubator at 37 °C and 10% CO_2 for further treatment.
15. Treat cells with 1 μM lapatinib after 24 h of incubation.
16. Incubate cells for 8 h in the incubator at 37 °C and 10% CO_2 .
17. Trypsinize cells and collect them in falcon tubes. Centrifuge the tubes at $300 \times g$ for 5 min.
18. Dissolve the pellet with DMEM media and collect the cells in eppendorf.
19. Centrifuge again at $300 \times g$ for 5 min.
20. Discard the supernatant and freeze the cells at -80°C until the lysis will be performed (*see* **Note 3**).

3.1.2 Western Blotting

1. Preparation of protein lysates and protein quantification (*see* **Note 4**)
 - (a) Lyse the frozen pellets with NP40 lysis buffer and protease inhibitor cocktail.
 - (b) Keep the lysates on ice for 20 min.
 - (c) Centrifuge the lysate at $12,000 \times g$ for 10 min at 4 °C to remove insoluble lysate material.
 - (d) Transfer the supernatant to clean eppendorf tube.
 - (e) Determine protein concentration by using the Pierce BCA Protein Assay kit according to the manufacturer's instructions. Briefly, pipette the samples into microplate wells and add the mix of Reagent A and B. Incubate for 30 min at 37 °C and then measure the absorbance at 562 nm in a Tecan Sunrise microplate absorbance reader.
 - (f) Determine protein concentrations by the equation of $\text{absorbance} \times 25 = \mu\text{g}/\mu\text{L}$.
2. SDS Polyacrylamide Gel Electrophoresis
 - (a) Cast a 6% acrylamide (v/v) resolving gel as mentioned in Table 1.
 - (b) After polymerisation of resolving gel, carefully rinse off all isopropanol using distilled water. Remove any residual water using filter paper.

- (c) Cast a 5% stacking gel as shown in Table 1.
 - (d) Immediately insert comb due to faster polymerization reaction. Stacking gel should be polymerized after approximately 10 min.
 - (e) Place the gel in an electrophoresis chamber and ensure comb side is facing inward.
 - (f) Fill chamber with SDS-running buffer up until fill line.
 - (g) Remove comb and use a syringe and needle to carefully flush out each well to remove any floating residual polyacrylamide.
 - (h) Using appropriately sized pipettes, load 4 μL of pre-stained protein ladder and the samples into each corresponding well.
 - (i) Connect the chamber after closing into a compatible power pack.
 - (j) Run gel for approximately 2 h at 90 V. Use bromophenol blue line as an indicator of electrophoresis progress.
3. Protein Transfer
- (a) Place a nitrocellulose membrane in transfer buffer.
 - (b) Soak two sponges and two whatman papers in transfer buffer.
 - (c) Assemble the sandwich cassette configuration as shown below in a tray filled with transfer buffer. Cassette > Sponge > Filter paper > Gel > Membrane > Filter paper > Sponge > Cassette.
 - (d) Carefully open the glass plate and cut off the stacking gel and place gel over the top of the first whatman paper.
 - (e) Place a labeled membrane on the top of the gel.
 - (f) Place second piece of filter paper on the top of the membrane followed by second sponge.
 - (g) Use a roller to carefully remove any bubbles in between layers, as this will lead to uneven protein transfer.
 - (h) Prepare wet transfer apparatus filled with transfer buffer and place sandwich cassette into the transfer apparatus with ice.
 - (i) Close lid and plug in transfer chamber. Now run the transfer for 90 min at 90 V.
 - (j) Remove the apparatus and transfer the membrane into a tray with ponceau solution.
 - (k) Temporarily stain membrane for 5–10 min to check ladder labeling.
 - (l) After labeling ladder, place membrane into TBS-T and wash on a shaker until membrane has no more ponceau.

4. Acetylated FOXO3 Detection

- Place nitrocellulose membrane in 5% (w/v) BSA for 30 min to inhibit nonspecific binding sites.
- Incubate membrane with rabbit anti acetylated FOXO3 (Lysine 242/245) antibody diluted at 1:1000. Keep overnight at 4 °C on a shaker.
- Wash membrane 3 times for 5 min. Each wash with TBS-T in order to remove unbound antibodies.
- Incubate membrane with anti-rabbit horseradish peroxidase-conjugated secondary antibody diluted at 1:2000 at room temperature for 1 h.
- Wash membrane 3 times for 5 min each wash with TBS-T to remove unbound secondary antibodies.
- Prepare membranes for autoradiography signal detection by mixing 1:1 ratio of ECL substrate kit (*see Note 5*).
- To activate horseradish peroxidase, cover membrane with substrate and with a plastic sheet and ensure no air bubbles are on the membrane. Transfer membrane into a developing cassette for film development (Fig. 1).

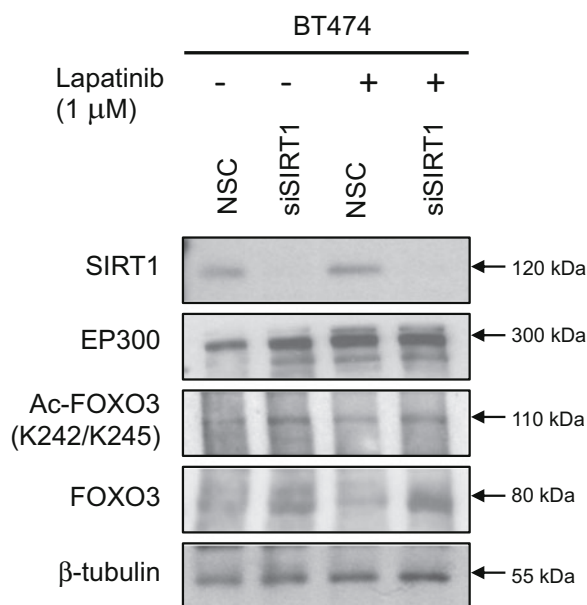


Fig. 1 BT474 cells were transiently transfected with SIRT1 siRNA and a nonspecific control (NSC) siRNA. The BT474 cells were treated with 1 μM lapatinib at 48 h post-transfection. Proteins obtained from whole cell extracts after 8 h of lapatinib treatment. Western blotting was performed using the protein lysates to assess the expression levels of SIRT1, EP300, FOXO3, and Ac-FOXO3 (K242/245). Beta-tubulin was used as a protein loading control

3.2 Immuno-precipitation (IP)

Immunoprecipitation (IP) is a widely used technique to enrich or purify a specific protein from a complex mixture, while co-IP is used to identify protein-protein interactions. The principle of the assay relies on the specific antigen/antibody interaction. The immune complexes are immobilized onto Protein A/G beads due to the specific binding of protein A/G to the FC fragment of the antibody. The immunoprecipitated proteins and their binding partners can then be separated by SDS PAGE for western blot analysis.

3.2.1 Lysate Preparation: Cell Harvesting/Cell Lysis/ Protein Quantification

1. Seed 2.0×10^6 cells in T75 flask.
2. When cells are at 70–80% of confluency, treat cells with sirtuin inhibitor (e.g., Sirtinol) for 24 h.
3. Trypsinize cells from all flasks and collect them in falcon tubes.
4. Centrifuge at $300 \times g$ for 5 min.
5. Dissolve the pellet with DMEM media and collect the cells in eppendorf tube.
6. Centrifuge at $800 \times g$ for 5 min.
7. Remove the supernatant and freeze the cells for further analysis.
8. According to the pellet's size add NP40 lysis buffer to lyse the cells. Incubate the lysates for 15–30 min on ice and vortex vigorously 4–5 times throughout the incubation time to facilitate cell lysis.
9. Centrifuge at $12,000 \times g$ for 10 min at 4 °C. Transfer the supernatants (protein lysates) to new 1.5 mL microcentrifuge tubes and discard the pellets.
10. Determine total protein concentration using the BCA Assay kit according to the manufacturer's instructions.
11. Use ~200–500 µg of cell lysate per IP sample and keep 1:10 of this amount for the input control.

3.2.2 Beads Preparation/ Cell Lysate Preclearing and Immunoprecipitation

1. Use 20 µL of Dynabeads A/G per sample. Wash the beads with 500 µL of ice-cold PBS with protein inhibitor (PI). Place the tube with the beads on the magnetic separation rack to remove the supernatant and discard. Repeat wash 2 times.
2. Add the washed beads in the lysates and incubate for 4–6 h at 4 °C under rotary agitation.
3. When the incubation time is completed remove the beads with the magnetic platform and transfer the precleared lysates to new 1.5 mL microcentrifuge tubes.
4. Prepare fresh Dynabeads A/G as aforementioned.
5. Add 20 µL of beads per sample to the precleared lysates.
6. Fill the lysates up to 300–500 µL with Lysis Buffer. Add 0.5–2 µg of antibody and incubate the lysate-beads-antibody mixture overnight at 4 °C under rotary agitation.

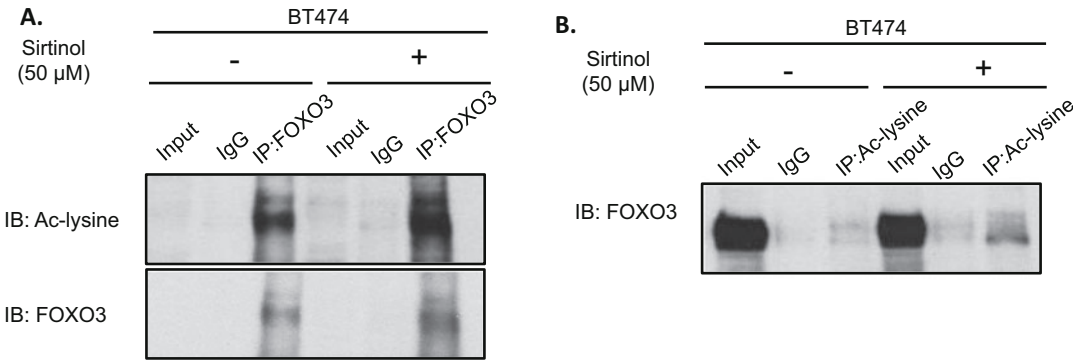


Fig. 2 Protein lysates of BT474 cells were obtained following treatment with or without 50 μ M sirtinol for 24 h. (a) Co-immunoprecipitation (Co-IP) was accessed with anti-FOXO3. Subsequent immunoblotting was performed using antibodies against Ac-Lysine and FOXO3. (b) A reverse Co-IP was performed with a pan-acetylated lysine antibody and immunoblotting with anti-FOXO3 antibody. The anti-IgG antibody was used as a negative control

7. Place the tubes on the magnetic separation rack and carefully pipette to remove the supernatant.
8. Gently wash the beads with 500 μ L of Lysis Buffer 3 times to remove nonspecific binding.
9. After the last wash centrifuge the beads gently ($200\text{--}800 \times g$ for 1 min at 4°C) to remove the excess of Lysis Buffer.
10. Resuspend the bead pellets in $2\times$ Sample Loading Buffer and vortex.
11. Boil the samples at 100°C for 5 min to denature the proteins and spin down.
12. Place the tubes on the magnetic separation rack to collect the samples. Transfer the supernatant in new 1.5 mL microcentrifuge tubes.
13. Load the samples on SDS-PAGE gel and analyze them by western blot or store them at -20°C until further use (Fig. 2).

3.3 Duolink In Situ Proximity Ligation Assay (PLA)

This assay is used to detect, visualize, and quantify protein expression, protein interactions, and specific posttranslational protein modification. In the cases of detecting protein interactions and modification, two primary antibodies are required and each raised from different species. The secondary antibodies are called PLA probes (PLA probe MINUS and PLA probe PLUS), which each conjugated with unique oligonucleotides. When the two PLA probes are in close proximity (<40 nm), the oligonucleotides will hybridize and join to a closed circle by adding enzymatic ligation solution. The ligated circle as a template is then amplified through rolling circle amplification and generating several-hundredfold repeated sequence product subsequently. Since the amplification

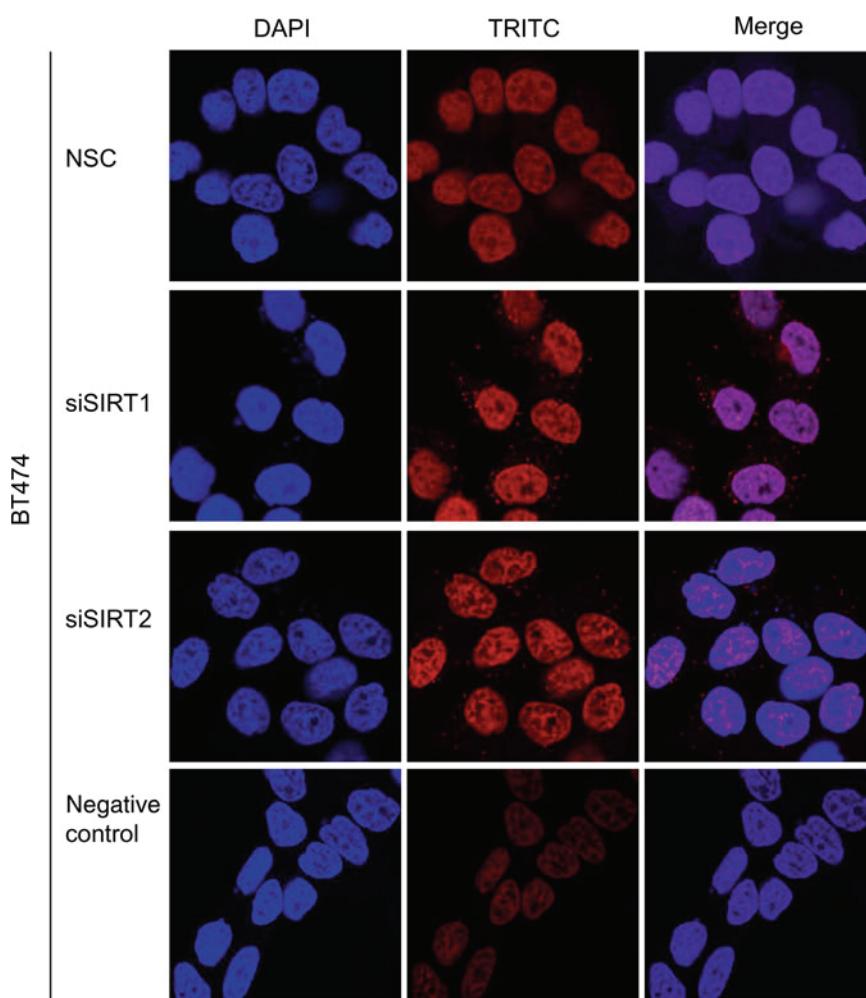


Fig. 3 Duolink proximity ligation assay (PLA) for detection of FOXO3 and its posttranslational modification of acetylated-lysine in BT474 cells. Cells were transiently transfected with SIRT1 and SIRT2 siRNA and fixed at 48 h post-transfection. Cells were transfected with nonspecific siRNA used as negative control. Samples were visualized with a fluorescence confocal microscope equipped with a 63 $\times$ oil immersion objective and LAS-AF software. Each red spot represents for a single detection and DNA as stained with DAPI in blue. Cells without blotting with primary antibodies represent as negative control

solution consists of nucleotides, polymerase, as well as fluorescently labeled oligonucleotides, the replicated product can be easily visible as distinct fluorescent spot when viewed with a fluorescence microscopy (Fig. 3).

3.3.1 Fixation and Permeabilization

1. Seed 15,000 cells per well in 8-well chamber slide.
1. Remove media from the 8-well chamber slide (*see Note 6*).
2. Rinse each well with 200 μ L 1 $\times$ PBS 3 times for 5 min.
3. Fix in 80 μ L 4% paraformaldehyde/PBS pH 7.4 at room temperature for 10 min.

4. Rinse each well with 200 μL 1 $\times$ PBS 3 times for 5 min.
5. Permeabilize cells with 80 μL 0.1% Triton X-100 for 10 min.
6. Rinse each well with 200 μL 1 $\times$ PBS 3 times for 5 min.

3.3.2 Blocking and Incubation with Primary Antibodies (see **Note 7)**

1. Add one drop of blocking solution to each well and ensure to cover the whole surface.
2. Incubate the slides for 30 min in incubator at 37 °C.
3. Dilute primary antibodies 1:100 in the provided Antibody Diluent.
4. Remove the blocking solution from the slides, ensure the samples are not dried before adding primary antibodies.
5. Add 80 μL primary antibody solution to each well.
6. Incubate at 4 °C overnight.

3.3.3 PLA Probes

1. Dilute the two PLA probes 1:5 in Antibody Diluent, e.g., 16 μL PLA probe PLUS, 16 μL PLA probe MINUS, and 48 μL Antibody Diluent to ensure the final volume is 80 μL .
2. Tap off the primary antibody solution.
3. Wash the slides gently in 1 $\times$ Wash buffer A 2 times for 5 min on a shaker.
4. Add 80 μL previous prepared PLA probe solution to each well.
5. Incubate the slides for 1 h in incubator at 37 °C.

3.3.4 Ligation

1. Tap off the PLA probe solution and wash the slides gently in 1 $\times$ Wash buffer A for 2 times for 5 min in a shaker.
2. Dilute the ligation stock 1:5 in high purity water, e.g., for an 80 μL reaction take 16 μL of the 5 $\times$ ligation stock and 64 μL of high-purity water.
3. Add ligase to the ligation solution from the last step, at a 1:40 dilution and vortex, e.g., 2 μL ligase stock in 80 μL ligation solution.
4. Add 80 μL to each well.
5. Incubate the slides for 30 min in incubator at 37 °C.

3.3.5 Amplification

1. Tap off the ligation-ligase solution and wash gently with 1 $\times$ Wash buffer A 2 times for 2 min in a shaker.
2. Prepare the amplification stock 1:5 in high-purity water and mix, e.g., 16 μL amplification stock dilute in 64 μL high-purity water.
3. Add polymerase to amplification solution from the last step at 1:80 solution and vortex, e.g., add 1 μL polymerase to 80 μL amplification solution.

4. Add 80 μ L amplification-polymerase solution to each well.
5. Incubate the slides for 100 min in incubator at 37 °C.

3.3.6 Preparation for Imaging

1. Tap off the amplification-polymerase solution and wash the slides in 1 $\times$ Wash buffer B 2 times for 10 min in gentle shaker.
2. Wash the slides in 0.01 $\times$ Wash buffer B for 1 min.
3. Air dry the slides at room temperature in the dark.
4. Carefully remove the chamber from the slides with provided safety removal tool.
5. Add minimal volume of DAPI to each well and put cover slip without making any air bubbles.
6. Use nail polish to seal the edges.
7. Air dry the slides at room temperature in the dark.
8. Store the slides at -20 °C in the dark or proceed with the next step.
9. Use confocal fluorescent microscope and keep settings constant during an experiment.

4 Notes

1. Concentrations of DMSO brought in the final culture medium should be lower than 0.5% to avoid cell damage.
2. Formation of polyacrylamide is based on a polymerization process. Addition of APS and TEMED at the last moment is highly recommended.
3. For the protein preparation, lyse the cells as quickly as possible to avoid protein damage.
4. In order to avoid protein degradation, the samples should be on ice in most of the steps described above.
5. Develop the western blot membranes with ECL as soon as possible as the half-life of ECL is about 45 min.
6. For Duolink experiments, to avoid drying of the samples, prepare just a few slides at a time.
7. For Duolink experiments, the primary antibodies used should be from different hosts.

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Chapter 8

Transcriptional Activity of FOXO Transcription Factors Measured by Luciferase Assays

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Abstract

The Forkhead box O (FOXO) family of transcription factors translates environmental cues into gene expression. FOXO factors are crucial for the maintenance of cell homeostasis, with important roles in cell fate decisions and differentiation. Identification of FOXO target genes requires strict validation by several methods. Luciferase-based reporters are a valuable starting point for determining the transcription-promoting capacity of potential FOXO-binding sites in candidate genes. Luciferase, an enzyme found in bioluminescent organisms catalyzes oxidation of luciferin to produce oxyluciferin together with light, which can be easily detected and measured with a luminometer. Due to their many advantages, transcriptional assays based on luciferase activity are widely used; they are easy, highly reproducible, and very sensitive. Continued improvements in luciferase-based vectors and measurement reagents confer considerable versatility. Luciferase-based reporters are also a reliable approach in the search for unknown components in the signaling pathways that control FOXO factor activity.

We previously reported that FOXO transcription factors control expression of the enzyme diacylglycerol kinase α (DGK α) in T cells. DGK α consumes diacylglycerol, a lipid that activates several mitogenic pathways. Here, we describe the use of a luciferase-based promoter bearing the FOXO-binding sites of the DGK α gene to explore the relationship between the expression of this enzyme and stress conditions in NIH3T3 mouse fibroblasts. Our data support a role for FOXO factors in promoting high DGK α levels in conditions of growth factor deprivation. DGK α regulation by FOXO factors correlates with the reported alterations in DGK α expression during cell transformation and cancer progression.

Key words FOXO factor, Transcriptional regulation, Luciferase, Reporter gene, PI3K, AKT, Diacylglycerol kinase α

1 Introduction

Genetic reporters are widely used to monitor gene expression dynamics by determining the transcriptional activity of a specific gene. This “reporter gene” encode for a protein with a unique enzymatic activity that can be easily distinguished from the rest of the cell proteins [1]. The basic strategy for gene reporter use is to clone the DNA whose transcriptional function is to be evaluated, just upstream of the reporter gene to generate a chimeric construct;

in this way, the reporter gene expression is controlled by the cloned DNA [2].

Luciferase is one of the most-used reporter genes. Its reporter activity is based on bioluminescence, a natural process that occurs in certain organisms in which a series of luciferase enzymes catalyzes oxidation of specific substrates known as luciferins to form oxyluciferin, with concurrent emission of light. Luciferases from many bioluminescence-producing organisms have been cloned, but the first and most widely used is the monomeric 61 kDa protein from the firefly *Photinus pyralis* [3].

Luciferase reporter assays offer considerable advantages compared to other methods. The protein activity does not require posttranslational modifications, there is no background luminescence in host cells, and the enzyme reaction is very rapid and highly sensitive. In addition, the method is simple, and can be easily applied and combined with other methods to measure distinct signaling processes, which confer versatility [4, 5]. The most convenient method for detecting the light produced by luciferase is to use a plate-reading luminometer. Since most cells used in these experiments have no activity similar to that of luciferase, the sensitivity of the luciferase reporter gene is limited only by the background noise of the luminometer, which in modern equipment is very low.

Assays based on luciferase gene reporters have improved steadily. Incorporation of coenzyme A in the reagent containing the substrate improved reaction kinetics and increased light intensity [6]. A series of modifications in the sites leading to spurious luciferase expression, or that might interfere with expression have been introduced in the new generation of some reporter plasmids, which has reduced the risk of anomalous expression. Luciferase coding regions have been also modified, leading to more stable, more active versions, or with shorter enzyme life [7, 8]. The setting of a luciferase-based reporter assay nevertheless always requires prior experimental standardization to minimize background reporter activity and to validate its suitability for the study of an individual promoter DNA and/or pathway.

A second reporter can sometimes be included to improve experimental accuracy, particularly in the case of transient transfections. The amount of transfected reporter gene plasmid can vary between individual cells, and can lead to erroneous conclusions when comparing different conditions. A convenient means to control this variability is to co-transfect the experimental gene reporter with a control reporter whose activity provides a baseline response. The activity of the experimental reporter is then normalized to that of the control. By using this internal control, differences in cell number and viability can also be minimized, as well as in cell lysis.

Renilla, the 36 kDa luciferase from *Renilla reniformis* [9], is one of the most common control reporters and is often used with

the firefly luciferase-based reporters. Although both luciferase and renilla are bioluminescent reporters, they have distinct evolutionary origins and thus differ in their structures and substrate requirements. In the widely used dual-luciferase reporter assays, the firefly luciferase is read first, then quenched and renilla activity is measured [10]. Since there could be trans-effects between the promoters of the co-transfected plasmids, it is important to ensure the independent genetic expression of both plasmids and to optimize their ratio [11]. Several factors can modify renilla expression, invalidating its use as a constitutively expressed control, such as dexamethasone, interferons, phorbol esters, androgens, etc. [12]. Review of the literature during experimental planning, and posterior standardization are necessary to validate the use of renilla in the desired experimental conditions. There is a series of plasmids encoding renilla under the control of different promoter types to facilitate optimal renilla expression in different cell types and to avoid possible interference with co-transfected plasmids [13].

Luciferase promoter assays are a convenient starting point to demonstrate the promotion of transcriptional activity of putative regulatory DNA elements in a gene, commonly in regions surrounding the coding region. Luciferase promoter assays are also used to validate differential expression of genes identified by microarray analysis, massive cDNA sequencing and/or RNA-seq studies. Due to their robust results, luciferase promoter assays can be applied in high-throughput screening for functional genomics and drugs [14, 15].

Luciferase reporter-based assays are reliable tools, mainly because they are cell-based assays that allow more physiological conditions than binding or *in vitro* biochemical assays [16]. Luciferase gene reporters can be used as powerful readouts of signaling events, because transcription is an end-point coupled to different signal transduction processes [4]. This advantage is particularly useful for the study of the FOXO (Forkhead transcription factors) axis, which is controlled by numerous parameters. Assays can take into account different environmental cues, culture conditions, or drugs that modify FOXO protein levels, subcellular localization, and/or activity. The measure of FOXO transcriptional functions can be used as a readout of the pathway studied or to identify new FOXO target genes.

In a previous study, we used this technique to show that FOXO transcription factors bind to the 5' regulatory region of the diacylglycerol kinase α (DGK α) gene [17]. DGK α transforms diacylglycerol (DAG), a bioactive lipid, into phosphatidic acid. DGK α activity attenuates T lymphocyte activation [18]. Our initial characterization of the 5' structure of the DGK α gene indicated the presence of three FOXO-binding sequences in this region that were highly conserved in mouse and human ([17, 19]; Fig. 1). We tested the transcription-promoting capacity of these sites by generating

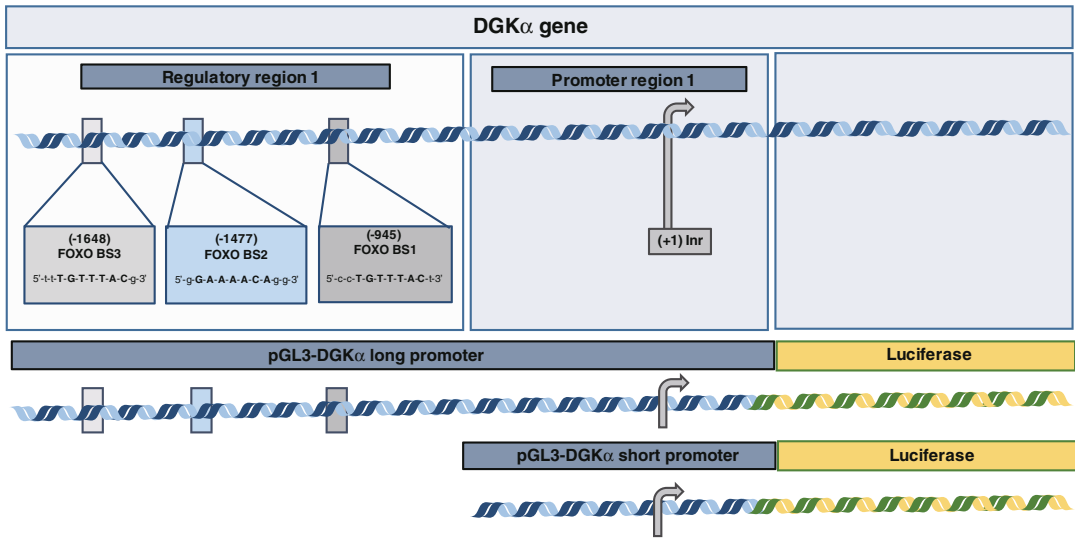


Fig. 1 Cartoon depicting the localization of the FOXO-binding sites in the regulatory region of the *DGKα* mouse gene, according to [17]. The transcription initiation site of the promoter 1 is indicated (arrow); the +1 position is assigned in the Inr element. The three FOXO-binding sites and their relative position are indicated. The structure of the pGL3-DGKα promoter luciferase constructs is shown. The pGL3-DGKα long promoter contains the three FOXO-binding sites, whereas the pGL3-DGKα short promoter lacks of that region

distinct luciferase reporter constructs using a commercial promoterless vector (pGL3, Promega). Together with several additional techniques, we identified *DGKα* as a component of the transcriptional programs orchestrated by the PI3K (phosphatidylinositol 3 kinase)/AKT/FOXO pathway in T cell differentiation [17].

Several studies suggested that *DGKα* expression also varies during the distinct cell differentiation states of other cell types [20]. *DGKα* was identified as one of the genes repressed by the PI3K/AKT/FOXO pathway during fibroblast proliferation [21]. Reduced *DGKα* gene expression as a result of oncogenic activation of the PI3K/AKT pathway would lead to loss of the *DGKα*-DAG-break functions during transformation, which coincides with studies suggesting that *DGKα* has tumor suppressor properties in healthy epithelia [20, 22].

Here, we evaluate *DGKα* regulation in fibroblasts in serum deprivation culture conditions. To this end, we describe in detail a protocol used to evaluate the transcriptional promoter activity of FOXO proteins, using a luciferase-based promoter that contains the three FOXO-binding sites of the mouse *DGKα* gene in NIH3T3 fibroblasts. Our data indicate a role for FOXO factors in promoting high *DGKα* levels in conditions of growth factor deprivation.

2 Materials

All solutions are prepared using ultrapure water. PBS, passive lysis buffer, and plasmids are stored at 4 °C, luciferase assay reagents at –20 °C, or –80 °C for long-term storage. All waste disposal regulations must be followed when eliminating materials.

2.1 Transient Cell Transfection

1. NIH3T3 cells. Cells can be obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine (*see Note 1*) at 37 °C in a 5% CO₂ atmosphere. Cells are maintained at 70–80% confluence; the culture is passaged routinely twice per week (usually 1:8–1:16 dilutions). We generally avoid the use of antibiotics in cultures and always use mycoplasma-free cultures with a minimum of 10 passages to facilitate transfection (*see Note 2*).
2. pGL3-Basic Vector (Promega) (*see Note 3*).
3. pGL3-DGK α short promoter and pGL3-DGK α long promoter, which contain none and three FOXO-binding sites, respectively ([17]; *see Fig. 1*).
4. Renilla luciferase vector (Promega).
5. pECE-FKHRL1 (also known as FOXO3a ([23]; Addgene, #1787).
6. Opti-MEM (1X) Reduced Serum Medium.
7. Plus Reagent.
8. Lipofectamine 2000 (*see Note 4*).

2.2 Preparation of Cell Lysates

1. Phosphate-buffered saline (PBS), pH 6.8.
2. Luciferase lysis buffer (LLC): 25 mM Tris-HCl pH 7.5, 15% glycerol, 8 mM MgCl₂, 1% Triton X-100, mM dithiothreitol (*see Note 5*).

2.3 Measurement of Luciferase and Renilla

1. Dual-Luciferase Reporter Assay System (Promega).
2. Opaque 1.5 ml eppendorf tubes.
3. Assay plates: 96-well, white, flat-bottom, untreated, no lid, polystyrene plates.
4. Luminometer.

3 Methods

3.1 Transient Cell Transfection

1. Seed NIH3T3 cells in 6-well cell culture plates such that they reach 50–70% confluence by the time of transfection (usually the next day). No plate pretreatment is necessary for NIH3T3

cells. Use three wells per luciferase reporter and condition. Culture size can be scaled down to 12-well plates if there is sufficient activity and the lysates are not needed for further use.

2. Pre-complex the DNAs with the Plus reagent by diluting 0.5–1.5 µg DNA each from the luciferase reporter constructs, 1 µg of the FOXO-encoding plasmid, and 50 ng DNA of the renilla luciferase vector into 100 µl reduced-serum medium (Opti-MEM I). Mix the Plus reagent before use; add 3 µl Plus reagent to diluted DNA, mix again, and incubate (room temperature, 15 min).
3. Dilute 3 µl Lipofectamine 2000 into 50 µl reduced-serum medium in a second tube. Mix.
4. Combine pre-complexed DNA (from **step 2**) and diluted Lipofectamine 2000 (from **step 3**). Mix and incubate (room temperature, 15 min).
5. While complexes are forming, replace cell culture medium with 2 ml of fresh medium.
6. Add DNA-Plus-Lipofectamine 2000 complexes (from **step 4**) to each well of cells containing fresh medium. Mix complexes into the medium gently.
7. Incubate the cells for 12–20 h (37 °C, 5% CO₂).
8. Incubate the cells in the distinct conditions. Three culture conditions are applied to cells transfected with the empty vector and to the cells transfected with the pGL3-DGKα promoters: (1) normal medium, (2) serum-starvation, and (3) serum-starvation plus serum. For condition 1, medium is replaced with fresh culture medium, while in conditions 2 and 3, the wells are washed three times with serum-free medium, in which they are maintained for 20 h. After this time, medium in the wells for condition 3 is replaced by fresh normal medium. After 24 h, cells are processed to measure luciferase activity.

3.2 Preparation of Cell Lysates

1. Remove growth medium from the cultured cells and gently add 1 ml of PBS to the well. Swirl the plate briefly and completely remove the rinse solution.
2. Into each culture well, dispense 200 µl LLB to completely cover the cell monolayer (*see Note 5*).
3. Place the culture plate on a rocking platform, with gentle rocking to ensure complete, even coverage of the cell monolayer with LLB. Rock culture plates at 4 °C (20–30 min).
4. Homogeneous lysates can be prepared by manually scraping the cells from culture wells with a cell scraper, cleaning it between uses if it used to prepare more than one sample. Tilt the plate and scrape the lysate down to the lower edge. Pipette

the accumulated lysate several times to obtain a homogeneous suspension.

5. Transfer lysate to a 1.5 ml eppendorf tube for further handling and/or storage. Clear the lysate samples by centrifugation at top speed in a previously cooled microcentrifuge ($15,000 \times g$, 15 min). Transfer cleared lysates to a new tube prior to reporter enzyme analyses or protein quantification for other purposes (e.g., western blot, *see* **Note 5**).

3.3 Preparation of the Reagents to Measure Luciferase

3.3.1 Luciferase Assay Reagent II (LAR II)

1. Suspend the kit-supplied lyophilized Luciferase Assay Substrate in 10 ml of Luciferase Assay Buffer II following the manufacturer's protocol.
2. Store the resulting reagent in 0.5–1 ml aliquots at -80°C in opaque 1.5 ml eppendorf tubes.
3. Thaw aliquots at room temperature before use.
4. Mix the thawed reagent by inverting the vial several times or by gentle vortexing; the thawing process generates both density and composition gradients within LAR II.
5. Avoid loss of assay performance due to repeated freeze-thaw cycles.

3.3.2 Preparation of Stop and Glo Reagent

1. Stop & Glo Buffer is stored in 0.5–1 ml aliquots at -80°C as the Stop & Glo Substrate $50\times$.
2. Thaw both at room temperature prior to use.
3. Stop and Glo substrate is supplied at a $50\times$ concentration; add 1 volume of Stop and Glo substrate $50\times$ to 50 volumes of Stop and Glo buffer immediately before reagent use.
4. Prepare an adequate volume for the desired number of assays (25 μl reagent per assay).

3.4 Dual-Luciferase Reporter Assay

1. Before the assay, thaw and gently vortex stored aliquots of LAR II and Stop and Glo reagents at room temperature.
2. Dispense 30 μl of cell lysates per well in a 96-well, flat-bottom white plate, with at least two wells per sample.
3. Program the luminometer to read luciferase and renilla labels with the following parameters:
Shaking (orbital) Duration: 10 s, amplitude 1 mm.
Mode: Luminescence.
Attenuation: None.
Integration time: 400 ms.
Settle time: 0 ms
4. Initiate reading of luciferase activity by manually adding the reagents or using the luminometer injectors. First dispense

25 μ l of LAR II reagent into each well for the luciferase reaction. Initiate reading of luciferase activity by manually adding the reagent or using the luminometer injectors.

5. Then add 25 μ l of Stop and Glo reagent to quench the luciferase and read the renilla activity (*see* **Note 6**).

3.5 Data Analysis

1. Correct the reporter activity by calculating the luciferase/renilla ratio for each sample. Luciferase activity is reported as relative units of luciferase activity (RLU).
2. Analyze the data by using GraphPad Prism 5 software or similar. Data can be shown as mean $\pm$ SEM; ANOVA and Bonferroni post-test analyses are applied to determine statistically significant differences.

Here, we showed the luciferase activity driven by two constructs that contain different regions of the DGK α promoter (Fig. 1), the DGK α long promoter construct contains three FOXO-binding sites. Figure 2a showed the luciferase activity obtained after transfecting different amounts of the DGK α long promoter construct together with a constant amount of the renilla reporter. The luciferase activity of the promoter construct increased in proportion with the amount of plasmid transfected. The Luciferase activity of the promoter normalized to that of the renilla is shown in Fig. 2b.

The luciferase activity driven by the two DGK α promoter constructs was next evaluated in cells that were cultured in media with serum or maintained in media without serum for 20 h (Fig. 2c). Differently from the DGK α short promoter construct, the transcriptional activity of the long construct increased significantly during serum deprivation or starving. The effect of serum deprivation was also demonstrated in Fig. 2d, where the luciferase activity of the DGK α long promoter in cells that were serum starved diminished after the addition of serum.

The effect of FOXO transcription factors over the expression of DGK α was evaluated directly by co-transfection of the promoter constructs together with a plasmid encoding for a FOXO protein (Ha-FKH1RL1/FOXO3a). FOXO co-transfection increased the activity of the two DGK α promoter constructs, with a higher significant increase in the case of the DGK α long promoter construct (Fig. 2e). A version of the DGK α long promoter construct with mutations in the FOXO-binding sites showed diminished transcriptional activity when compared with the wild-type version (Fig. 2f). Together these data indicate a role for FOXO factors in regulating DGK α expression in conditions of growth factor deprivation.

3.6 Additional Suggested Experiments

The data may be validated in the luciferase promoter assays using functional modifications in FOXO proteins, either by co-transfection with plasmids encoding mutants FOXO protein,

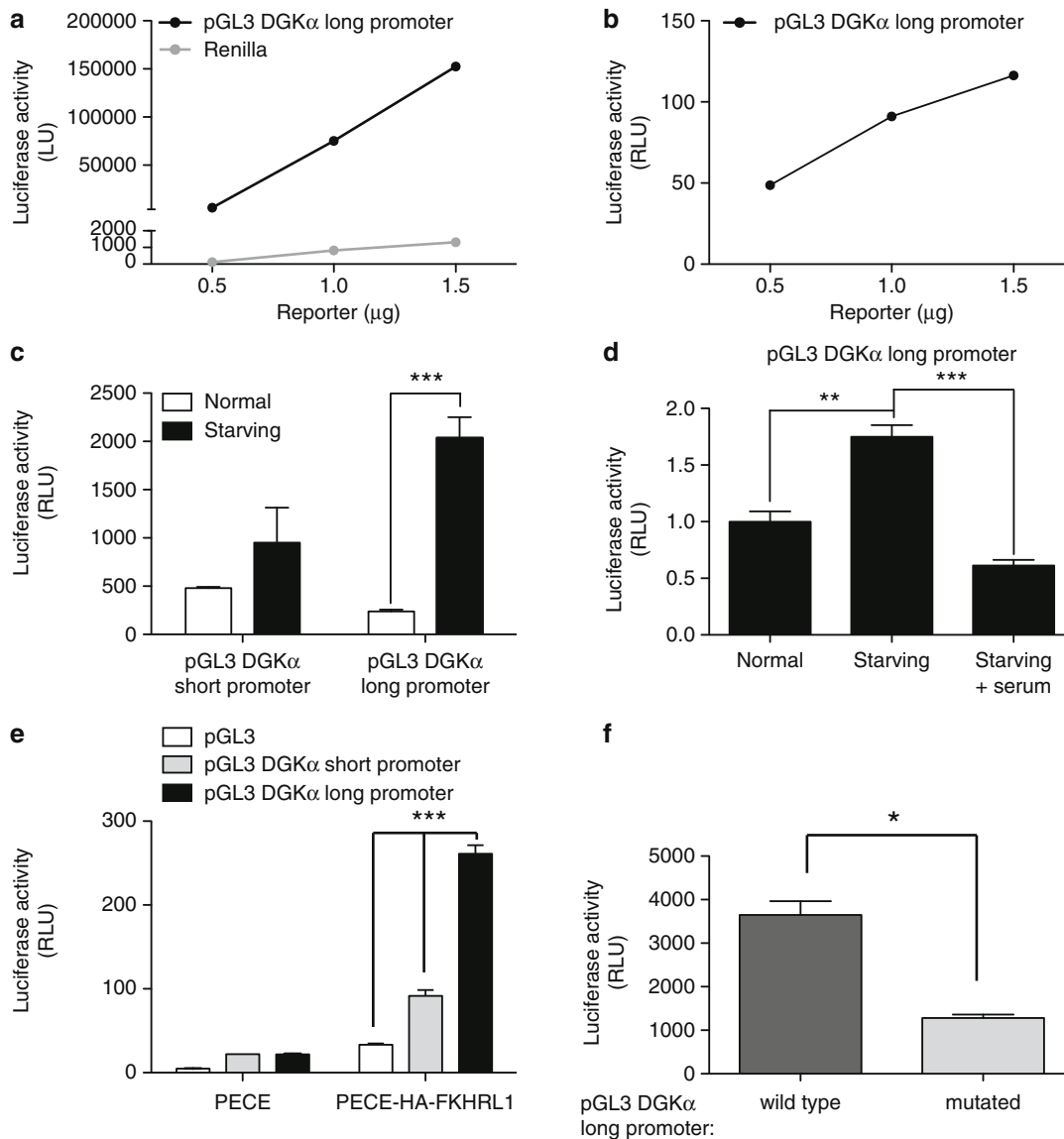


Fig. 2 Luciferase activity in NIH3T3 cells transfected with the indicated pGL3-DGK α constructs. **(a)** NIH3T3 cells were transfected with the pGL3-DGK α long promoter construct and 50 ng of renilla. The light units of each construct are indicated. **(b)** The activity of the pGL3-DGK α long promoter was relativized to that of renilla and indicated as relative light units (RLU). **(c)** Cells were transfected with 1.5 μ g of either the pGL3-DGK α short or the long promoter construct and 50 ng of renilla, and cultured in the indicated conditions. **(d)** The activity of the pGL3-DGK α long promoter construct was analyzed as in **c**, but a set of cells that were first serum starved and then serum stimulated (20 h) was included. **(e)** Cells were transfected with pGL3 or the pGL3-DGK α promoter constructs together with renilla and either, pECE plasmid or pECE-Ha-FKHRL1. **(f)** Cells were transfected with either the pGL3-DGK α long promoter construct or with a version that has the three FOXO-binding sites mutated, and together with renilla and the pECE-Ha-FKHRL1. In **c–f**, RLU indicate the luciferase/renilla ratio (mean $\pm$ SEM of three independent transfections). Data were analyzed using *t*-test (**f**) or ANOVA and Bonferroni post-test (**c–e**). In all the cases the results are representative of at least three independent experiments

by silencing these transcription factors, or by environmental conditions that modify their activity. To corroborate FOXO-mediated transcription of the gene studied, additional types of experiments must be performed, including chromatin immunoprecipitation (ChIP) assays and/or gene expression by real-time quantitative PCR. Western blot can also be used to monitor the levels and phosphorylation of the endogenous or overexpressed FOXO proteins in the distinct experimental conditions.

4 Notes

1. To evaluate FOXO activity using luciferase promoter assays, it is critical that the cell line used be suitable for this purpose: the cell line must maintain a functional PI3K/AKT/FOXO pathway, as is the case of the NIH3T3 fibroblasts. Cell lines as Jurkat T cells, which have no PTEN, are not suitable for this application. In our hands, the HEK293 cell line did not yield consistent results for measuring FOXO function in luciferase assays.
2. NIH3T3 cells are more easily transfected if they had been subcultured many times. Mycoplasma reduce transfection efficiency; it also can provide false results by interfering with normal cell signaling, thus providing false results. The use of antibiotics in the culture can reduce cell viability after transfection.
3. When constructing the reporter plasmid, it is important to select the most appropriate version of the vector to facilitate expression of the reporter gene under the transcriptional promoting activity of the DNA to be studied. If the DNA has a minimal promoter in addition to the transcriptional regulators, a “basic, promoterless” version of the vector is appropriate. This is the case of the construction we use, which contains promoter 1 of the murine DGK α gene (Fig. 1). In contrast, if the DNA under study has no minimal promoter, the versions of the vector containing minimal or constitutive promoters should be used.
4. NIH3T3 are easily transfected cells. Lipofectamine can be replaced by less expensive transfection reagents. In our laboratory, homemade liposomes also work with this cell line. In the case of Lipofectamine, the use of Plus greatly improves transfection efficiency.
5. When calibrating the experimental conditions, cell extracts can be useful for monitoring the similarity of duplicates and cell health (luciferase activity can also be normalized by considering protein content), as well as the activity state of the PI3K/AKT

pathway and expression of endogenous or overexpressed FOXO proteins. There are several buffers that allow luciferase activity to be read and can vary in effectiveness in extracting nuclear proteins such FOXO. Using LLB, we obtain suitable luciferase activity levels and detect FOXO in western blot.

6. During setup of the experimental conditions and if sample number is small, the luciferase reagents can be added manually, thus reducing wastage. Use of injectors is recommended for reading a larger number of samples. Scaling from p96 to p384 plates is recommended for a larger number of samples and/or automatization.

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Chapter 9

Monitoring the Transcriptional Activity of FOXO Transcription Factors by Analyzing their Target Genes

Vanessa Henriques, Susana Machado, Wolfgang Link, and Bibiana I. Ferreira

Abstract

FOXO proteins represent a subfamily of transcription factors that belong to the forkhead family. The study of FOXO target genes can be performed using Real-Time PCR (RT-PCR). The RT-PCR is a sensitive method that allows the detection and quantification of minute amounts of nucleic acids. In RT-PCR the accumulation of the amplicon is detected and measured as the reaction progresses. Here, we describe the application of RT-PCR technique to monitor the transcriptional activity of FOXO transcription factors.

Key words FOXO, Transcription factor, Target genes, Real-time PCR, Fluorescent reporter, Quantitative

1 Introduction

FOXO proteins represent a subfamily of transcription factors that belong to the forkhead family, first described in *Drosophila* [1, 2]. In mammals there are four *FOXO* genes: *FOXO1*, *FOXO3*, *FOXO4*, and *FOXO6*, involved in crucial cellular processes like regulation of stress resistance, metabolism, cell cycle arrest, and apoptosis [3–6]. The control of several cellular processes requires the activation and repression of target genes, carried out in a large part by transcription factors [7]. The study of signaling networks regulated by FOXO transcription factors is essential to understand its functions. One of the first steps to identify target genes of a given transcription factor includes the overexpression or the knockdown of the transcription factor in study and the analysis of the resulting changes in gene expression [8]. Several *FOXO* target genes are already identified: *PTEN* [9], *p21* [10], *p27* [11], *FasL* [12], *TRAIL* [13], *CyclinD1* [14], *PUMA* [15], *Cited2* [16], or *BAX* [17]. Real-Time Polymerase Chain Reaction (RT-PCR) can be used to analyze gene expression of FOXO target genes. RT-PCR

was first described in 1993 by Higuchi R. et al., where they describe the detection of the amplicon using a DNA-binding dye Ethidium Bromide (EtBr) [18]. RT-PCR proved to be a powerful tool to quantify gene expression and is considered the gold-standard method to analyze gene expression [19]. The difference between RT-PCR and regular PCR relies, mainly, on the fact that in PCR the amplified DNA product is detected in the end-point analysis, and in RT-PCR the accumulation of the amplification product is measured in real time, as the reaction progresses with the product quantification after each cycle [20]. The RT-PCR includes several processes. First, the RNA is isolated from the tissue/cell samples and transcribed into a complementary DNA (cDNA) by reverse transcriptase from mRNA. Then, cDNA is used as a template for the RT-PCR reaction. The reaction is detected by the use of fluorescent reporters that enables the detection of the amplicon as the reaction progresses [20]. The amount of amplification product in each cycle is calculated by the measurement of the increase in the fluorescent signal (from DNA-binding fluorophores like SyberGreen) [21–23]. The amount of the amplification product is given by the threshold cycle (Ct) in a PCR reaction. The Ct can be defined as the cycle number in which the fluorescence emission exceeds a chosen threshold (detectable signal is achieved) [21, 22]. The higher the mRNA concentration, the lower the Ct [22]. RT-PCR is an extremely accurate, fast, cost-effective method and allows qualitative and quantitative analysis [19, 24]. The chapter that follows describes a RT-PCR protocol to analyze the expression of FOXO target genes, using comparative Ct method ($2^{-\Delta\Delta C_t}$) [25].

2 Materials

Benches and material should be clean using RNaseZap. Use RNase and DNase-free pipettes and pipettes tips (filtered) and gloves. Waste material should be disposed according to waste disposal regulations (*see* **Note 1**).

2.1 RNA Extraction Using E.Z.N.A. Total RNA Kit

1. RNaseZap.
2. TRK Lysis Buffer: add 20 μ L of 2-mercaptoethanol (2-mercaptoethanol should be stored in the refrigerator and used only in the extraction hood) per 1 mL of TRK Lysis Buffer.
3. 70% Ethanol prepared with RNase-free water.
4. DNase (NZYtech): keep it on ice. Prepare DNase in a DNase and RNase-free eppendorf: $\sim$ 80 μ L of DNase buffer per sample, add DNase.
5. RNA Wash Buffer I.
6. RNA Wash Buffer II.

7. RNase-free water.
8. Ice.
9. HiBind® RNA spin columns.
10. Vortex.
11. Centrifuge.
12. Sterile RNase-free pipette tips and microcentrifuge tubes.

2.1.1 RNA Quantification and Storage

1. NanoDrop ND-2000.
2. RNase and DNase-free pipettes and pipette tips.
3. RNase-free water.

2.2 cDNA Synthesis Using cDNA Synthesis Kit, NZYTech

IMPORTANT: Keep all the reagents on ice.

1. NZYRT Enzyme Mix (Includes NZY Reverse Transcriptase and NZY Ribonuclease Inhibitor).
2. NZYRT 2× master mix (includes oligo(dT)18, random hexamers, MgCl₂, and dNTPs).
3. NZY RNase H (*E.coli*).
4. DNase and RNase-free water.
5. Thermal Cycler.

2.3 Real-Time PCR Using LuminoCt Syber Green (Sigma Aldrich)

1. Ice box.
2. DNase and RNase-free PCR tubes.
3. DNase and RNase-free water.
4. Primers Forward and Reverse for each gene to be analyze (Table 1).
5. cDNA.
6. DNase and RNase-free PCR tubes.
7. DNase and RNase-free pipettes and pipette tips.
8. Plates and optical caps for specific thermal cycler.

Table 1
Primer sequences for real-time PCR

Gene of interest	Sequence (5' → 3')	
BAX	Forward	GTGGCAGCTGACATGTTTTTC
	Reverse	GGAGGAAGTCCAATGTCCAG
p27	Forward	CCGGCTAACTCTGAGGACAC
	Reverse	CTTCTGAGGCCAGGCTTCTT
TRAIL	Forward	TTCACAGTGCTCCTGCAGTC
	Reverse	ACGGAGTTGCCACTTGACTT

9. Real-time quantitative thermal cycler.
10. LuminoCt SYBR Green qPCR ReadyMix.
11. Centrifuge.

3 Methods

3.1 RNA Extraction Using E.Z.N.A. Total RNA Kit

All procedures must be performed on a clean workspace. Before starting, carefully wipe the workspace and the material with RNaseZap (solution that destroys RNases on contact).

1. Add TRK Lysis Buffer (mix 20 μ L of 2-mercaptoethanol in 1 mL of TRK Lysis Buffer) to each sample to lyse cells or tissues. The volume of TRK Lysis Buffer to be added depends on the sample size. If the number of cells is $\leq 5 \times 10^6$ add 350 μ L, or 700 μ L if the cell number is $\geq 5 \times 10^6$. Mix carefully with a 200 μ L pipette using up and down movements.
2. Add the same volume of 70% ethanol to each sample (according to the volume used in **step 1**) and vortex. A precipitate might appear after adding ethanol, vortex again.
3. Transfer the entire sample to a HiBind® RNA spin column, with the spin column inside a 2 mL collection tube. The maximum capacity for this column is 750 μ L. If the volume is higher you must repeat the procedure (**step 3**). Spin the samples at $10,000 \times g$ for 30–60 s at room temperature. Discard the supernatant and the collection tube in the extraction hood (remember that TRK Lysis Buffer contains 2-mercaptoethanol).
4. Place a new 2 mL collection tube in each spin column. Add 250 μ L of RNA wash buffer I to each column. Spin the samples at $10,000 \times g$ for 30–60 s. Discard the supernatant and the collection tube. Place a new 2 mL collection tube in each spin column.
5. DNase treatment (*see Note 2*). Important: DNase must always be on ice. Prepare DNase solution: in RNase-free microcentrifuge tubes add 70 μ L of DNase buffer and 10 μ L of DNase per sample. Add 80 μ L of the solution to each sample (*see Note 3*). Incubate for 15 min.
6. Add 500 μ L of RNA Wash Buffer I. Spin the samples at $10,000 \times g$ for 30–60 s. Discard the supernatant but do not discard the collection tube.
7. Add 500 μ L of RNA Wash Buffer II (must be diluted with absolute ethanol before use). Spin the samples at $10,000 \times g$ for 30–60 s. Discard the supernatant and keep the collection tube for **step 8** (*see Note 4*).
8. Repeat **step 7**.

9. RNA elution. Transfer the columns to sterile RNase-free microcentrifuge tubes. Elute the RNA with 50–100 μL RNase-free water. Centrifuge for 1 min at the maximum speed.

3.1.1 RNA Quantification and Storage

Eluted RNA (in RNase-free water or in an elution buffer such as TE) can be quantified using a Nanodrop2000 or a spectrophotometer. During these procedures always use clean gloves to avoid contaminations. Nucleic acids purity is measured by two ratios: absorption at 260–280 nm (A_{260}/A_{280}) and absorption at 260–230 nm (A_{260}/A_{230}). A_{260}/A_{280} ratios below 1.8 are indicative of phenol contamination or low concentration of the nucleic acids while a low 260/230 ratio, below 2, indicates EDTA or carbohydrates contaminations, or residual phenol.

1. Wipe the Nanodrop2000 pedestal with a clean and dry paper.
2. Select RNA as the sample type. Pipette 1 μL of RNase-free water (blank, solution where RNA is diluted). Click on blank to calibrate the Nanodrop2000 for your samples measurements.
3. Wipe the pedestal. Pipette 1 μL of your sample and click measure. The software will measure the RNA concentration and the two ratios 260/280 and 260/230 (*see Note 5*).
4. Repeat **step 3** for the other samples. Wipe the Nanodrop2000 pedestal with a clean and dry paper.
5. RNA samples must be storage at $-80\text{ }^{\circ}\text{C}$ since RNA is highly unstable. Avoid multiple freezing and thawing cycles.

3.2 cDNA Synthesis Using cDNA Synthesis Kit, NZYTech

The following procedures must be performed on ice. Use clean gloves.

1. Prepare and label nuclease-free PCR tubes. Include 2 tubes for negative control (*see Note 6*).
2. Each sample requires 20 μL of reaction. Pipette for each sample tube: 10 μL of master mix, 2 μL of enzyme, 2 μg of RNA sample, and DNase and RNase-free water up to 20 μL (*see Note 7*).
3. Program the thermo cycler with the following protocol: $25\text{ }^{\circ}\text{C}$ for 10 min, $50\text{ }^{\circ}\text{C}$ for 30 min, and $85\text{ }^{\circ}\text{C}$ for 5 min. Add 1 μL of RNaseH to each sample tube (*see Note 8*) and continue the reaction at $37\text{ }^{\circ}\text{C}$ for 20 min. Add a final step at $4\text{ }^{\circ}\text{C}$ to keep your samples refrigerated.
4. Dilute part of your cDNA in DNase and RNase-free water to tenfold (*see Note 9*). Keep part of your original cDNA stock, and freeze both (diluted and original stock of CDNA) at $-20\text{ }^{\circ}\text{C}$ (*see Note 10*).

3.3 Real-Time PCR
Using LuminoCt Syber
Green (Sigma Aldrich)

RT-PCR or q-PCR is a quantitative method that allows the analysis of gene expression. Depending on the total number of reactions (considering the number of your samples and the number of genes to analyze) you can use 96 or 384-well PCR plates (*see Note 11*). We recommend to perform at least 3 technical replicates and include in the analysis 2 or 3 housekeeping genes (genes constitutively expressed in a tissue) (*see Note 12*).

1. Prepare and label DNase and RNase-free PCR tubes for each gene/sample and for each of the master mixes (per gene). For each gene analyzed include a negative control containing no cDNA (non-template control). Make the calculations of each reagent needed (*see Note 13*).
2. Each reaction has 10 μ L of total volume. Pipette the correct water volume to each pool master mix of each gene: 3,2 μ L of water per reaction. Select the adequate oligos (oligos must be diluted at 10 μ M). Place them on ice. Pipette the correct volume of primers to the corresponding pool master mix, final concentration 0,4 μ M (*see Note 14*).
3. Thaw cDNA samples on ice.
4. Prepare the PCR plate according to the planned layout (example in Fig. 1).
5. Pipette 5 μ L LuminoCt per each reaction to the pool master mix.
6. From each gene master mix pipette 9 μ L to each sample well.

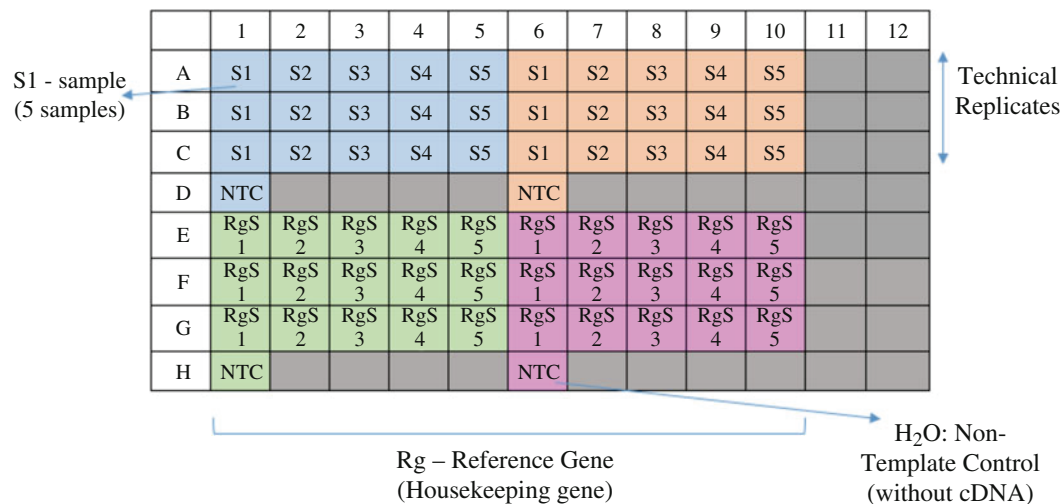


Fig. 1 Representation of a 96 well plate layout. Different genes are represented in different colors. Each gene is being assessed in five different samples using three technical replicates. The two housekeeping genes are represented in green and pink and labeled as Rg. NTC refers to the non-template control that should be included for each gene that is being analyzed

7. Pipette 1 μL of cDNA (diluted 1:10) to each correspondent well (*see* **Note 15**).
8. Seal the PCR plate using optical caps for specific thermal cycler (*see* **Note 16**).
9. Spin the PCR plate.

3.3.1 Program Setup

Bio-Rad CFX Manager is a program used for the detection of PCR and Real-Time PCR.

1. Start the CFX Manager program.
2. Select a protocol and click the EDIT button and define the protocol accordingly: 20 s at 94 °C, 3 s at 94 °C for denaturation, 20 s at 56 °C for annealing/extension and the final step at 4 °C. Select the number of cycles: 40.
3. Open the thermal cycler lid and place the PCR plate.
4. Close the lid and click RUN.

3.3.2 Data Analysis

Data can be analyzed manually using exported Excel files or using Bio-Rad CFX Manager software. Here, we describe a comparative Ct method ($2^{-\Delta\Delta C_t}$) to analyze RT-PCR data. With the comparative Ct method it is possible to calculate the relative gene expression levels between different samples using the *Ct values* generated from RT-PCR [25].

Data Analysis Using Comparative Ct Method

Export all datasheets to Excel files.

1. Open the Excel file containing the *Ct* values. This analysis can be performed using Microsoft Office Excel or other statistical program.
2. Calculate the ΔC_t for each target sample and for each reference sample. $\Delta C_t = C_t (\text{target gene}) - C_t (\text{reference gene})$. The target gene refers to your gene of interest and the reference gene refers to a standard housekeeping gene.
3. Calculate $\Delta\Delta C_t = \Delta C_t (\text{target sample}) - \Delta C_t (\text{reference sample})$. The result of this equation is the fold change of the target gene expression in the target sample, relative to a reference/control sample, normalized to the housekeeping genes (*see* **Note 17**) and do the graph (Fig. 2).

4 Notes

1. Use clean gloves while preparing all reagents and during RNA extraction, to avoid possible contaminants. Prepare purified deionized water, absolute ethanol, and 70% ethanol using purified deionized water. This is essential from the beginning to

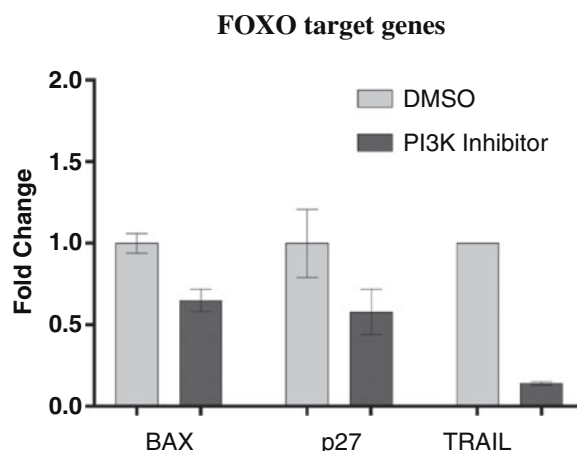


Fig. 2 Gene expression analysis of different FOXO target genes. Light grey bars represent U2OS cells treated with DMSO and dark grey bars represent U2OS cells treated with a PI3K inhibitor. mRNA expression levels were evaluated using RT-PCR and data was analyzed using Bio-Rad CFX manager 3.1 software. Y axis represents the fold change. GAPDH was used as a housekeeping gene

the end of work. RNA extraction is a critical step; RNA is quickly degraded by RNase, an enzyme present ubiquitously.

2. DNase treatment is an optional step that will remove any contaminant DNA present in your RNA samples. For this reason, we strongly advise to perform this step.
3. Pipette the 80 μ L carefully, do not touch with the pipette tip on the column's membrane.
4. To avoid ethanol contamination on your RNA samples, **carefully** tap the collection tube on a filter paper sheet to remove the remaining wash buffer. Additionally, we also recommend an extra spin without adding any reagent, to guarantee that there is no ethanol in the sample.
5. Low 260/280 ratio usually indicates phenol contamination or low concentration of the nucleic acids. The Nanodrop2000 can also be contaminated from previous samples (e.g., Proteins). In this case we advise to repeat the procedure from **step 1**.
6. Include two negative controls: (1) one tube where no enzyme is added and (2) one tube where RNase-free water is used instead of RNA. This set of controls will guarantee that there is no contamination with genomic DNA.
7. We recommend performing one Master Mix in order to reduce pipetting errors. Calculate the amount of Master Mix needed for all samples and then pipette the correct volume of Master Mix to each sample. Following, calculate the volume equivalent to 2 μ g of RNA for each of your samples and subtract this amount to 20 μ L – this value refers to the amount of water that you need to pipette to make up a total volume of 20 μ L per reaction.

8. RNase H must be added in order to eliminate any remaining RNA following the reverse transcription reaction. The presence of remaining RNA may bind to cDNA and inhibit DNA synthesis. The inhibition of DNA replication might affect the Ct values.
9. It is recommended to dilute the cDNA at least five to tenfolds in order to avoid inhibitory effects on real-time PCR due the presence of non-cDNA contaminants (often carried out from impure RNA), or components of cDNA synthesis.
10. It is suggested to maintain the original cDNA stock and the diluted cDNA. If there is the need to run the samples again you use the same dilution and avoid thawing the original stocks unnecessarily.
11. The recommended volume for 96-well PCR plates is 15 μ L and for the 384-well PCR plates is 10 μ L. We recommend placing the PCR plate on a dark sheet of paperboard to increase the contrast and to help you keep track of the wells that have been loaded.
12. Prepare a scheme/layout of your PCR plate in Microsoft Office Excel discriminating the exact position of each sample as well as the primers needed for each one, as shown in Fig. 1. The scheme will help you while pipetting and designing the plate setup on the software latter.
13. We recommend making a master mix for each individual gene. To calculate the volumes needed for the pool master mix you have to consider the total number of reactions per gene (e.g., if you have five different samples and you perform three technical replicates and a non-template control, the total reactions per gene is 16). Add an 8% volume increment to each component of the master mix to account for pipetting errors. For a total volume of 10 μ L reaction you must pipette: 0,40 μ L of Primer Forward (at 10 μ M concentration), 0,40 μ L of Primer Reverse (at 10 μ M concentration), 3,2 μ L of DNase and RNase-free water, and 5 μ L of Luminoc SYBR green and 1 μ L cDNA (diluted 1:10). Considering the previous example, if you have 16 reactions per gene, you must prepare a master mix with 6.9 μ L Primer Forward, 6.9 μ L Primer Reverse, 55.3 μ L water, 86.4 μ L LuminoCt (8% error included), and then pipette 9 μ L of this master mix per well, and pipette 1 μ L cDNA per well. LuminoCt must be protected from light since it is light sensitive.
14. Carefully mix primers prior to use using a 200 μ L pipette.
15. Use a new tip for each well that is being pipetted.
16. After sealing the PCR plate use a spatula to make sure that the plate is totally sealed and cut the excess.

Table 2
Representation of the comparative Ct method

	Target gene	Reference gene
Target sample—Treatment with drug	A	GAPDH 1
Reference /control sample	C	GAPDH 2

$$\Delta Ct = Ct(\text{target gene}) - Ct(\text{reference gene})$$

$$\Delta Ct(\text{target sample}) = Ct(A) - Ct(\text{GAPDH 1})$$

$$\Delta Ct(\text{Reference sample}) = Ct(C) - Ct(\text{GAPDH 2})$$

$$\Delta\Delta Ct = \Delta Ct(\text{target sample}) - \Delta Ct(\text{Reference sample})$$

17. Comparative Ct method is a useful method to calculate relative gene expression levels using Ct values generated by RT-PCR. Here we will present an example to help your data analysis. Imagine the following situation: analyze the expression of gene X before and after a treatment with drug A. In this case the target sample will be the sample after treatment, and the reference or control sample, the sample without treatment (Table 2). The housekeeping gene used was GAPDH.

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Chapter 10

Using ChIP-Based Approaches to Characterize FOXO Recruitment to its Target Promoters

Neeraj Kumar and Arnab Mukhopadhyay

Abstract

Chromatin immunoprecipitation (ChIP) coupled to quantitative real-time PCR (ChIP-qPCR) or Next-Generation Sequencing (ChIP-seq) enables us to study the dynamics of chromatin recruitment of transcription factors (TFs). The popular model system *Caenorhabditis elegans* has provided us with fundamental understanding of the role of Insulin/IGF-1-like signaling (IIS) in metabolism and aging. The FOXO TF DAF-16 is the major output of the pathway that regulates most of the phenotypes associated with the IIS pathway. Here, we describe a ChIP protocol to study FOXO recruitment dynamics in whole *C. elegans* extracts. We discuss detailed practical procedures, including optimization, growth, harvesting, formaldehyde fixation, sonication of worms, TF immunoprecipitation for further downstream processing using qPCR as well as NGS for the analysis of FOXO-bound DNA.

Key words Chromatin immunoprecipitation, Quantitative real-time PCR, Next-generation sequencing, Transcription factor, FOXO, DAF-16, Gene promotor, *C. elegans*

1 Introduction

Chromatin immunoprecipitation (ChIP) is an invaluable tool for probing in vivo interactions between a specific protein and its genomic DNA targets that has made an incredible impact on the field of modern molecular biology [1]. It is routinely employed to discern the Transcription Factor (TF) interactions with its target genes, at specific genomic locations [2–4]. In most ChIP procedures, formaldehyde is used as a reversible crosslinking agent to generate protein-protein and protein-DNA complexes [5]. The cross-linked chromatin complex is then cleaved either using nuclease digestion or by sonication to generate TF-protected fragments of DNA of nearly 400 base pairs [6, 7]. An aliquot of the fragmented DNA is set aside for use as a reference (input) sample before the preclearing and immunoprecipitation steps. After preclearing to reduce nonspecific immunoprecipitation, the cross-linked, fragmented protein-DNA complexes are immunoprecipitated using a

specific antibody raised against the protein of interest or its tag. After rigorous washing steps, the immunoprecipitated complexes are eluted from the protein A- or protein G-agarose resin. Thereafter, both the eluted ChIP as well as the input samples are treated with proteases and heated overnight to reverse cross-link the protein-DNA complex [8]. TF-specific fragmented DNA are extracted by standard protocol [9] and further utilized for various downstream processes, such as quantitative real-time PCR (qPCR), microarray or by high-throughput sequencing [10].

The FOXO transcription factor DAF-16 is the major output of the Insulin-IGF-1-like signaling (IIS) pathway in *C. elegans* [11, 12]. Most of the phenotypes associated with the IIS pathway, like longevity, metabolism, stress resistance, and dauer formation, are dependent on DAF-16. DAF-16 also takes inputs from the germline signaling pathway to affect metabolism and longevity [13]. How a single transcription factor can process signal information from multiple pathways and differentially regulate gene expression to modulate diverse phenotypes is indeed a remarkable scientific problem to study. The answer to this lies in deciphering the recruitment dynamics of DAF-16 under different physiological conditions like lowered IIS or defective germline signaling. In this context, establishing consistently performing ChIP experiments in *C. elegans* is of utmost importance.

ChIP has been used as a robust and versatile technique in different model organisms, despite challenges of its inherent variability [10]. A great degree of optimization of several steps is required, like crosslinking time and temperature, chromatin quantification, sonication parameters, amount of resin used, antibody specificity, reverse-crosslinking, elution and DNA extraction, as they affect the reproducibility of the experimental outcomes of ChIP [8]. ChIP in *C. elegans* poses further challenges in terms of reproducibility due to difficulty in disrupting the outer cuticle and underlying epidermal layers, the contaminating DNA from the bacterial food, and the inherent sample variability among the biological replicates due to staging. Therefore, use of ChIP in *C. elegans* research is not as widespread as compared to cell culture-based studies, but has helped in elucidating a wide range of cellular mechanisms [14–21].

Here, we describe an optimized ChIP protocol that can be efficiently performed using *C. elegans* larva as well as adults. The output of the ChIP protocol may be visualized by qPCR as well as high-throughput sequencing.

2 Materials

Prepare all solutions using analytical grade reagents with ultrapure water and store them at room temperature (unless mentioned otherwise). Make proper aliquots of sterile solutions to prevent

contamination. Avoid multiple freeze-thaw of protein or antibodies solutions; it is often better to maintain them in one-time-use aliquots. Filter buffer solutions using a 0.4 μ m filter to eliminate insoluble contaminants.

2.1 Worm Culture

1. *C. elegans strains*: Many strains can be procured from the *Caenorhabditis* Genetics Centre (CGC; <https://cgc.umn.edu/>) or National Bioresource Project (NBRP; <http://shigen.nig.ac.jp/c.elegans/>).
2. *M9 Buffer (1 $\times$)*: Dissolve 3 g KH_2PO_4 , 6 g Na_2HPO_4 , 5 g NaCl, 1 ml of 1 M MgSO_4 in 1 L of water. Autoclave it and store in aliquots that may be discarded after use.
3. *Cholesterol (10 mg/ml)*: Dissolve 100 mg cholesterol powder in 10 ml of 100% ethanol.
4. *Luria Bertani broth*: Dissolve 10 g Tryptone, 5 g Yeast extract, 10 g NaCl in 1 L of water and autoclave it.
5. *Potassium phosphate Buffer (1 M)*: Dissolve 98 g anhydrous KH_2PO_4 and 48 g anhydrous K_2HPO_4 in 900 ml deionized water (pH should be 6.0). Minor adjustments may be made with 1 M solutions of KH_2PO_4 or K_2HPO_4 . Autoclave and store at room temperature (RT).
6. *Nematode Growth Medium (NGM) agar*: Dissolve 3 g NaCl, 17 g agar powder, 2.5 g Bactopectone in 975 ml of deionized water and autoclave it. Add 1 ml of 1 M CaCl_2 , 1 ml of 1 M MgSO_4 , 25 ml of Potassium Phosphate Buffer (PPB), and 0.5 ml of cholesterol (10 mg/ml) after cooling the media down to 60 $^\circ\text{C}$. Let media solidify at RT after pouring. You will need 60 mm, 90 mm, as well as 150 mm petri plates for the experiments.
7. *Preparation of OP50 bacteria and seeding NGM agar plates*: Inoculate a single colony of OP50 into 100 ml of LB broth and grow for 16 h at 37 $^\circ\text{C}$ with shaking (200 rpm). Drop enough on a NGM plates so that the culture is spread evenly. Allow plates to dry at RT. Use seeded plates within a week.
8. *Bleaching solution*: Commercial bleach (6.15% sodium hypochlorite) is used for bleaching worms.
9. *Instruments*: To maintain *C. elegans* cultures in the laboratory, instruments required include BOD incubators, stereomicroscope with diascopic illumination, autoclave, hotplate, magnetic stirrer, vacuum pump assembly, liquid dispensing system, refrigerated table-top centrifuge, vortex and laminar flow cabinet.

2.2 Chromatin Immunoprecipitation

1. *PBS (1 $\times$)*: Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 , 0.24 g KH_2PO_4 in water to a final volume of 1 L. Adjust the pH to 7.4 with HCl and store at RT after autoclaving.

2. *TE* (1×): Mix 1 ml of 1 M Tris–Cl, pH 8.0 and 0.2 ml of 0.5 M EDTA in 100 ml of water. Autoclave and store at RT.
3. *Cross-linking buffer* [CB; 1% formaldehyde (vol/vol)]: Mix 1 ml formaldehyde (37%) and 36 ml of 1× PBS. This solution must be made fresh each time.
4. *Protease inhibitor cocktail*: Dilute the protease inhibitor as specified by the manufacturer and store diluted aliquots in 50 µl volume at –20 °C. We use Sigma (Cat.no. P2714) protease inhibitor cocktail and dilute it in 10 ml Milli Q water.
5. *Glycine* (2.5 M): Dissolve 98.75 mg Glycine in 500 µl of water. Make fresh every time.
6. *SDS lysis buffer*: Mix 10 ml SDS solution (10%), 2 ml EDTA solution (0.5 M, pH 8.0), and 5 ml Tris–Cl solution (1 M, pH 8.0) in water to make a final volume of 100 ml and store at 4 °C. Add 10 µl of the diluted protease inhibitor cocktail per ml of SDS lysis buffer just before use.
7. *ChIP dilution buffer*: Mix 55 ml Triton X-100 (10%), 1.2 ml EDTA solution (0.5 M, pH 8.0), 8.35 ml Tris–Cl solution (1 M, pH 8.0), 16.7 ml NaCl solution (5 M) in water to make a final volume of 500 ml and store at 4 °C. Add 10 µl of diluted protease inhibitor cocktail per ml of SDS lysis buffer just before use.
8. *Salmon sperm DNA/protein “A” agarose beads*: The beads are supplied as a 50% (wt/vol) solution in 70% (vol/vol) ethanol that should be resuspended by vortexing. Pipette out 100 µl of the solution in a microcentrifuge tube and wash by adding 1 ml chilled 1× PBS. Mix thoroughly by inverting 5–6 times, centrifuge at 500× *g* for 1 min and quickly remove the supernatant without disturbing the beads. Repeat the washing step two more times and finally resuspend the beads in 50 µl of 1× PBS containing protease inhibitor cocktail.
9. *Low salt wash buffer*: Mix 1 ml SDS solution (10% wt/vol), 10 ml Triton X-100 (10% vol/vol), 400 µl EDTA solution (0.5 M, pH 8.0), 20 µl Tris–Cl solution (100 mM, pH 8.0), 3 ml of NaCl solution (5 M) in water to make a final volume of 100 ml and store at 4 °C.
10. *High salt wash buffer*: Mix 1 ml SDS solution (10% wt/vol), 10 ml Triton X-100 (10% vol/vol), 400 µl EDTA solution (0.5 M), 20 µl Tris–Cl solution (100 mM, pH 8.0), 10 ml NaCl solution (5 M) with water to a final volume of 100 ml and store at 4 °C.
11. *LiCl wash buffer*: Mix 2.5 ml LiCl solution (1 M), 1 ml sodium deoxycholate solution (10% wt/vol), 20 µl EDTA solution (0.5 M), 1 ml Tris–Cl solution (100 mM, pH 8.0) with water

to a final volume of 100 ml. Make it fresh each time to avoid any precipitation and keep in ice for 20–30 min before use.

12. *Elution buffer*: Mix 100 μ l of 10% (wt/vol) SDS and 100 μ l of 1 M NaHCO_3 to a final volume of 1 ml with water.
13. *Additional reagents*: Glycogen (1 mg/ml), ethanol (100% vol/vol), Liquid nitrogen and phenol-chloroform-isoamylalcohol (25:24:1), pH 7.7–8.3.
14. *Instruments*: A 7 ml glass dounce homogenizer (for example, Item# 357542, Wheaton, USA), waterbath ultrasonicator (we used Bioruptor Plus, Diagenode, Belgium), tube rotator, rocking shaker, and dry bath are required for processing of ChIP samples.

2.3 Real-Time PCR

1. *Primer design*: Primers may be designed using online tools like Primer3. To ensure that the ChIP experiment has worked successfully, the binding of DAF-16/FOXO to the promoter of its conserved transcriptional target *sod-3* may be evaluated. For this, primers encompassing the predicted DAF-16/FOXO-binding site on the *sod-3* promoter may be considered. Primers should also be designed from the 3'UTR region of *sod-3* where no binding of the transcription factor is expected. Users can design other primers as per their gene of interest (see **Note 1**). We also used *daf-16/FOXO* null, *daf-16 (mgdf50)*, as a negative control for ChIP experiments.
2. *Oligonucleotide primers*: Resuspend the lyophilized primers in 10 mM Tris–Cl (pH 8.0) to prepare a 100 μ M stock. This is further diluted to make a 2.5 μ M working stock. The forward and reverse primer working ratio as well as specificity must be checked before setting up final experiments (described later). Depending on the optimum ratio, a primer mix is prepared for use in the qPCR reactions.
3. *SYBR Green*: Any qPCR master mix containing SYBR Green dye can be used as per the manufacturer's instruction.
4. *Optically clear tubes or plates* can be used for qPCR as per requirement.
5. *Real-time PCR machine* of any brand can be used after proper calibration.

2.4 High-throughput Sequencing

Any appropriate NGS platform can be used for sequencing ChIP DNA. However, users are advised to choose a system that can generate maximum number of low-error reads (around 100 bp or more). Read number can vary from 5–10 M to 20–40 M, depending upon the expected number of sites a protein of interest can bind. Available platforms are Illumina NextSeq, HiSeq or NovaSeq, Ion Torrent (Life Technologies), 454 (Roche), SOLiD (Life Technologies), PacBio (Pacific Biosciences) etc.

3 Methods

3.1 Worm Culture

For ChIP experiments, worms of a particular developmental stage or mixed stage worm cultures can be obtained from solid as well as liquid medium. Mixed stage cultures are generally considered when the expression status of the transcription factor-of-interest against which the antibody is raised is not well characterized. It is also a desired condition when one is interested in elucidating all likely binding sites of the transcription factor. Therefore, growing mixed stage culture is described here.

1. Aseptically pour fixed volume of NGM agar into sterile 60, 90, or 150 mm plastic petri plates using a peristaltic pump and let them dry for 2 days at RT.
2. Spread OP50 liquid culture on the dried NGM plates using a pipette, under sterile conditions in a biosafety cabinet.
3. Incubate the plates at room temperature for 2–3 days for the bacteria to grow.
4. Transfer well-fed worms of desired strain of *C. elegans* to 60 mm plates and let them grow at 20 °C for a few days to obtain sufficient gravid worms on the plates.
5. Bleach the worms to obtain eggs and remove any contamination (*see Note 2*).
6. Transfer eggs on to fresh 90 mm plates seeded with OP50 and let them grow at 20 °C in an incubator. In a few days, you will expect to obtain worms in various developmental stages, without starvation.
7. Cut chunks of agar from a well-fed plate containing 5–10 gravid adults along with other stages and keep inverted on 150 mm plates.
8. Grow these plates in an incubator at 20 °C for 6–8 days till they consist of all the stages of well-fed worms (*see Note 3*). Observe the plates every day to prevent starvation. Starved out areas may be replenished with concentrated *E. coli* OP50.

3.2 Cross-Linking and Sample Preparation

1. Harvest the worms from 10 to 15 plates (150 mm) by washing using 4–5 ml of 1× PBS buffer (thrice) and transfer them into 15 ml centrifuge tubes (*see Note 4*).
2. Centrifuge the worms at $650 \times g$ for 1 min and discard the supernatant. Collect the pellets from all tubes into a single 15 ml tube by resuspending with small volume of 1× PBS.
3. Centrifuge and wash the pellet four times with 1× PBS to remove excess bacteria.
4. After the final wash and centrifugation, remove most of the supernatant from the tube.

5. Resuspend the worm pellet with 3 ml of crosslinking buffer (CB), spin down at $650 \times g$ for 30 s, discard the supernatant, and then again resuspend in 3 ml of fresh CB.
6. Transfer the resuspended worms to a 7 ml glass dounce homogenizer and apply 10 strokes with a 1/3 turn after each stroke.
7. Collect the partially lysed worms in the same 15 ml tube. Rinse the homogenizer with one additional ml of CB and add it to the 3 ml partially lysed worm suspension to make a total volume of 4 ml.
8. Incubate it at room temperature on a tube rotator (20 rotations per minute) for 15 min (*see Note 5*).
9. Add 200 μ l of 2.5 M glycine after 15 min and incubate tubes for an additional 5 min on the tube rotator to quench the crosslinking reaction.
10. Pellet worms by centrifugation at $650 \times g$ for 2 min and discard the supernatant.
11. Wash the pellet four times with 10 ml of $1\times$ PBS containing proteinase inhibitor cocktail.
12. Remove buffer as much as possible after the last wash.
13. Instantly freeze the cross-linked pellet in liquid nitrogen and then it can be stored indefinitely at -80°C for downstream processing.

3.3 Sonication

1. Thaw worm pellet from -80°C by incubating on ice and then resuspend the pellet in 2 ml of chilled SDS lysis buffer containing protease inhibitor (*see Note 6*).
2. Remove the supernatant after centrifugation at $650 \times g$ for 2 min at 4°C .
3. Resuspend the pellet again in 2 ml SDS lysis buffer containing protease inhibitor and incubate on ice for 10 min.
4. Transfer the suspension into a 10 ml tube compatible with a water bath-based ultrasonicator.
5. Individual user will be required to empirically standardize the conditions of sonication to obtain sufficient DNA yield, fragment length, and reproducibility. We found the target size of 400 bp (fragment spread from 150 to 1200 bp) after 18 cycles (30 s on/1 min off) on high power setting (*see Note 7*).

3.4 Chromatin Preclearing and Immunoprecipitation

1. Transfer the sonicated lysate from 10 ml ultrasonication tubes to 2 ml microcentrifuge tubes.
2. Centrifuge the sonicated lysate at $16,000 \times g$ at 4°C for 20 min to pellet the debris. Transfer the supernatant to a fresh tube without disturbing the pellet.

3. Quantify the amount of protein in the supernatant using a protein estimation assay (*see* **Note 8**).
4. Take 2.5 mg of total protein in 15 ml centrifuge tube and make the volume equal in all the tubes by adding SDS lysis buffer. Then, dilute the total protein five times with ChIP dilution buffer. ChIP experiments should be performed by taking at least three technical replicates for individual samples (*see* **Note 9**).
5. After dilution, take aliquots of 50 μ l each from the three technical replicates for individual samples. Mark them as “input aliquot” and store at -80°C (*see* **Note 10**).
6. Add 50 μ l of prewashed salmon sperm DNA/protein-A agarose beads and 20 μ l of preimmune serum for preclearing. Incubate at 4°C for 1 h on a rocking shaker (20 rocking per minute).
7. Centrifuge the tubes at $400 \times g$ for 3 min at 4°C and carefully transfer the supernatant to fresh tubes. Discard the pellet of beads.
8. Add 35 μ l of anti-DAF-16 antibody [21] in each tube and incubate overnight at 4°C on a rocking shaker (20 rockings per minute) (*see* **Note 11**).

3.5 Washes and Elution

1. Centrifuge the tubes at $400 \times g$ for 2 min at 4°C , discard the supernatant, and transfer the beads to 2 ml microcentrifuge tubes by resuspending in 1 ml of low salt wash buffer. Place the tubes on a rocking shaker (30 rocking per minute) for 5 min at RT.
2. Centrifuge the 2 ml tubes at $400 \times g$ for 1 min at RT, aspirate the supernatant, and add 1 ml of high salt wash buffer and place the tubes on a rocking shaker for 5 min at RT.
3. Wash the beads once with LiCl wash buffer as described in **step 2**.
4. Wash the beads three times using $1\times$ TE, as described in **step 2**.
5. After the last wash, aspirate and discard the supernatant as much as possible, without disturbing the pellet.
6. The protein-DNA complex is eluted from beads twice with 250 μ l of elution buffer (a total of 500 μ l) and pooled in a 2 ml microcentrifuge tube. For this, add the buffer to the beads, mix well, incubate at RT for 10 min, centrifuge at $400 \times g$, and collect the supernatant.

3.6 Reverse Cross-Linking

1. Take one 50 μ l “input aliquot” out of the three technical replicates for individual samples (stored at -80°C in **step 5**

of Subheading 3.4, chromatin preclearing and Immunoprecipitation).

2. Add 450 μ l of elution buffer in each “input aliquot.”
3. Add 50 μ l of 5 M NaCl in the diluted “input aliquot” as well as in the eluted 500 μ l protein-DNA complex (from **step 6** of Subheading 3.5, Washes and Elution).
4. Reverse cross-linked the complexes by incubating overnight at 65 °C, in a dry bath.

3.7 DNA Extraction

1. To the mixture, add 10 μ l of 0.5 M EDTA, 20 μ l of 1 M Tris-Cl (pH 6.5), and 10 μ l of proteinase K (20 mg ml⁻¹).
2. Incubate the mixture at 45 °C for 2 h in a dry bath.
3. Add 600 μ l of phenol-chloroform-isoamylalcohol to the tubes. Vortex for 10 s and then centrifuge at 14,000 $\times g$ for 10 min at RT.
4. Transfer the upper aqueous phase of each sample to a clean tube without disturbing the lower organic phase.
5. Add 1 ml of ethanol and 1 μ l of 1 mg/ml glycogen to each tube and mix. Incubate at -80 °C for 30 min.
6. Centrifuge at 14,000 $\times g$ for 10 min at 4 °C to pellet the DNA. Carefully aspirate and discard the supernatant.
7. Add 1 ml of 70% ethanol to each tube and centrifuge at 14,000 $\times g$ for 10 min. Aspirate the supernatant carefully and air dry the pellet, but avoid over-drying.
8. Resuspend the input and ChIP DNA in 200 μ l and 25 μ l of 10 mM Tris-HCl, pH 8.5 respectively. Store the DNA at -20 °C till further downstream processing.

3.8 Real-Time PCR

1. Mix forward and reverse qPCR primer pair, in required ratio, in the same tube following optimization. The forward and reverse primers working ratio and specificities must be checked before setting up final experiments (Table 1) (*see Note 12*).
2. Serially dilute the input DNA ranging from 1:5 to 1:160 with 10 mM, Tris-Cl pH 8.5.
3. Set up qPCR reactions in triplicate with the diluted input DNA as per Table 2.
4. Vortex the samples briefly to mix and centrifuge for 10 s.
5. Perform qPCR as per conditions mentioned in Table 3 (*see Note 13*).
6. Save and export the threshold cycle (Ct) data as an “excel file” format for analysis.
7. Calculate the efficiency for each primer pair [$10^{(-1/\text{slope})}$] by plotting the log (concentration of template) against the

Table 1

[illegible]

Table 2
QPCR reactions to determine primer efficiency using input DNA

Component	Amount per reaction
Primer mix (optimized ratio)	x μ l
Diluted 'input' DNA (1:5 to 1:160)	1 μ l
Nuclease-free water	5.5–9.5 μ l
SYBR green mix	12.5 μ l
Total	25 μl

x = Number of μ l depending on the primer ratios as determined in Table 1

Table 3
PCR cycling conditions for quantitative ChIP-qPCR

	Stage 1	Stage 2	
Temperature	95.0 °C	95.0 °C	60.0 °C
Time	10.00 min	15 s	1.00 min
Cycles	1	40	

Table 4
Final reaction setup for ChIP-qPCR

Component	Amount per reaction
Primer mix (optimized ratio)	x μ l
Sample DNA (X)	1 μ l
Nuclease-free water	5.5–9.5 μ l
SYBR green mix	12.5 μ l
Total	25 μl

average Ct values of the three technical replicates for each dilution. The efficiency of the primer pair should ideally be 2.0 (range from 1.8 to 2.2 are also acceptable) (*see Note 14*).

- Set up the qPCR reactions for each ChIP DNA and the diluted input sample as per Table 4 and perform qPCR as per conditions mentioned in Table 3 (*see Note 15*).
- Save and export the threshold cycle (Ct) data to “excel file” format for further analysis.
- Calculate the Δ Ct value for each sample as [Ct (sample) – Ct (input)].

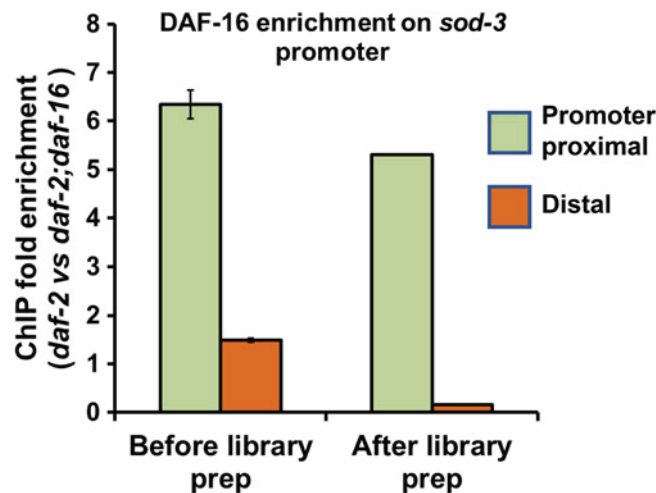


Fig. 1 Validation of a ChIP experiment using anti-DAF-16 antibody. ChIP was performed in *daf-2(e1370)* where DAF-16 is activated as well as in *daf-16(mgdf50);daf-2(e1370)*, where DAF-16 is absent. Binding to either the *sod-3* promoter-proximal DAF-16-binding site or binding to *sod-3* 3'UTR was determined using qPCR in the two strains. Fold enrichment of DAF-16 binding is shown after ChIP as well as after library preparation

11. Calculate the $\Delta\Delta Ct$ (ΔCt (experimental sample) – ΔCt (negative control)). For DAF-16 ChIP, a null mutant of *daf-16*, like *daf-16(mgdf50)*, may act as a negative control.
12. Calculate the fold difference between experimental sample and negative control by using the equation- $2^{\Delta\Delta Ct}$. (see **Note 16**, Fig. 1 over here).

3.9 High-throughput sequencing

NGS may be performed on one of several platforms, as per user preference. The basic principles and protocols vary among the different platforms and it is not reasonable to discuss all of them in the current chapter. Moreover, most of the users prefer to outsource the samples for high-throughput sequencing. Therefore, we are providing the details of the basic steps required to assess the size, quality, and quantity for ChIP samples, irrespective of the sequencing platform employed.

1. Determine the approximate concentration of the input DNA samples using a spectrophotometer (see **Note 17**).
2. Use a high-sensitivity fluorometer (for example, Qubit Fluorometer, Thermo Fisher Scientific, USA) to accurately measure the concentration of the fragmented DNA molecules. For this, first, calibrate the machine using nucleic acid standards provided by the manufacturer. Following calibration,
 - (a) Take 1 or 2 μ l each of the ChIP or the diluted input samples and mix in the buffer (provided by the

- manufacturer) to make the volume to 200 μ l in 0.6 ml centrifuge tubes.
- (b) Briefly vortex and then centrifuge the samples.
 - (c) Incubate them in the dark for 2 min and measure absorbance.
 - (d) Determine the DNA concentration in ng/ μ l.
3. To further validate the exact size and quality, use of Bioanalyzer (Agilent, USA) is advised.
 4. For this, dilute the samples appropriately in the range of 250–350 pg/ μ l for Agilent High Sensitivity DNA chip-based electrophoresis.
 5. Run 1 μ l of each diluted ChIP and input samples to verify the quality and fragment size distribution.
 6. We obtained the DNA fragment spread from 0.15 to 1.2 kb with maximum fragments size near to 400 bp. DNA is ready for in-house NGS library preparation or outsourcing as per user preference.
 7. One may use qPCR, as described above, to ensure that the enrichment is retained following ChIP-seq library preparation (Fig. 1).

4 Notes

1. Design primers for the promoter of interest by using the default parameters mentioned in the Primer3 software (<http://bioinfo.ut.ee/primer3/>). Choose a region encompassing the predicted TF-binding site, with an amplicon size range of 50–150 bp. As a control, design another primer pair in the 3'-untranslated region (3'-UTR) of the same target gene or any intergenic region where the TF may not bind.
2. Harvest *C. elegans* from NGM agar plates containing many gravid adult worms into a 10 ml centrifuge tube, using sterile 1 $\times$ M9. Centrifuge and remove supernatant and add fresh 1 $\times$ M9. Repeat these **steps 3–4** times and finally, add water to make the volume to 3.5 ml. Add 0.5 ml of 5 N NaOH and 1 ml bleach (sodium hypochlorite). Shake well or vortex the tube for around 5 min. Visually monitor the tube under the microscope to ensure complete worm lysis and extend the time to at the most 6 min, if required. Centrifuge for 30 s at 1300 $\times g$ and aspirate the supernatant. Wash the pellet with 1 $\times$ M9 and collect eggs by centrifugation as above. Repeat the washing 3–4 times. Transfer the sterile eggs on to a sterile NGM plate seeded with OP50.

3. Always ensure a fixed number of gravid adult worms (e.g., 1200 gravid adults per 90 mm plate) along with other stages. Keeping constant ratio of different stages of worms on the plates will minimize experimental variations. Never use contaminated plates for downstream processing.
4. The number of plates of worms that should be used may need to be standardized depending on the amount of ChIP-ed DNA required. When a large number of promoters are to be assessed for binding, or if the downstream application is ChIP-sequencing, one will need greater number of 150 mm plates to start with. Small-scale experiments can be done with twelve 150 mm plates. It is also wise to start with larger number of plates as some may be lost due to infections.
5. The standardization of crosslinking time in CB at a defined temperature (22–23 °C) is critical in achieving optimum signal-to-noise ratio. For initial standardization, one should perform the ChIP procedure with different crosslinking times to determine the optimum signal (promoter) to noise (3'UTR or intergenic region) enrichment. We observed 15 min incubation time is the most suitable for our experiments with DAF-16 antibody, but this should be empirically determined for each antibody.
6. SDS tends to precipitate in the buffer on cooling. Before use, dissolve the SDS properly in the buffer by mixing.
7. The density of the cross-linked worms in the SDS buffer should be the same for each experiment. This is required to ensure that the same number of cycles and pulse time will deliver similar DNA fragment length in successive experiments.
8. Detergent (SDS) present in the buffer may interfere with protein estimation. The SDS may be removed by precipitating the protein using chilled acetone. We used CB X™ protein assay kit (G-Biosciences, USA) that uses similar principle.
9. Amount of lysate (sonicated sample) required for each ChIP experiment should be determined empirically for each antibody. After standardization, we used nearly 2.5 mg of sonicated lysate for each technical replicate for ChIP experiments with DAF-16 antibody.
10. To check for the efficiency of sonication, one of the three “input” aliquots is processed for DNA extraction (Subheading 3.7) after the Chromatin preclearing and Immunoprecipitation steps. The extracted DNA may be run on a 1% agarose gel. DNA fragment spread from 1.2 to 0.15 kb with maximum enrichment at around 400 bp may be considered optimum sonication. The other input sample is processed by reverse crosslinking, along with the ChIP-ed samples. The third aliquot is retained frozen and serves as a standby.

11. The amount of antibody added should be determined empirically for each batch or TF. Select the antibody concentration that gives the best signal-to-noise ratio.
12. First add the forward and reverse primer (in nM) in different ratios (100:100, 100:200, 100:300, 200:100, 200:200, 200:300, 300:100, 300:200, 300:300) from the 2.5 μ M stock to standardize the working ratio. Accordingly, add water to equalize volume in each qPCR tube. Then, add diluted input DNA and SYBR green as given in Table 1. Following qPCR, select the primer ratio that produces a single sharp dissociation peak [22].
13. First, make master mix of forward and reverse primers in the standardized ratio. We also recommend making master mix of common reagents during qPCR, if possible. Because of the low volumes involved, DNA sample should not be pipetted into the tubes first.
14. The efficiency of the primer pair should ideally be 2.0 (range from 1.8 to 2.2 is also acceptable). Ct values >35 should not be considered. Ct values should ideally be in 20–30 range and can be adjusted by taking more concentrated ChIPed DNA samples. The Ct of the technical replicates should not differ by more than a value of 0.5.
15. Dilute the “input samples” in the range of the Ct obtained for the ChIP DNA to avoid errors. It will be used later to normalize the experimental samples.
16. A deletion or loss of function mutant in the protein-of-interest should ideally be the negative control. If it is not available, then immunoprecipitation with pre-bleed serum or a no-antibody control can be used as negative control.
17. To start with, a spectrometric evaluation provides a quick estimation of both concentration and purity of sample. But for exact quantification of very low concentration of intact dsDNA, fluorescent dye-based methods (e.g., Qubit Fluorometer, Thermo Fisher Scientific, USA) offer relatively high specificity and sensitivity.

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Chapter 11

RNAi-Mediated Silencing of FOXO Factors

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Abstract

RNAi-mediated silencing of a particular gene is an important tool in the field of biology. Knocking down experiments has revealed the role of different proteins and their downstream targets. Here, we describe the procedure of silencing transcription factor of Forkhead box class “O” (FOXO) using shRNA.

Key words FOXO, shRNA, Competent cell, Transformation, Transfection

1 Introduction

RNA interference (RNAi) has emerged as an important tool in the field of molecular biology. It is widely used to study the gene function by knocking down the expression of a particular gene. Use of this technique has helped in better understanding of the role of different genes in disease pathogenesis. The RNAi works on the sequence-specific degradation of the host mRNA [1]. Over time a number of methods of RNAi have been discovered. The simplest one is transfection of chemically synthesized short interfering RNA (siRNA) and short hairpin RNA (shRNA) oligonucleotide directly into the cytoplasm.

In case of siRNA delivery, the siRNA duplex is directly delivered into the cytosol of target cell. On the other hand, DNA-based strategies are used for shRNA delivery where siRNA are produced by the intercellular processing of longer hairpin transcripts by Dicer enzyme [2]. The shRNAs are transcribed in nucleus and transported to the cytoplasm where they are processed by Dicer to produce the effector siRNA.

FOX (Forkhead Box) proteins are a family of transcription factors that play an important role in the regulating the expression of genes involved in cell growth, proliferation, differentiation, longevity, and apoptosis. Many fox proteins are important in embryonic development. FOX proteins also have pioneering transcription activity by being able to bind condensed chromatin during cell

differentiation processes. The defining feature of fox proteins is the forkhead box, a sequence of 80–100 amino acids forming a motif that binds to DNA. This forkhead motif is also known as winged helix due to the butterfly-like appearance of the loops in the protein structure of the domain. Forkhead genes are subgroups of helix-turn-helix class of proteins.

In mammals, the class of Forkhead box class “O” (FOXO) transcription factors consists of four members—FOXO1 [3], FOXO3a [4], FOXO4 [5], FOXO6 [6]. FOXO1, FOXO4, FOXO3a are orthologs to *C. elegans* Daf 16 and *Drosophila melanogaster* dFOXO. FOXO6 is expressed only in brain. FOXO family proteins are involved in multiple important biological processes, such as cell cycle arrest [7], DNA repair [8], apoptosis [9], glucose metabolism [10], aging and autophagy [11]. Because of this broad impact of FOXO protein family on mammalian cell function, the effect of FOXO activity is associated with aging, neurodegeneration, cancer, infertility, and immune system dysfunction.

We and others have shown the important and necessary role of these transcription factors in neurodegenerative diseases. A number of pro-apoptotic genes relevant in neurodegeneration are directly controlled by FOXO [12–14]. Apart from neurodegenerative diseases, FOXO have been implicated in various other diseases.

Silencing of FOXO using shRNA involves the following steps:

- Selection of a target sequence,
- Designing oligonucleotides,
- Synthesizing the oligonucleotides,
- Cloning of the shRNA in suitable vector,
- Transforming the plasmid in bacterial cell for amplification,
- Isolation of plasmids and
- Transfection in neuronal cells.

2 Materials

2.1 For Ligation

Linearized pSIREN vector.

T4 DNA Ligase.

T4 DNA ligase Buffer.

BSA (10 mg/ml).

Nuclease free H₂O.

2.2 For Competent Cell Preparation

PIPES (1 M) pH: 7.0: (10 ml).

3.024 gm of PIPES was added to water. The pH was adjusted by HCL or NaOH. The volume was made 10 ml with nuclease-free water.

CaCl₂ solution (60 mM) pH: 7.0: (100 ml).
 665.88 mg CaCl₂ (60 mM final concentration).
 15 ml glycerol (15% final concentration).
 1 ml of 1 M PIPES pH: 7.0 (10 mM final concentration) were taken and the volume was adjusted to 100 ml with water. The solution was autoclaved before use.

2.3 For Transformation

SOC medium.
 Luria Broth.
 Competent *E coli* cells.

2.4 For Transfection

Neuronally differentiated PC12 cells.
 Primary hippocampal neuron.
 Dulbecco's modified Eagle's medium (DMEM).
 Horse serum (HS).
 Lipofectamine 2000.
 Complete medium of neuronal PC12 cells (DMEM, 1% HS, Nerve Growth Factor (50 ng/ml)).
 Complete medium for hippocampal neuron (neurobasal containing 1% B27).

3 Methods

3.1 Selecting Target Sequence

Selecting a target sequence is most crucial in the preparation of a shRNA. Usually the target sequence selected should be between 19 and 25 bp. There is no guarantee of effective gene silencing for a particular shRNA until proved by experiment. Randomly designed siRNAs that target different parts of a gene may have very different results. There are rules that govern siRNA sequence preference. Researchers have postulated rules for selecting a target sequence that are most effective [15–18]. One such set of rules indicate that a siRNA that satisfies all the following is capable of effective gene silencing: (a) the antisense strand having A/U at the 5' end, (b) the sense strand having G/C at the 5' end, (c) At the 5' terminal, one third of the antisense strand having at least five A/U residues, (d) no GC stretch of more than 9 nucleotide in length [9].

The target sequence used by us to knock down FOXO was 5'-AAGGATAAGGGCGACAGCAAC-3' [18–20] (*see Note 1*). This sequence is common for FOXO1, FOXO3a, and FOXO4 of rat. We have also used a shFOXO3a that specifically knocks down rat FOXO3a. The target sequence was 5'-CAACCTGTCACTGCACAGC-3'.



Fig. 1 Design of shRNA oligonucleotide. The hairpin loop sequence has been used to generate shRNAs using Clontech knockout RNAi system. Poly (T) tract is used as termination signal. 5' BamH I and 3' EcoR I overhangs are necessary for directional cloning into RNAi-Ready pSIREN vectors using Clontech knockout RNAi system

3.2 Designing Oligonucleotides

For the preparation of shRNA, two complementary oligonucleotides for each target sequence are needed. The sequence of oligonucleotides should include the following:

1. A 5'-BamH I restriction site overhang on top strand and a 5'EcoR I restriction site overhang on the bottom strand. This will enable directional cloning of the double stranded oligonucleotides.
2. In the top strand, the restriction site is followed by a 19 base oligonucleotide sense sequence (the target sequence).
3. The target sense strand is followed by a nucleotide hairpin loop of 9 nucleotides. We use the sequence 5'-TTCAAGAGA-3' as recommended by the manufacturer's protocol.
4. The hairpin loop is followed by antisense sequence of the target.
5. Following the antisense sequence 5–6 nucleotide long poly (T) sequence (this is RNA Pol III terminator sequence) is to be added.
6. Besides the restriction site overhang, the bottom strand is the complementary sequence of the top strand (*see* Fig. 1).

3.3 Cloning of shRNA into pSIREN Vectors

pSIREN-Zs Green vector was used as the cloning vector. The Vector was from BD Biosciences.

3.3.1 Annealing of Oligonucleotides

1. Both of the oligonucleotides (oligonucleotide for the top and bottom strands) were resuspended in TE buffer at a concentration of 100 μ M.
2. The oligonucleotides were mixed in the ratio of 1:1 to get a concentration of 50 μ M of double stranded oligonucleotides.
3. This mixture was heated at a temperature of 95 $^{\circ}$ C for 30 s.
4. The mixture was then heated at 72 $^{\circ}$ C for 2 min.
5. The mixture was then heated at 37 $^{\circ}$ C for 2 min.
6. The mixture was then heated at 25 $^{\circ}$ C for 2 min (*see* **Note 2**).
7. The mixture was stored on ice. (The double stranded oligonucleotides are now ready for ligation into the vector.)

3.3.2 Ligation of Double Stranded Oligonucleotides into RNAi Ready pSIREN

1. The annealed double stranded oligonucleotides in previous step (Subheading 3.3.1, step 7) were diluted with TE buffer to a final concentration of 0.5 μM .
2. For ligation the following things were added:

Reagent	Stock concentration	Amount (in μl)
Linearized pSIREN vector	25 ng/ μl	2
Diluted, annealed oligonucleotides	0.5 μM	1
T4 DNA Ligase Buffer	10 $\times$	1.5
BSA	10 mg/ml	0.5
Nuclease-free H_2O	–	9.5
T4 DNA ligase	400 U/ μl	0.5
Total		15

3. Incubate the reaction mixture at room temperature for 3 h for ligation.
4. Following ligation the vector is ready for transformation.

3.4 Competent Cell Preparation and Transformation

3.4.1 Procedure of Competent Cell Preparation

1. A single colony of *E. coli* was inoculated into 50 ml of Luria Broth (LB) and grown for overnight at 37 °C incubator with shaking.
2. Next day, 2 ml of the above culture was added to 200 ml of freshly autoclaved LB and allowed to grow in 37 °C incubator to an OD of 0.375 measured at 590 nm.
3. The culture was aliquoted into 4, 50 ml prechilled sterile polypropylene tubes and left on ice for 5-10 min. The tubes were then centrifuged at 1600 $\times g$ at 4 °C for 5 min.
4. The supernatant was gently decanted and the pellet was resuspended in 10 ml of ice-cold CaCl_2 solution. The mixture was centrifuged for 5 min at 1100 $\times g$ at 4 °C.
5. The supernatant was decanted and the pellet was resuspended in 10 ml CaCl_2 . The resuspended cells were kept in ice for 30 min. The cells were then centrifuged at 1100 $\times g$ at 4 °C for 5 min.
6. Each pellet was resuspended in 2 ml of ice-cold CaCl_2 solution. 250 μl of the solution were aliquoted in prechilled sterile polypropylene micro centrifuge tubes.
7. The cells were stored in –80 °C.

3.4.2 Transformation

1. The competent cells were thawed on ice.
2. 125 µl of the competent cells were taken and 2–4 µl of DNA was added (*see Note 3*).
3. The tubes containing the mixture of competent cells and DNA were placed on ice for 30 min.
4. Following the incubation, heat shock was given (at 42 °C for 30 s). Immediately after heat shock the microcentrifuge tube was placed on ice (*see Note 4*).
5. The microcentrifuge tube was incubated on ice for at least 2 min.
6. 300 µl of SOC medium was added in the microcentrifuge tube (*see Note 5*).
7. The microcentrifuge tube was incubated at 37 °C for 1 h under shaking condition (*see Note 6*).
8. After the initial incubation the solution was plated on agar plates containing ampicilline (100 µg/ml) (*see Note 7*).
9. The plates were incubated at 37 °C overnight.

3.5 Plasmid Isolation

1. A single colony from the agar plate was inoculated in a test tube containing sterile Luria Broth. Antibiotic should be added before inoculation (*see Note 8*).
2. The test tube was incubated at 37 °C for 4–5 h under shaking condition for startup culture.
3. After 4–5 h, about 200 µl from the startup culture was added in a conical flask containing 200 ml of Antibiotic containing Luria Broth. The flask was then incubated at 37 °C overnight under shaking condition (*see Note 9*).
4. The plasmids were isolated using the Plasmid isolation Maxi kit from Qiagen following the manufacturer's protocol.
5. These plasmids were checked and used for transfection.

3.6 Transfection

The transfection method described here is in respect to neuronally differentiated PC12 cells and hippocampal neuron. This protocol can be applied on any neuronal cells with minor modification in the medium used. The generalized protocol is as follows:

1. For PC12 cells, equal amounts of serum-free medium were taken in two microcentrifuge tubes. The tubes were marked 1 and 2. In tube 1, plasmid DNA containing the shRNA was added and mixed. In tube 2, Lipofectamine 2000 was added and mixed (the amounts of serum-free medium, plasmid DNA, and lipofectamine 2000 are mentioned in Table 1).
For hippocampal neurons, the serum-free medium should be replaced by neurobasal medium without B27 supplement.
2. After adding plasmid DNA and lipofectamine tubes 1 and 2 were incubated at room temperature for 5 min.

Table 1
Components of the transfection reaction

	For one well of a 24-well plate		For one well of a 6-well plate	
	Tube 1	Tube 2	Tube 1	Tube 2
Serum-free medium	50 μ l	50 μ l	200 μ l	200 μ l
Plasmid DNA	0.5 μ g	–	2.5 μ g	–
Lipofectamine 2000	–	1.5 μ l	–	7 μ l

3. The contents of tube 1 was transferred to tube 2 and mixed gently. The tube 2 is now incubated for 20 min at room temperature.
4. The plated cells were taken out of the incubator and the medium is replaced by serum-free medium (400 μ l for one well of 24 well and 1500 μ l for one well of 6-well plate).
5. The entire mixture of tube 2 is added to the wells.
6. The cells were incubated for 4 h in cell culture incubator at 37 °C with 5%CO₂.
7. After 4 h, the medium containing DNA and lipofectamine was removed and re fed with complete medium (for composition of complete medium *see* Subheading 2).
8. The expression of pSIREN ZsGreen was observed after 48 h of transfection (*see* Fig. 2a, b) (*see* Note 10).

3.7 Checking Efficacy of the shRNA

The efficacy of the shRNA was shown by its ability to substantially reduce expression of endogenous FOXO3a in neuronal PC12 cells.

1. PC12 cell were transfected with shFOXO and shRand (shRand is a shRNA used as transfection control. It contains a random shRNA that does not affect the expression of any gene in mammalian cell).
2. Transfected cells were maintained for 48 h for optimal expression of the transfected constructs.
3. Cells were then harvested and the FOXO3a levels were analyzed by western blotting. The levels of FOXO3a were significantly less in shFOXO transfected cells than that of the cells transfected with shRand, indicating that the shRNA have effectively blocked the FOXO expression (*see* Fig. 2c). The result clearly shows that shFOXO successfully reduce the expression of FOXO3a in neuronally differentiated PC12 cells.

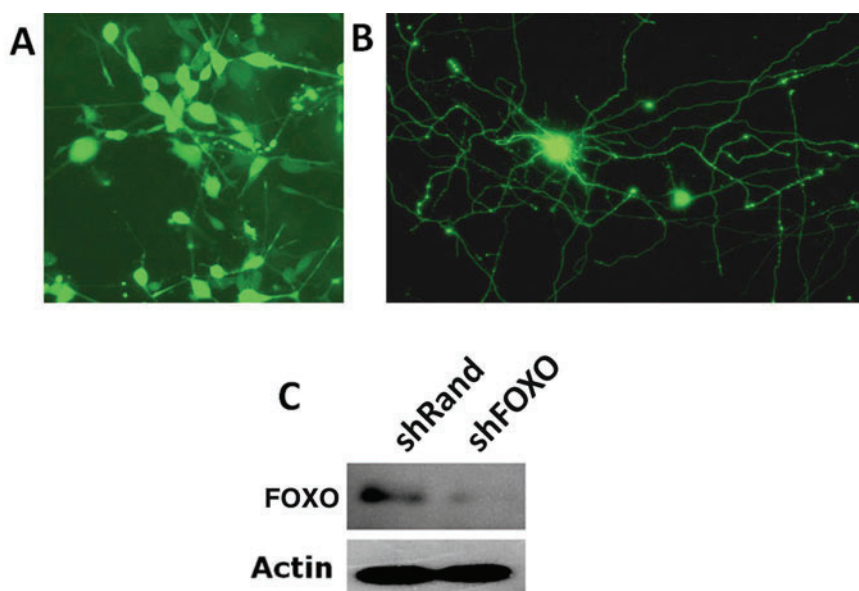


Fig. 2 (a) A fluorescence micrograph of differentiated PC12 cells transfected with shFOXO. The green fluorescence of the cells indicates expression of the Zs Green tag present in the pSIREN vector. (b) Fluorescence micrograph of primary cultured hippocampal neurons transfected with shFOXO. The green fluorescence of the neurons indicates expression of the Zs Green tag present in the pSIREN vector. (c) Neuronally differentiated PC12 cells were transfected with shFOXO and shRand. 48 h post transfection, the cells were harvested and processed for western blotting analysis. Result shows a visible reduction in the FOXO protein level in cells transfected with shFOXO in contrast to cells transfected with shRand. Actin was used as a loading control

4 Notes

1. It is advisable to target more than one sequence, prepare shRNA and transfect. Following transfection the efficacy of the shRNAs should be checked. The shRNA that blocks the expression of the protein should be used for experiments.
2. The **steps 3–6** should be performed in a thermal cycler.
3. The DNA should be in elution buffer. If the DNA is immobilized on paper, first the DNA containing paper need to be placed in a micro centrifuge tube and slowly elution buffer to be added till the paper is completely wet. The paper should be soaked for overnight and then solution can be used for transformation.
4. The time and temperature is very important for proper transformation. Any deviation from this condition may hamper the efficiency of transformation.
5. In place of SOC medium Luria Broth can also be used.
6. At this stage no antibiotic should be added. This will allow the multiplication of all the bacterial cells with and without the plasmids.

7. The antibiotic used should be the one whose resistant gene is present in the plasmid transformed. This will allow the selection and growth of the transformed bacteria. As we have used pSIREN as our cloning vector the antibiotic used was ampicillin as the pSIREN vector contains an ampicillin resistant gene.
8. Inoculation may be done using a heat sterilized platinum loop or a sterile tip.
9. For optimal growth of the bacteria 200 ml of Luria Broth should be taken in a 500 ml conical flask.
10. As the shRNA containing plasmid used here have a fluorescence tag, the expression of this plasmid can be visualized under fluorescence microscope.

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Part II

Imaging Approaches



Chapter 12

Immunofluorescence Analysis by Confocal Microscopy for Detecting Endogenous FOXO

Francisco Castillo, Thomas A. Mackenzie, and Bastien Cautain

Abstract

Cancer cells are known to inactivate tumor suppressor proteins by triggering their anomalous subcellular location. It has been well established that the aberrant location of FOXO proteins is linked to tumor formation, progression of the same, or resistance to anti-neoplastic treatment. Furthermore, the abnormal location of FOXO has also been considered a potential biomarker for diabetic complications or longevity in different organisms. Here, we describe the immunodetection of endogenous FOXO by confocal microscopy, which can be used as a chemical tool to quantify FOXO expression levels, its cellular location, and even its active/inactive forms with relevant antibodies.

Key words Biomarker, FOXO proteins, Nuclear translocation, Immunodetection, Confocal microscopy

1 Introduction

In the 20th century, a diversity of tinctorial techniques emerged to spot discrete elements within cells and tissues due to the discovery of genuine stains that produced a detectable signal at the site of interest. Later, the ability to produce labels as monoclonal specific antibodies, recognizing particular proteins, gave birth to the modern immunohistochemistry [1].

These days, the immunohistochemistry technique is mainly about the immunologic fluorescent detection of a targeted protein/antigen, visualized with a photonic microscope, as a reliable readout to determine both its location and its expression levels. Therefore, immunohistochemistry has made a significant impact in the field of cell signaling. General procedures involve the fixation of the relevant cells before being processed. Then, the immunologic detection includes the blocking of nonspecific activities, the incubation with the primary antibody, and the labeling of the antigen-antibody reaction, to make the antigen visible. Finally, confocal microscopy is preferentially used either to monitor the detection

in real time or to collect the desired images when the fluorescent reaction reaches the equilibrium [2].

The optimization of the antigen-antibody reaction encompasses numerous variables, such as the nature of the antigen (proteins, glycoproteins, small exogenous biological molecules), the quality of the primary antibody (in terms of specificity and sensitivity), and even the fluorescent properties of the secondary antibody/dye (i.e., compatibility with other fluorescent probes that may be required for the assay). Furthermore, there are some issues that are cell-line dependent, such as the previous permeabilization and fixation of the sample, or the fluorescent imaging of the cell [3].

FOXO immunodetection has been extensively applied to shed light on many disease-related topics, such as the link between oxidative stress response and life span through MST signaling [4], tumorigenesis via HDM2 degradation [5], or, more recently, the understanding of how AKT hyper activation contributes to many pathophysiological conditions [6].

Here, we show that endogenous FOXO transcription factors can be efficiently detected in a relevant U2OS osteosarcoma cell-line using commercial antibodies [7]. Fairly similar procedures have been described for other cell lines such as β TC-3 [8] or BT474 [9] and even for E12.5 full-body embryos [10].

2 Materials

1. U2OS human osteosarcoma cell line (American Type Culture Collection, Manassas, VA, USA).
2. T75 culture flasks.
3. Antibiotics: 100 UI penicillin, 100 μ g/mL streptomycin. Dissolve powder up to 10,000 UI (penicillin) and 10,000 μ g/mL (streptomycin) in complete medium as a 100 $\times$ stock. Filter the solution with a 0.22 μ m filter, store at 4 or -20° . Add it to the Growth media when required (1:100 w/v, see below).
4. Growth media: Commercial Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and penicillin-streptomycin.
5. Trypsinization solution: TrypLE™ Express (Gibco life technologies).
6. Microplates: imaging 96-well plates (Greiner bio-one).
7. 1 $\times$ Phosphate-buffered saline (1 $\times$ PBS): 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer solution (pH 7.4 at 25 $^{\circ}$ C). Commercial PBS ready-made tablets are recommended for the sake of reproducibility *see* **Note 1** below.

8. 1× PBS 0.3 M glycine buffer: Combine 11.1 g Glycine-HCl powder with 800 mL 1× PBS. Adjust pH to 7.4 and bring volume to 1 L with 1× PBS. Store aliquots at -20° if desired.
9. Permeabilization/blocking solution: 1× PBS 0.3 M glycine, 1% BSA, 10% normal goat serum, 0.1% Tween 20. To prepare 10 mL, add 1 mL normal goat serum to 9 mL 1× PBS 0.3 M glycine buffer and mix well. While stirring, add 0.1 g of BSA and 10 μ L Tween 20. Make fresh and use straight after preparation.
10. Antibodies: FOXO1 or FOXO3a as primaries (Cell Signaling Technology) and DyLight[®]594 donkey anti-rabbit as secondary antibody (Abcam) *see* **Note 2** below.
11. 1:200 antibody solution: dilute, 1:2000 (v/v), the relevant primary or secondary antibody in 1× PBS, 1% BSA, 0.3% Tween 20 buffer. Make fresh and use straight after preparation *see* **Note 3** below.
12. Nuclear staining solution: dissolve DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) powder to a maximum of 1.43 μ M in 1× PBS buffer *see* **Note 4** below.
13. Imaging hardware: Leica SPE confocal imaging system.
14. Imaging software: Leica software.

3 Methods

A flow diagram summarizing the steps described in Subheadings 3.1–3.3 can be found in Fig. 1.

3.1 Sample Preparation

1. Turn the water bath to 37° before starting.
2. Defrost cell aliquot from liquid nitrogen in the water bath, gently.
3. Add 1 mL of thawed cells to 11 mL of pre-warmed Growth media.
4. Transfer the resuspended cells to a T75 culture flask or similar.
5. Put the culture flask into the incubator at 37°C with 5% CO_2 . Let cells adhere to the flask bottom for at least 12 h.
6. Make sure the trypsinization solution is pre-warmed for the next step and then remove the growth media, carefully.
7. Remove the adherent cells from culture surface by incubating with 5 mL of the trypsinization solution, for 5–10 min, at 37°C .
8. Inactivate the trypsinization reaction with 10 mL of pre-warmed growth media.

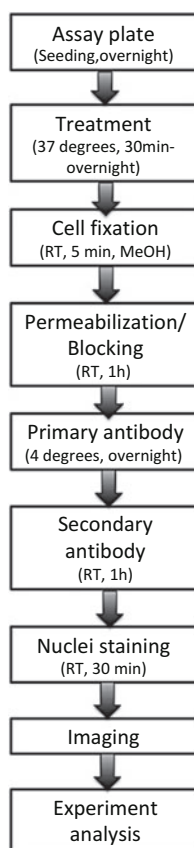


Fig. 1 Assaying strategy to identify FOXO translocation compounds. A flow diagram of the experimental steps required for small molecules as FOXO relocators. Depending on the incubation time of the molecule under investigation, the whole experiment can be performed between 4 and 5 days. Additional time course experiments, together with cell viability assays, are recommended if the mechanism of action of the small molecule of interest is unknown

9. Count the cells and make two separate stocks for subsequent steps. (a) Next cell passage. (b) Cells for the assay (20,000 cells/well). In both cases cell cultures are maintained in a humidified incubator, at 37 °C with 5% CO₂, and passaged again when confluent using relevant trypsinization solution.
10. If desired, cells can be treated with active molecules after **step 8**. More precisely, after 12 h (overnight) of incubation at 37 °C with 5% CO₂ in assay plates, add each test compound (ideally ≤0.5% DMSO, final volume). Cells can be exposed from 30 min up to overnight, depending on the expected kinetics and toxicity of the tested molecules. If such information is not available, an additional cell viability assay (MTT or similar), compound solubility tests, and a time course optimization are strongly recommended *see* **Note 5** below.

11. Wash the assayed cells with $1\times$ PBS, at least twice, gently.
12. Fix the cells with methanol for 5 min, at room temperature.
13. Wash with $1\times$ PBS, at least twice, to get rid of the methanol leftovers.

3.2 Antibody Staining

1. Remove $1\times$ PBS from previous step.
2. Add the permeabilization/blocking solution for 1 h at room temperature.
3. Remove the permeabilization/blocking solution carefully.
4. Wash with $1\times$ PBS to remove the blocking solution leftovers.
5. Add the 1:200 primary antibody solution and incubate at 4°C , with gentle shaking, overnight.
6. Wash with $1\times$ PBS to remove the blocking solution leftovers.
7. Incubate with 1:2000 fluorochrome-conjugated secondary antibody at 37°C for 1 h, or 4°C overnight. Keep plates covered with foil to prevent fading of fluorochrome.

3.3 Imaging

1. Stain with $1.43\ \mu\text{M}$ DAPI (cell nuclei in blue, *see* Fig. 2) for 30 min at room temperature.
2. Wash twice with $1\times$ PBS to remove the DAPI staining solution excess. This is important, so the blue DAPI signal does not interfere with the fluorescent signal coming from the antibodies. Endogenous FOXO is detected in the red channel (*see* Fig. 2).
3. Fluorescent signal is temperature dependent. Setting temperature control might be relevant for getting high-resolution images. 22°C is recommended according to our previous experience.

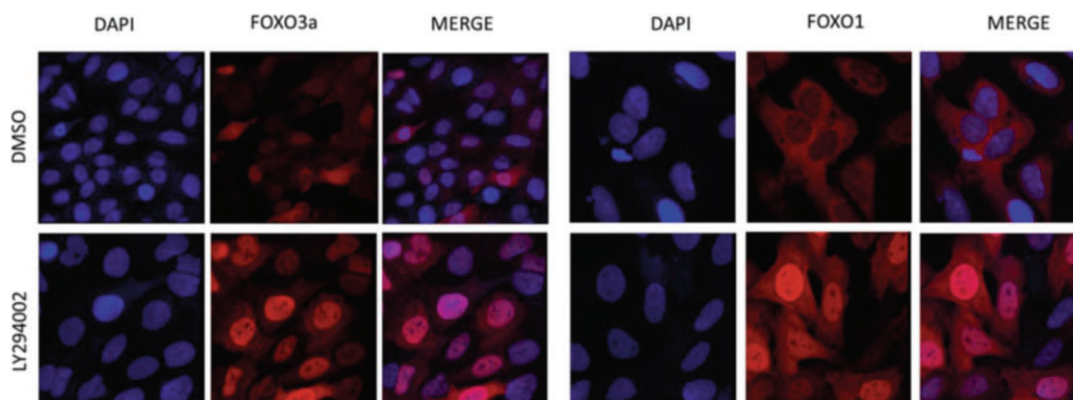


Fig. 2 Representative example of how a positive control compound induces the nuclear translocation of FOXO proteins. Cells were seeded in black-wall clear-bottom tissue culture plates and allowed to attach overnight. Attached cells were subsequently treated with compound LY294002 for 30 min before fixation and DAPI staining. Afterwards, endogenous FOXOs were immunodetected with specific antibodies, whose signal was finally visualized by a confocal microscope

4. Read the assay plate using 63× water immersion lenses on the confocal imaging system.
5. Before analyzing and discussing the data, *see* **Notes 6** and **7**.

3.4 Concluding Remarks

In this present chapter, we describe how to monitor the subcellular location of FOXO proteins by immunohistochemical detection. Compounds capable of reactivating FOXO are considered very attractive anti-cancer therapeutics [11–13] and potentially interesting to prevent or treat age-related diseases [14, 15]. Other transcription factors such as YAP, NFAT, NF- κ B, MEF2, or Nurr1, whose aberrant subcellular relocation is at the origin of different diseases [16, 17], can be equally immunodetected. Therefore, the importance of the protocol described here lies in that it is an accurate method to detect other transcription factors either as biomarkers, or even to further validate the activity of new molecules, by using the adequate combination of commercial antibodies and cell lines. Additionally, the same procedure we describe here for the subcellular location of FOXO proteins could be used to precisely confirm the activity of a variety of direct upstream regulators such as AKT1–3, SGK 1–3, MST-1, CK1, Cdk2, DKYR1a, and IKK β or indirect AKT activators, such as PI3K, PDK1 [18, 19], and NFKB2 [11, 20].

4 Notes

1. Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade sterilized water.
2. NFKB2 and p100/p52 primary antibodies (Cell Signaling Technology[®]) are recommended as additional reporters (see above, Concluding Remarks).
3. 5% (w/v) BSA, 1× PBS, 0.1% Tween20 can be equally used as antibody dilution Buffer.
4. Hoechst 33342 (Trihydrochloride, Trihydrate) can be used instead of DAPI to label the nuclei if desired or if any DAPI interference is detected whatsoever.
5. The nuclear/cytoplasmic fluorescence intensity is a bona fide parameter to quantify where the endogenous FOXO protein is mainly located under the desired experimental conditions. LY294002 (Cell Signaling Technology[®]) strong inhibitor of PI3K, and Leptomycin B (Calbiochem) can be used during Subheading 3.1, **step 9** as cytoplasmic and nuclear positive controls, respectively.
6. It might be worth automatizing all liquid handling for compound treatment, washing, fixing, and staining as a way of improving the reproducibility of the results.
7. All experiments should be performed at least in triplicate.

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Chapter 13

High-Throughput Image-Based Screening to Identify Chemical Compounds Capable of Activating FOXO

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Abstract

FOXO proteins are transcription factors with important roles in the regulation of the expression of genes involved in cell growth, proliferation, differentiation, and longevity. FOXO proteins are active in the nucleus but, upon post-translational modification they form a docking site for 14-3-3 proteins and are translocated to the cytoplasm where they are inactive.

We make use of this regulatory mechanism of FOXO proteins to develop an image-based high-throughput screening platform to detect compounds that regulate FOXO3 subcellular localization. This system has proven a powerful tool to isolate inhibitors of proteins upstream of FOXO, such as PI3K inhibitors.

Key words Forkhead box O proteins–FOXO, Nucleus-cytoplasm translocation, High-content screening

1 Introduction

The Forkhead Box O family of transcription factors is comprised of four members in mammals: *FOXO1*, *FOXO3*, *FOXO4*, and *FOXO6*. All of them share a common Forkhead domain of ~100 aminoacids that binds to a consensus sequence called DBE (DAF-16 family protein binding element: 5'-TTGTTTAC-3') [1]. FOXO proteins are implicated in many cellular and physiological functions, affecting cell growth, survival, metabolism, aging, and cancer [1, 2].

Mechanistically, FOXO members are regulated by their subcellular localization: they are active while remaining in the nucleus, and phosphorylation by the kinase AKT, a target of the insulin-PI3K pathway, creates a docking site for 14-3-3 proteins that translocate FOXO factors to the cytoplasm and inactivate them [3]. Therefore, FOXO subcellular localization is a reliable readout of its activity, and modulators of this translocation can affect FOXO activity [4–6]. This property has been used in different approaches

by our laboratories and others to screen for FOXO-modulating compounds [7–10] using high-content screening (HCS) techniques. HCS constitutes a very powerful tool [11], combining the efficiency of high-throughput techniques with cellular image-based acquisition of quantitative data from complex biological systems.

In this chapter, we perform a thorough description of a protocol to generate a robust and sensitive platform for high-throughput/high-content screening of FOXO3 subcellular localization. The potential of this platform has been proven by the identification of chemical compounds [7–10, 12], such as PI3K inhibitors that enhance FOXO nuclear translocation and have shown strong and potentially useful metabolic [13, 14] and antitumoral [10, 12] effects.

2 Materials

2.1 Cell culture

- *Cells*: U-2 OS human osteosarcoma cell line (ATCC® HTB-96™). Culture medium: Dulbecco's modified Eagle's Medium (DMEM) with 25 mM glucose (high glucose), 4 mM Ultraglutamine I, containing 10% heat inactivated fetal bovine serum (FBS). FACS medium: DMEM supplemented with 2.5% FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 0.25 µg/ml Amphotericin B and 3 mM EDTA. Freezing medium: 10% DMSO in FBS. Trypsin-EDTA (200 mg/l EDTA, 170,000 U/l trypsin). Transfection reagent: Fugene 6 or similar. For clone selection: G418 disulfate salt. Well surface coating: 0.1 mg/ml poly-D-lysine hydrobromide in water. Plates: 384-well plates with clear optical plastic bottom and black walls. For PI3K inhibition: BYL719 or another well-known PI3K inhibitor. Nuclei staining: 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI).

2.2 Plasmids

pEGFP-C3-FOXO3, kindly provided by T. Finkel [9].

2.3 Equipment

- *Flow cytometry*: Cell sorter with a set of lasers suitable for DAPI and EGFP discrimination, such as INFLUX™ (BD Biosciences).
- *Confocal microscopy*: High-throughput wide-field or confocal microscope, with filters and/or lasers suitable for DAPI and GFP detection with a motorized stage that allows for 384-well plates acquisition such as Opera LX (Perkin Elmer).

2.4 Software

The required image analysis software must be able to segment nuclei from DAPI signal and find or estimate the cytoplasm. Aca-pela v2.0 (Perkin Elmer) or Cell Profiler are valid options for these purposes. Software packages like Microsoft Excel, SPSS or similar should be used for: statistical analysis, calculation of nucleus vs cytoplasm ratio and threshold, data mining and graph imaging.

3 Methods

3.1 Generation of a U-2 OS Cell Line Stably Expressing EGFP-Foxo3

3.1.1 Reverse Transfection and Selection of Cells with G418

Mix DMEM without serum or antibiotics with 12 μ l of Fugene in a 1.5 ml tube. Leave for 5 min at RT. Adjust initial volume of DMEM so that the final volume is 200 μ l. Add 2 μ g of the plasmid encoding EGFP-FOXO3 (pEGFP-C3-FOXO3 [9]), ratio DNA/Fugene 1:6. Mix and incubate for 20 min at room temperature. In the meantime, trypsinize the U-2 OS cells and count them. After the incubation time, mix 4 ml of culture medium containing 330,000 cells with the DNA/Fugene complexes and seed in a 6 cm plate. In parallel, seed the same density of cells in another 6 cm plate. We will use this last plate as a non-transfected control to monitor the selection of the transfected cells. This procedure is applicable to Fugene 6. If another transfection reagent is used, follow manufacturer's instructions.

After 48 h, check for EGFP expression and, if positive, start the selection of the transfected cells with G418 (1 mg/ml) (*see Note 1*). Add G418 at the same concentration to the plate of non-transfected cells. It will take approximately 1 week to complete the selection of the transfected cells (*see Note 2*).

3.1.2 U-2 OS-EGFP-FOXO3-Cells Clone Isolation

Once the selection has finished (approximately day 8 of G418 treatment), when cells are still growing as single colonies, pick several clones of U-2 OS-EGFP-FOXO3 cells into 96-well plates. To do that, first mark target, EGFP-positive colonies using a fluorescence microscope. Wash the plate once with PBS, aspirate all the PBS to dryness, and add a drop of trypsin on every target colony. Incubate at 37 °C for 5 min. With the help of a 200 μ l-micropipette, using approximately 50 μ l of medium, recover as many cells as possible from the colony (*see Note 3*). Plate cells into the 96-well plate into a final volume of 200 μ l with 1 mg/ml of G418. Expand clones from 96-well plates to 12-well plates and from these to 6 cm plates. To select the best clone, first discard any clone that presents a nuclear localization of EGFP-FOXO3 in basal conditions. Then, check for EGFP-FOXO3 nuclear translocation after treatment with a known PI3K inhibitor (PI3Ki). To do that, treat 8,000 cells in 96-well plates with 100 μ l of normal medium or 100 μ l of 6 μ M BYL719, a well-known PI3Ki. Check in a fluorescence microscope the nuclear localization of EGFP-FOXO3 at different time points (1, 2, 4, 6 h).

Select for clones with high intensity of the EGFP signal and positive for their nuclear translocation after inhibition of PI3K. At this step, freeze a part of the chosen clone for safety and expand to two 15 cm plates. Keep G418 at 250 μ g/ml to maintain the selection pressure.

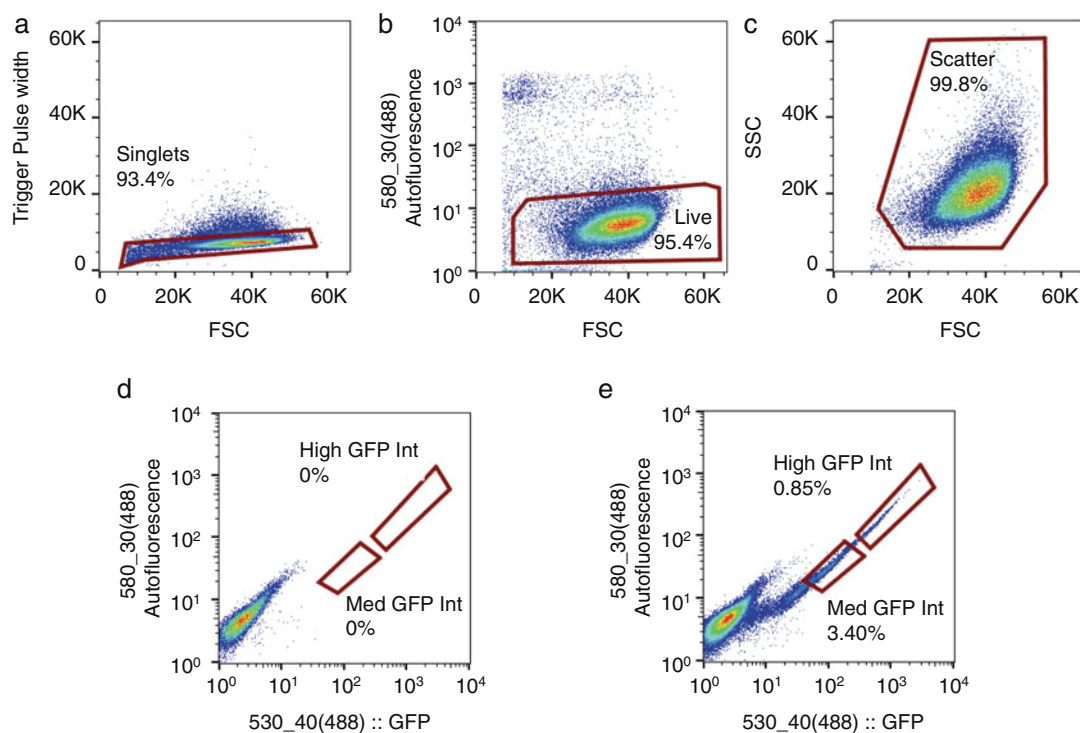


Fig. 1 FACS-sorting of EGFP-FOXO3 positive cells. Stably transfected cells expressing the chimera construct EGFP-FOXO3 are analyzed by flow cytometry to select for single cells (a), DAPI-negative live cells (b), and non-debris (c). Once this preliminary study is performed, a negative control is analyzed (d) to define the EGFP-expressing cells in the positive clones (e). High-EGFP and Medium-EGFP cells from this last panel are FACS-sorted separately, to determine which subpopulation is the most suited for the following assays

3.1.3 Cell Sorting

Trypsinize and resuspend cells in FACS medium to a final concentration of $10\text{--}15 \times 10^6$ cells/ml. Add $1 \mu\text{g}/\text{ml}$ DAPI for viability discrimination. After proper selection of single, live cells (*see* Fig. 1), collect the cell population with the 5% highest EGFP expression (*see* Fig. 1) and seed them in an appropriate plate (*see* Note 4). The following day check for cell density and expand if necessary with $250 \mu\text{g}/\text{ml}$ G418. Keep cells growing with antibiotics at least 3 days, until confirmation that sterility was preserved after sorting, and withdraw the antibiotics for further culture. Expand sorted cells to generate a large frozen stock (*see* Note 5).

3.2 Setup of the Screening Platform

3.2.1 Culture of Cells

- Coat wells with $20 \mu\text{l}$ of $0.1 \text{ mg}/\text{ml}$ poly-D-Lysine for 5 min (*see* Note 6).
- The day before the treatment, plate cells (*see* Note 7) to obtain a 70–80% confluency at the day of the treatment. We recommend plating 3,500 cells per well in a 384-well plate.

- (c) Do not plate cells in the two outer lanes of wells. Instead, keep them filled with PBS to minimize the edge effect (more evaporation in the wells closer to the border of the plate).

3.2.2 Treatment of Cells

We recommend performing the screening in 384-well plates. The volumes indicated are calculated for this format; if using larger wells, please escalate the volumes.

The following conditions must be considered for cell treatment:

- (a) Maximum solvent concentration: it indicates the maximum concentration of the solvent used to dissolve the tested compounds that do not affect FOXO3 nuclear translocation (*see Note 8*). To obtain this parameter a curve of serial dilutions of the solvent must be tested in the same conditions of the screening.
- (b) Time of incubation with the compounds: the translocation time will vary depending on the kinetics of FOXO3 activation for each compound. 1 h is enough for compounds with quick kinetics, but we recommend incubating for longer times (up to 5 h) not to discard compounds with slower kinetics.
- (c) Volume, concentration, and dispensation of the compounds: the volume of the test compound is defined by the concentration of the stock solutions, the maximum solvent concentration, and the volume limit of the well. In our hands, treatments with 20 μ l of test compounds on 80 μ l of culture medium were optimal (*see Note 9*).
- (d) Fixation method: to better preserve cell structure, we recommend fixing the cells with 4% formaldehyde this way: remove half the volume of culture medium (50 μ l of medium in wells filled with 100 μ l, for example) of each well and add that same volume (50 μ l, in our example) of 8% formaldehyde in PBS. Mix by pipetting softly.
- (e) DAPI staining: after fixation, cell nuclei must be stained. For this, add 20 μ l of DAPI to a final concentration of 1 μ g/ml and leave for 10 min at room temperature. Wash with PBS.

3.2.3 Acquisition and Image Analysis Optimization

Once cells are cultured and treated with the previous conditions, they can be analyzed in the confocal microscope.

- (a) Use a 20 $\times$ objective and appropriate cell confluency (*see Subheading 3.2.1, step b*), to ensure that 50–75 cells are obtained per field. Since the optimum number of acquired cells for a robust statistical power is between 1,000 and 1,500, we recommend acquiring 20 fields per well. Consider 500 as the minimum number of cells in the 20 fields acquired in a given well, and discard any well with fewer cells.

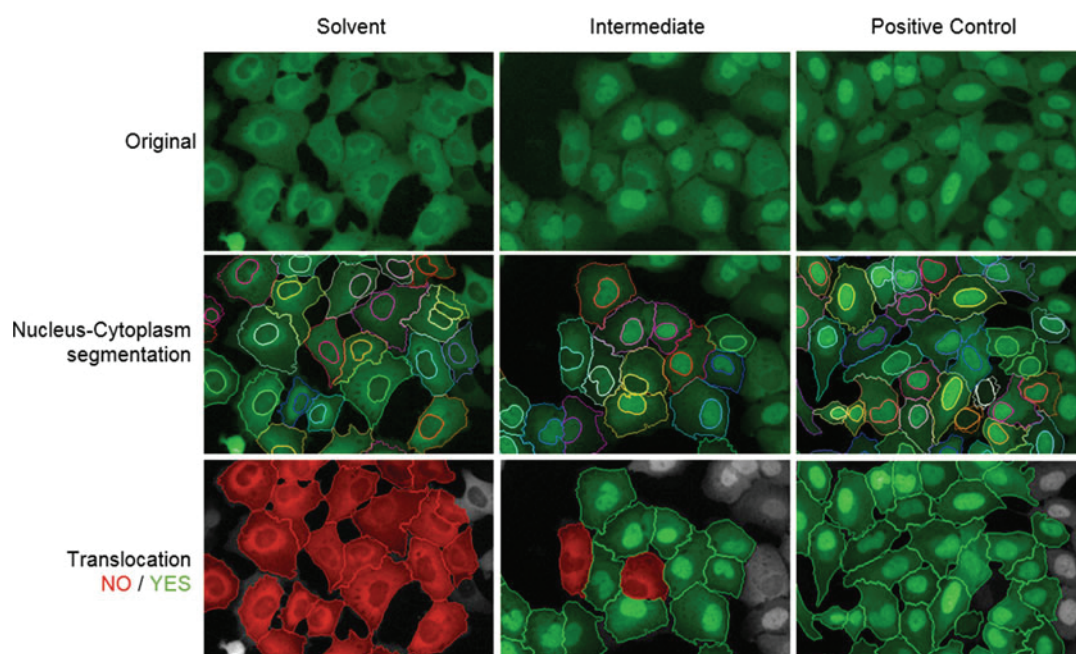


Fig. 2 Acquisition and image analysis. Cells stably expressing the construct EGFP-FOXO3 are imaged by a HTS confocal microscope with $20\times$ amplification. The raw images (top panels), including the DAPI signal for the nuclei (not shown in this figure for clarity purposes), are analyzed to define the cytoplasm and the nucleus of each cell (central panels), allowing for the calculation of nuclear to cytoplasmic ratio of EGFP signal for each cell. The median value of the cellular nuclear to cytoplasmic ratios in solvent-treated cells is used to calculate the threshold of activation. This value determines which cells are positive (higher ratio than the threshold of activation, green cells in bottom panels) or negative (lower ratio than the threshold of activation, red cells in bottom panels) for FOXO3 nuclear translocation. A final value of the percentage of positive-green cells can then be calculated for each well, and determine which treatment induces a net increase in the nuclear translocation of FOXO3 and can be considered a hit

- (b) The image analysis routine should be able to identify nuclei, based on DAPI signal; and cytoplasm, based on EGFP cytoplasmic signal (*see* Fig. 2, center panels). For nucleus and cytoplasm detection consider parameters such as size and contrast. Nucleus detection will improve by adding roundness. Cytoplasm can be estimated by generating a ring around the nucleus but the signal quantification will have more error. Results will improve by segmenting the whole cytoplasm with the proper algorithms. Test and check which option fits better with your system.
- (c) It is important to filter out non-EGFP positive or too small cells; for this, green intensity and cell size calculations are also needed. Thresholds for these filters will vary depending on magnification and EGFP expression.
- (d) After this image analysis process, the ratio of green fluorescent signal intensity between nucleus and cytoplasm for each cell

can be quantified. This ratio is used to calculate two crucial parameters: the threshold of activation, defined as the basal ratio of nuclear/cytoplasmic FOXO3 in cells treated with vehicle; and the sensitivity to activators of FOXO3 nuclear translocation, using positive control-treated cells (*see Note 10*). The threshold of activation is the median nucleus/cytoplasm ratio plus 2 times the standard deviation (Threshold = Median Ratio Nuc/Cytopl + 2 × SD) in the vehicle-treated cells. For each well, the levels of FOXO3 activity will be represented by the percentage of cells with their nuclear FOXO3 above the threshold of activation (*see Fig. 2*, bottom panels).

3.2.4 Quality Control

Before proceeding to test the library of compounds, the robustness of the platform must be validated. For this, several parameters have been designed in the literature. We recommend using the *Z* factor, a simple and widely used parameter [15].

The *Z* factor is calculated using this formula:

$$Z\text{-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

where σ_p = standard deviation of the positive control; σ_n = standard deviation of the negative control; μ_p = mean of the positive control; μ_n = mean of the negative control. A theoretically perfect platform would yield a *Z* factor = 1, and a HTS platform is considered acceptable when its *Z* factor is higher than 0.5 [15] (*see Note 11*).

3.3 Screening

To test your compounds of interest, follow these steps:

- (a) Coat wells with 20 µl poly-D-Lysine for 5 min and wash once with PBS. Remove the PBS and plate 3,500 U-2 OS cells overexpressing EGFP-FOXO3 per well in a final volume of 80 µl.
- (b) Twenty four hours after cell plating, add 20 µl per well of a 5 × concentrated solution of the tested compounds in complete medium. The final volume will be 100 µl. Keep the treatment during your time of choice (*see Subheading 3.2.2, step b*). Fix cells with 4% formaldehyde (*see Subheading 3.2.2, step d*). Leave for 20 min and wash with PBS. Add 20 µl of DAPI (1 µg/ml) and leave for 10 min at room temperature. Wash with PBS. Acquire images at the HTS confocal and analyze (*see Note 12*).

Important points to take into account:

1. For a HTS experiment, the physical position of the treatments can have a strong influence in the final outcome due to position effects. To monitor this effect in each plate, we recommend

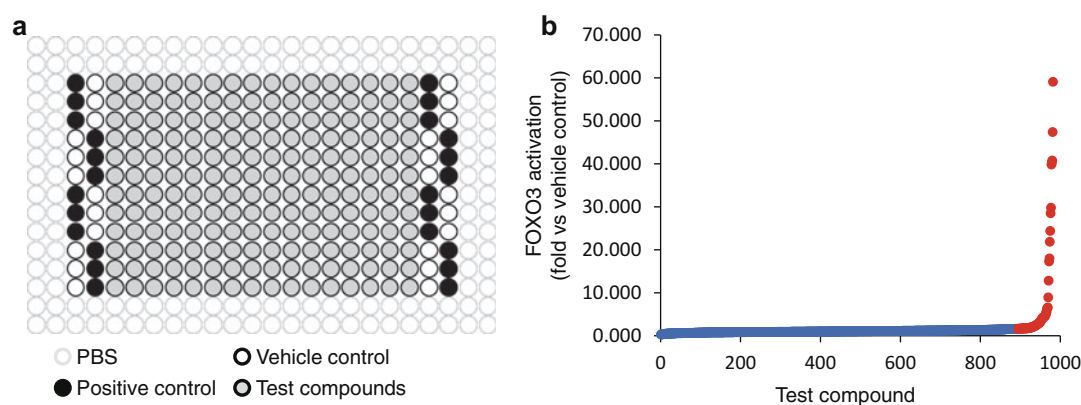


Fig. 3 High-throughput screening. **(a)** Cells are seeded in 384-well plates and treated in triplicate with the indicated controls and the tested compounds. Note the symmetrical disposition of the controls, intended to minimize the position effect. Also, note that the two most external lanes are not used for plating cells but, instead, they are filled with PBS to minimize the edge effect. **(b)** Once the percentage of nuclear FOXO3-positive cells are calculated for each compound, the fold increase over vehicle control is calculated. Only compounds with a certain fold increase will be selected as first hits (red dots). These first hits must then be retested on the same platform to confirm their FOXO3-activating effects and to establish a final, more restrictive list of hits for further characterization

placing controls in the side columns (accounting for the side effect) and alternating positions, so that readouts of controls can be informative about a possible position effect [16]. For an example, see Fig. 3a.

Also, to minimize pipetting errors, we recommend using triplicates of every compound.

2. Concentration of the treatments: we recommend using as a first approach a high concentration of the tested compounds, limited by the maximum solvent concentration defined in Sub-heading 3.2.2, step a. If toxicity or excessive effects are found, a second validating assay with lower concentrations can be performed to discriminate the actual FOXO3-activating compounds.
3. Quantification of nuclear translocation of EGFP-FOXO3: in order to establish in which cells EGFP-FOXO3 has translocated to the nucleus, a threshold of activation must be first defined. We fixed this threshold as the median nucleus/cytoplasm ratio plus 2 times the standard deviation of the intensity of nuclear EGFP-FOXO3 in the vehicle control-treated cells. Each cell will be annotated as positive (above the threshold of activation) or negative (below the threshold of activation) for nuclear translocation of FOXO3 (see Fig. 2 bottom panels), and a percentage of positive cells in each well will be calculated. An average of the percentage of positive cells from the three replicate wells will be obtained for each tested compound.

Finally, a value of FOXO3 nuclear translocation as fold increase versus the vehicle-treated cells can be calculated for each compound (*see* Fig. 3b).

4. Hit definition: the exact threshold value of fold increase of positive cells that will define a “positive” or “hit” compound is not a fixed number. We recommend being rather permissive for the first round of the HTS, reaching up to 10% of “first hits” from the total number of tested compounds. Then, perform a second round of validation with the first hits on the HTS platform to exclude compounds that do not replicate. In this second round of validation, a much more restrictive selection can be applied to choose the final compound to explore further.
5. Once hits are validated, further analysis should be performed: curves of concentration to evaluate the IC₅₀, and biochemical assays in different cell types/platforms to monitor the robustness of the effect of the chosen hits.

4 Notes

1. Perform a selection curve for your own batch of U-2 OS cells to optimize the G418 concentration.
2. Split the cells to avoid confluency during the selection week. Expand from the 6 cm plate to a 10 cm plate and, if necessary, to a 15 cm plate, doing always the same with the control plate for selection. Expanding into larger plates allows for the generation of the maximum number of individual colonies.
3. Add the 50 μ l of medium and recover quickly right after to avoid dispersion of the cells, recovering as much medium as possible. Also, pipet up and down to detach cells from the plate.
4. As a reference, seed 50,000 cells in a 12-well plate, or 250,000 cells in a 6 cm plate.
5. Make enough number of aliquots to use the same batch of cells for all the screening plates, to avoid variations in the cellular response.
6. Poly-D-Lysine is not necessary for proper attachment of growing cells. Instead, it greatly improves the maintenance of cell shape and attachment after fixation.
7. Culture cells in enough volume of medium (no less than 60 μ l medium in 384 wells plates, or equivalent volumes in larger wells) to avoid excessive evaporation.
8. At first, to define the maximum solvent concentration, FOXO3 nuclear translocation can be assessed by fluorescence

microscopy, without a precise quantification. Once the precise parameters for the analysis of images have been set up, a more precise maximum solvent concentration can be calculated.

9. We recommend not using very small volumes of test compounds to improve accuracy and allow proper mixing.
10. The precise percentage of basal nuclear FOXO3 in vehicle-treated cells, and of activated nuclear FOXO3 in positive control-treated cells, will vary between clones. The threshold of basal nuclear FOXO3 localization must be defined for each clone and will be used to define cells with activated (increased nuclear localization) FOXO3. Importantly, for the definition of this threshold, outliers must first be removed. In our hands, once this threshold is defined, the main criterion to choose the best clone for the HTS is its ability to detect FOXO3 nuclear translocation after treatment with weak activators.
11. Importantly, *Z* factors should be obtained using controls as strong as the expected hits for the screening.
12. If fluorescence cannot be measured in the 24–48 h after fixation, cells should be kept in PBS with 0.02% sodium azide, protected from light, for a few more days before acquisition.

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

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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, CMRI

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

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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 Ultraglutamine 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 μ l FuGENE, and 2 μ g of pRevMAPKKnesGFP (*see Note 2*) to a final volume of 200 μ l 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 μ g/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

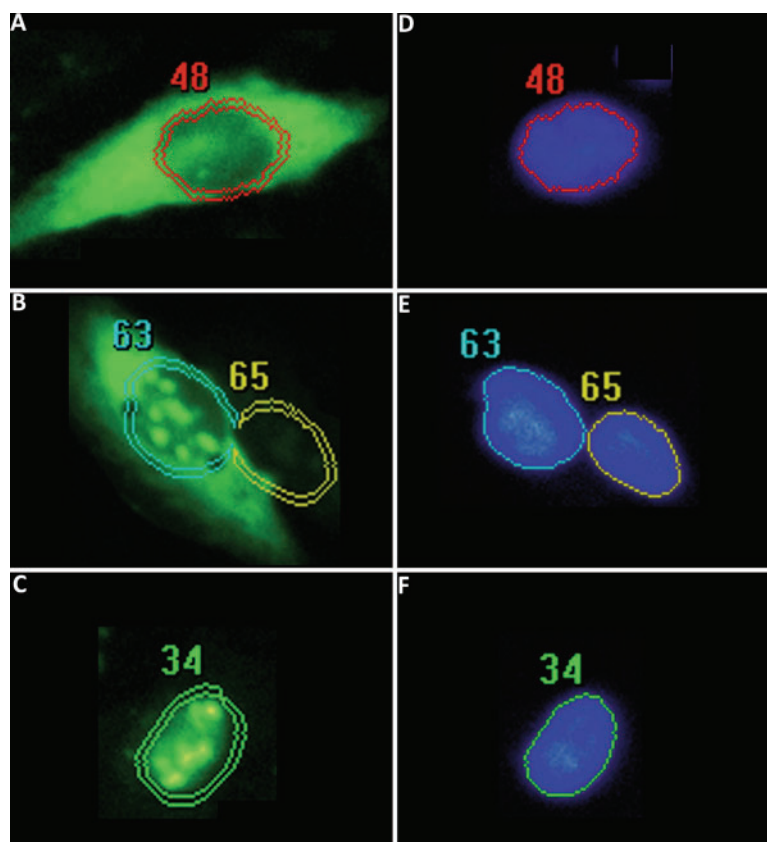


Fig. 1 Assay cells are capable of monitoring CRM-1-mediated nuclear export. (a–c) Images of cells taken in the GFP channel. (a) Well treated with DMSO, (b) Well treated with hit compound, (c) Well treated with 4 nM LMB, (d–f) Images of cellular nuclei taken in the DAPI channel

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:

$$Z' = 1 - (3SD_{LMB} + 3SD_{DMSO}) / |\text{Mean}_{LMB} - \text{Mean}_{DMSO}|$$

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

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.

3.3.1 Preparation of Mother Plates

- 3.3.2 Treatment of the Assay Cells** Transfer 2 μ l 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 μ l DMSO to wells A1 to D1 in the first row and to wells A12 to D12 in the last row and 2 μ l 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 N_2 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.

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Quantifying Tissue-Specific Overexpression of *FOXO* in *Drosophila* via mRNA Fluorescence In Situ Hybridization Using Branched DNA Probe Technology

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Abstract

While the highly conserved FOXO transcription factors have been studied in *Drosophila melanogaster* for decades, the ability to accurately control and measure their tissue-specific expression is often cumbersome due to a lack of reagents and to limited, nonhomogeneous samples. The need for quantitation within a distinct cell type is particularly important because transcription factors must be expressed in specific amounts to perform their functions properly. However, the inherent heterogeneity of many samples can make evaluating cell-specific FOXO and/or *FOXO* load difficult. Here, we describe an extremely sensitive fluorescence in situ hybridization (FISH) approach for visualizing and quantifying multiple mRNAs with single-cell resolution in adult *Drosophila* cardiomyocytes. The procedure relies upon branched DNA technology, which allows several fluorescent molecules to label an individual transcript, drastically increasing the signal-to-noise ratio compared to other FISH assays. This protocol can be modified for use in various small animal models, tissue types, and for assorted nucleic acids.

Key words Fluorescence in situ hybridization, FISH, *Drosophila melanogaster*, Heart tube, Dorsal vessel, Branched DNA, bDNA, ViewRNA, RNAscope

1 Introduction

Forkhead transcription factors, characterized by a conserved “Forkhead box (Fox)” or “winged helix” DNA-binding domain, were first identified in *Drosophila melanogaster* [1–3]. Since their initial discovery, 19 subfamilies, designated A–S, have been classified. The “O” subfamily is involved in numerous physiological and pathological processes, including aging, growth, cancer, and neurological and cardiovascular diseases [4–9]. Several features of *Drosophila* have rendered it essential in helping discern the biological roles of the FOXO subfamily since the beginning of the 21st century [6, 10, 11]. Flies possess a single *FOXO* gene, *dFOXO*, which helps simplify research [12]. The longest protein product of this

gene shares 89% and 88% Forkhead box domain identity with FOXO3 and FOXO1, respectively [13, 14], the two most abundant and highly studied FOXO proteins in mammals [15]. Generally, use of fly models affords researchers the benefits of easy, tissue-restricted manipulation of gene expression, a short life cycle, conserved aging and disease processes, and the capability of studying large, isogenous populations of offspring [16–18].

FOXO proteins control the expression of a vast array of genes, which have been studied in a variety of cell types [19, 20]. As with other transcription factors [21], subtle changes in *FOXO* expression or FOXO activity can result in context-dependent, beneficial or detrimental, cellular responses [22, 23]. In skeletal and heart muscle, for example, FOXO-mediated changes in proteostasis can have either adaptive or maladaptive functional consequences that seemingly correlate with the transcription factor's dose [22–26]. Therefore, precisely quantifying these slight yet critical differences in expression is essential for understanding downstream gene regulation. Accurate quantitation, however, is frequently hampered by low expression levels characteristic of many transcription factors [27, 28], non-optimal reagents or techniques, and heterogeneity of the tissue- or cell-types in the samples being studied.

Different approaches for assessing protein and RNA levels are well-established. Relative protein load can be determined via quantitative western blotting [29]. While valuable, this method can be accompanied by impediments. Monoclonal antibodies are the gold-standard in protein identification experiments, but their production is expensive and laborious [30, 31], and availability is often limited to human, murine, or other vertebrate proteins. Polyclonal antibodies have a higher probability of nonspecific cross-reactivity, which complicates data interpretation, and reproducibility is influenced by greater lot-to-lot variation [29]. To circumvent some of the drawbacks of antibody use and/or to complement protein analysis, methods such as quantitative reverse transcription polymerase chain reaction (qRT-PCR) have been developed and optimized to similarly examine mRNA abundance in cells and tissue. qRT-PCR is a common and sensitive means to determine RNA levels and transcriptional differences in tissue samples under diverse conditions [32]. A key obstacle that regularly faces both total protein and mRNA quantitation, however, is tissue heterogeneity within samples, potentially leading to contamination by undesired cells together with the cells of interest [33]. Moreover, both western blotting and qRT-PCR become increasingly difficult, and results more variable, when the amount of tissue is limited. These problems can be bypassed by alternative approaches that allow direct identification of mRNA exclusively in chosen cells. Highly sensitive in situ detection of RNA allows evaluation of multiple transcripts within nonhomogeneous samples without the need to

first isolate specific cell types or extract, purify, and amplify an mRNA of interest.

Early iterations of in situ hybridization (ISH) detected specific DNA and RNA sequences using radiolabeled, nucleic acid-based probes [34–37]. Over the past few decades, protocols for probe design and in situ visualization of RNA have been developed [38–42] and adapted for use in the majority of model systems, including *Drosophila*. Techniques have been improved upon, including the use of fluorescent probes (fluorescence in situ hybridization, FISH) [43, 44], to significantly increase safety and to maximize the probe signal while minimizing background signals (*see Note 1*). However, these approaches are adversely influenced by nonspecific primary probe binding, which causes an increase in background signal and thereby reduces contrast. High signal-to-noise ratios are particularly vital when investigating modestly expressed transcripts or subtle changes in transcription [28].

Here, we outline two similar FISH approaches for analyzing transcript levels in individual cells within an entire organ using branched DNA (bDNA) technology [40, 45]. bDNA signal amplification assays eliminate most background and allow for high-specificity detection of mRNAs (Fig. 1). Specifically, we tested several probes for different messages in *Drosophila* cardiac tissue, which has not been especially amenable to conventional RNA labeling methods. The *Drosophila* heart is a complex organ made up of many cell types, including ostia and valve cells [46–48], and is intimately associated with pericardial, fat, and non-cardiac muscle cells. The difficulty of working with such a small (~80 cells) organ paired with a lack of antibodies makes standard protein quantitation approaches rather elusive. Thus, our modified FISH protocols, based on the commercially available ViewRNA (ThermoFisher Scientific) and RNAscope (Advanced Cell Diagnostics) kits, allow mRNA analysis of modestly-expressed transcription factors, including *FOXO*, in distinct cells, such as individual cardiomyocytes, while disregarding undesired cells. The bDNA approach comprises multiple amplification steps, in addition to the initial complementary probe hybridization, to produce fluorescent signals with exceptionally high contrast (Figs. 1A and 2) [40, 45]. The FISH methods described here are practical as an independent means for quantifying cellular mRNA in heterogeneous samples and as supplements to the other techniques discussed above.

2 Materials

2.1 Manipulation of Gene Expression in the *Drosophila* Heart

1. Anesthetizing pad connected to CO₂ tank or triethylamine (FlyNap).
2. *tinCΔ4-GAL4* fly line (cardiac-specific driver line) [49].

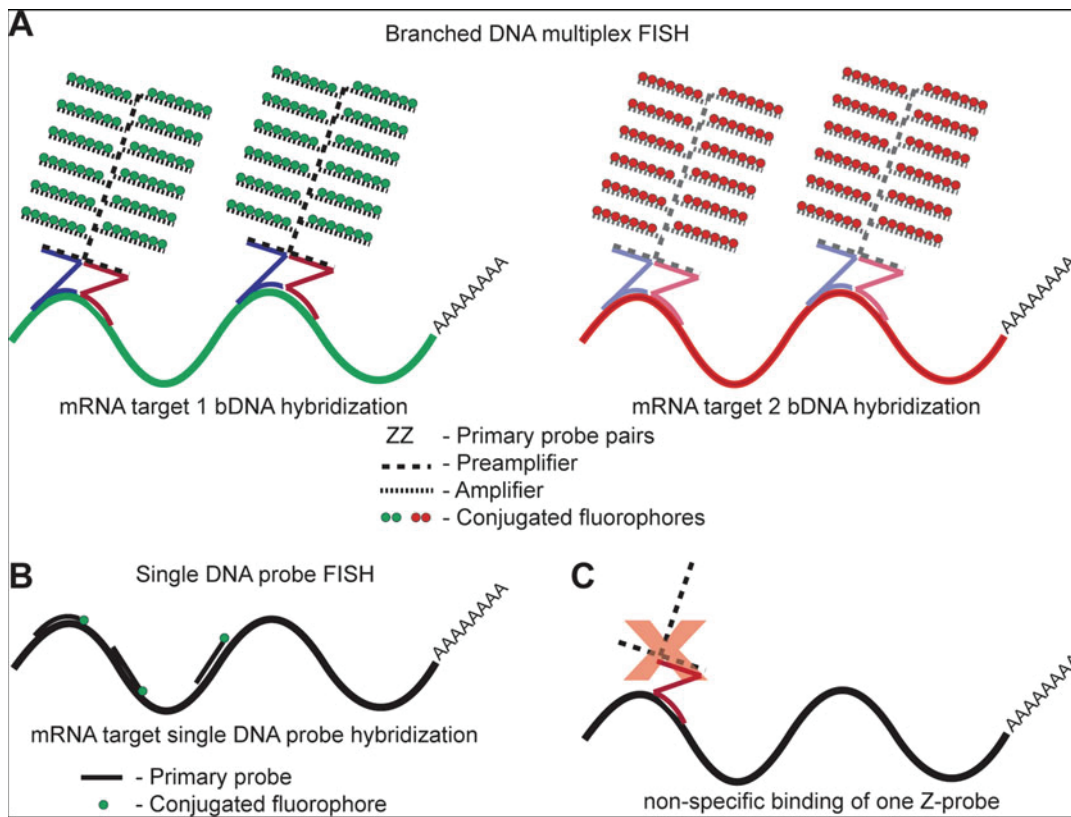


Fig. 1 Overview of the branched DNA approach. **(A)** In bDNA assays, pairs of primary probes (ZZ) identify and hybridize to a specific gene product of interest. Preamplifiers and amplifiers then bind the probe pairs to form tree-like structures. Fluorescent label probes attach to respective amplifier “branches” to give up to 100× higher signals than approaches illustrated in **(B)** under equivalent imaging conditions. Both ViewRNA and RNAscope follow the same general cascade of hybridization events. Label probes for ViewRNA are pre-determined at the time of probe set design. Label probe combinations for RNAscope are determined at the time of the experiment according to Table 1. **(B)** Alternative FISH approaches include the direct hybridization of several short oligonucleotide probes with a single fluorophore, which require long transcripts to ensure high fluorescence intensity for detection [38, 41]. While direct fluorophore conjugation does not allow for signal-to-noise ratios as high as those afforded by bDNA assays, as described by Titlow et al., a benefit of this method is the capability of viewing protein localization in parallel with RNA localization [38]. **(C)** An advantage of using a bDNA approach for RNA in situ hybridization is the high specificity of probe pairs compared to individual oligonucleotide probes. If only one member of a probe pair hybridizes to an off-target RNA sequence, the preamplifier cannot bind, and therefore no fluorescent signal will result

3. *DMef2-GAL4* fly line (ubiquitous muscle driver line, Bloomington *Drosophila* Stock Center, BDSC, BL-27390).
4. *UAS-FOXO wt.m3-1* fly line (inducible *dFOXO* overexpression line) [50].
5. *yw* fly line (control background line).
6. *UAS-mCD8::GFP* (membranous GFP-reporter line, BDSC, BL-32184).

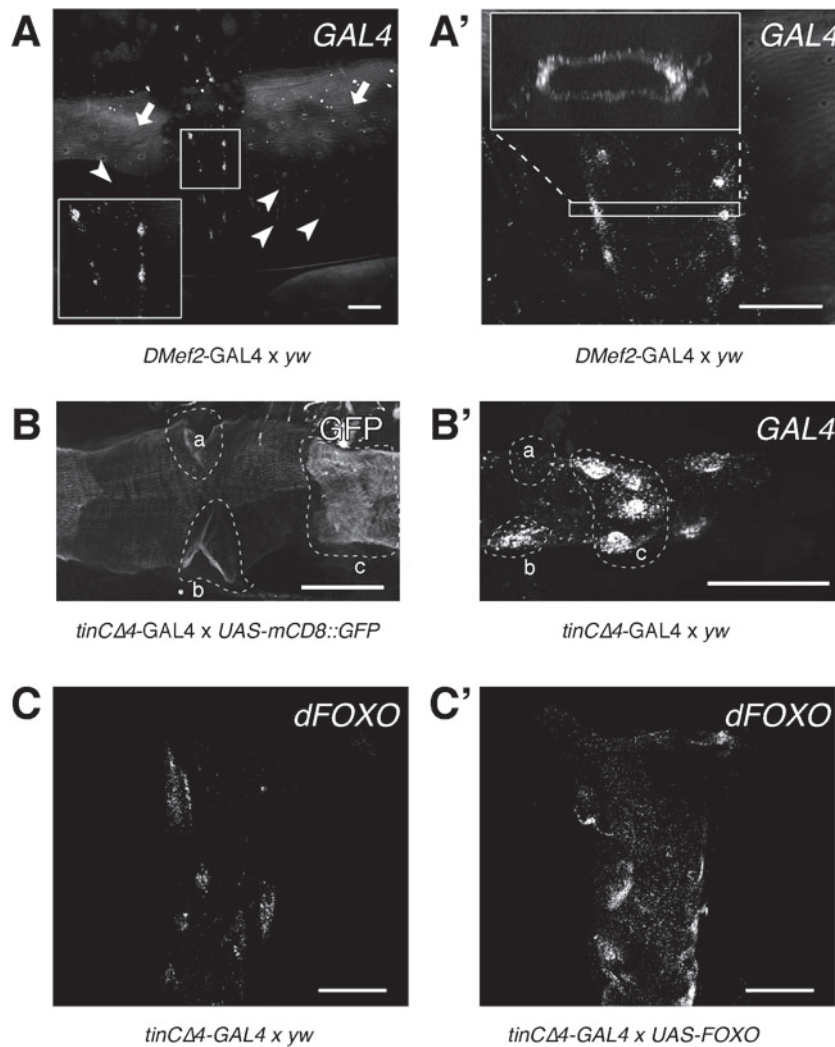


Fig. 2 Characterization of *GAL4* expression patterns in *DMef2-GAL4* and *tinCΔ4-Gal4* using RNAscope and ViewRNA. **(A)** Adult *Drosophila* abdominal carcass treated with a probe against *GAL4* shows expression of *GAL4* in abdominal body wall muscles (arrowheads) and in the cardiomyocytes of the heart (inset). Note the background fluorescence from the cuticle (arrows). **(A')** RNA molecules can be detected throughout the heart tube (cross-sectional view). **(B)** Activity of *GAL4* assayed via *mCD8::GFP* reporter gene expression. GFP can be detected throughout the entire heart tube, but shows stronger fluorescence in the ostia cells (a, b) and valve cells (c). **(B')** GFP intensity is mirrored by high levels of *GAL4* transcripts in the same cell types (a, b, and c, compare to **B**). **(C)** Endogenous *dFOXO* transcripts from control hearts (*tinCΔ4-GAL4 x yw*) were probed using ViewRNA. **(C')** *tinCΔ4-GAL4* drives *UAS-dFOXO* expression to a visibly greater extent than the endogenous *dFOXO* promoter in control hearts. All scale bars (white) represent 50 μm. **(C** and **C')** were adapted for use with permission from [23]

7. Standard *Drosophila* media: deionized water, molasses (9.10% v/v), agar (12.40 g/L), yeast (17.05 g/L), yellow cornmeal (68.50 g/L), tegosept (p-hydroxybenzoic acid methyl ester,

0.92% v/v), propionic acid (0.275% v/v), and phosphoric acid (0.033% v/v) to desired volume.

8. Vials for mating and collecting flies.

2.2 Adult *Drosophila* Heart Dissections (See Note 2)

1. A list of all required materials is available in [51].
2. First generation (F1) offspring from fly crosses described below in Subheadings 3.1 and 3.2.
3. Triethylamine (FlyNap).
4. Petri dishes (35 × 10 mm).
5. Petroleum jelly.
6. Curved microdissection spring scissors and microforceps.
7. Adult *Drosophila* hemolymph (AH): 108 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 8 mM MgCl₂, 15 mM Hepes pH 7.1, 1 mM NaH₂PO₄, 4 mM NaHCO₃, 10 mM sucrose, and 5 mM trehalose. Adjust to pH 7.1, and add sugars immediately before use [51].
8. Glass capillaries (100 µL) pulled to a maximum diameter of 40 µm.
9. Plastic tubing (1/16").
10. Vacuum or suction source.
11. Stereo dissecting microscope with light source and a dark base, ideally.

2.3 Fixation and Hybridization

1. Incubator set to exactly 40 °C (*see* Note 3).
2. 10 mM EGTA in AH.
3. Phosphate-buffered saline (1× PBS): 137 mM NaCl, 20 mM phosphate, 2.7 mM KCl, pH 7.4.
4. 1× PBST (PBS + 0.1% Tween-20).
5. 4% formaldehyde in 1× PBS.
6. Flat-bottom 96-well plate, white.
7. Foil or other material (such as a second, opaque 96-well plate placed on top) to protect fluorophores from light.
8. FISH assay kit: both kits include wash buffer, protease solution (s), reagents for amplification of the primary probes, color probes, and DAPI (Fig. 1a).
 - (a) ViewRNA ISH cell assay kit (ThermoFisher scientific, QVC0001): in addition to reagents listed above, this kit includes 10X PBS buffer solution, detergent solution, and diluent solutions.
 - (b) RNAscope® Fluorescent Multiplex Reagent Kit (Advanced Cell Diagnostics, Inc., 320850).

2.4 Mounting, Imaging, and Quantitation

1. Glass slides and ultra-thin cover slips (22 × 22 mm).
2. ProLong Gold with DAPI or other acceptable mounting media.
3. Clear nail polish for sealing slides.
4. Epifluorescent or confocal microscope with filters required for the chosen color probe types (*see* **Note 4**).
5. ImageJ software for quantifying punctae (transcripts) (*see* **Note 5**).

3 Methods

3.1 Manipulation of Gene Expression in the *Drosophila* Heart

3.1.1 Cardiac-Specific Overexpression of dFOXO

1. Collect virgin females and young males for all crosses.
2. When using the UAS-GAL4 bipartite system of gene manipulation, typically mate virgin females expressing GAL4 driven by a tissue-specific enhancer with young males homozygous for an allele containing the desired UAS-construct.
3. Place virgin *tinCΔ4-GAL4* females in vials containing molasses-cornmeal media with either young transgenic (e.g., *UAS-FOXO wt.m3-1*) or control (e.g., *ym*) males at 25 °C.
4. After 3 days, remove P1 (parental generation) adults from the vials.
5. 10 days after mating was initiated, collect adult progeny (F1), place on fresh media, and maintain at 25 °C.

3.1.2 Muscle-Specific Expression of GAL4 and Membranous GFP

1. Follow steps 1–3 listed in Subheading 3.1.1 using *DMef2-GAL4* virgin females and *UAS-mCD8::GFP* males.
2. Perform **steps 4 and 5** in Subheading 3.1.1.

3.2 Probe Design

bDNA probes are specifically designed to detect the transcript(s) of interest. The target region should cover ~1000 bases, which gives the maximum number of primary probe pairs per mRNA necessary to achieve the strongest signal (*see* **Note 6**). Since probe design is a proprietary process, the RefSeq number of the target sequence must be submitted to the probe vendor. They will assess if probe design is feasible, if the probes will be specific for the given transcript, and if alternate splice variants of the gene product may be detected. A number of primary probe sets are readily available, including those targeting glyceraldehyde 3-phosphate dehydrogenase (*GAPDH1*) as a positive control and yeast-*GAL4* as either a positive control in the *GAL4*-background or a negative control in the absence of *GAL4*. Probe sets targeting human or other vertebrate RNA may serve as a generic negative control for *Drosophila* tissue. Positive control(s) can help validate successful FISH, and negative control(s) can assist in determining the degree of

background or nonspecific binding. Additional thought must be given to how these probes will be combined, since each probe set of a specific type cannot be combined with a different probe set of the same type in a multiplex experiment (e.g., RNAscope C1 probes can only be used together with a C2 and a C3 probe, but not another C1 probe (Table 1), and ViewRNA probe type 1 can only be used together with type 4 and 6, but not another type 1) (*see Notes 7 and 8* for detailed examples of ViewRNA and RNAscope probe set and combination strategies, respectively).

3.3 Adult *Drosophila* Heart Dissections

1. A video depicting the protocol for semi-intact adult *Drosophila* heart preparations is available in [51].
2. Anesthetize flies using FlyNap.
3. Coat a thin layer of petroleum jelly on the bottom of a small petri dish. Immobilize ~six anesthetized flies dorsal side down by separating their wings and sticking them into the petroleum jelly.
4. For each fly, using curved microdissection spring scissors, remove the head and ventral portion of the thorax in one motion by placing the scissors at an angle under the fly's legs. Leave the dorsal portion of the thorax and wings to keep the preparation firmly stuck to the petroleum jelly. This cut should also remove a small segment of the anterior end of the abdomen.
5. Fill the petri dish with enough fresh AH to cover the preparations.
6. Again, for each fly, use the spring scissors and fine-tipped forceps to remove the posterior tip of the abdomen (segment eight). Make two cuts, one up each side of the abdominal cuticle, and remove the ventral cuticle, exposing the abdominal organs.
7. Carefully remove the internal organs, leaving the beating heart tube untouched and intact against the dorsal cuticle.
8. Use pulled microcapillaries connected to a suction source to carefully remove fat surrounding the heart tube to expose as much of the tissue as possible. Ensure the heart is not disturbed, as it is easily damaged.

3.4 Fixation

1. Replace the AH with enough 10 mM EGTA in AH to cover the preparations to arrest hearts within 1 min.
2. Exchange the EGTA solution with 4% formaldehyde in 1× PBS, and incubate with gentle rocking at room temperature for 20 min (*see Note 9*).
3. Wash 3× in 1× PBST at room temperature for 10 min each, and replace with PBS.

4. While the fixed samples remain in PBS, carefully cut off most of the remaining thoracic material, and trim the sides of each abdominal cuticle.
5. Delicately, transfer the preparations to appropriately marked well(s) of a 96-well plate filled with 150 μ L 1 $\times$ PBS, up to six specimens per well. Use as many wells as needed for a given experiment.
6. The samples may be stored in 1 $\times$ PBS at 4 °C overnight (*see Note 10*).

3.5 In Situ Hybridization

3.5.1 ViewRNA

1. Ensure the incubator is set to and maintains 40 °C.
2. Prepare wash buffer by combining wash buffer component 1 with water, and then add component 2 according to the manufacturer's protocol.
3. Warm all diluent buffers to 40 °C for 30 min before use.
4. Carefully aspirate the 1 $\times$ PBS from each well containing specimens (*see Note 11*), and replace it immediately with 100 μ L detergent solution included in the ViewRNA ISH Cell Assay kit. Cover the 96-well plate, and incubate at room temperature for 5 min.
5. Draw out the detergent solution, and rinse heart tube preparations 2 $\times$ with 150 μ L 1 $\times$ PBS at room temperature for 2 min each to remove all residual detergent solution.
6. Replace PBS with 100 μ L protease solution in each well (1:4000 in 1 $\times$ PBS, *see Note 12*) to expose the mRNA for hybridization. Cover the 96-well plate, and incubate at room temperature for 10 min with gentle agitation.
7. Remove the protease solution, and rinse 3 $\times$ with 150 μ L 1 $\times$ PBS at room temperature for 2 min each to remove residual protease.
8. Substitute PBS with 100 μ L working probe set solution (ViewRNA probes diluted 1:100 in probe set diluent buffer). This solution contains all probe sets used in the assay (Fig. 1a, ZZ probe pairs). Cover the 96-well plate, and incubate at 40 °C for 3 h.
9. Aspirate the working probe set solution, and wash 3 $\times$ with 150 μ L wash buffer at room temperature for 2 min each to remove residual probes. IMPORTANT: Do not soak samples in wash buffer longer than 30 min.
10. Exchange wash buffer with 100 μ L working pre-amplifier mix solution (pre-amplifier mix diluted 1:25 in amplifier diluent buffer) (Fig. 1a, dashed lines). Cover the 96-well plate, and incubate at 40 °C for 30 min.

11. Remove the working pre-amplifier mix solution, and wash $3\times$ with 150 μL wash buffer at room temperature for 2 min each to remove remaining pre-amplifier molecules. IMPORTANT: Do not soak samples in wash buffer longer than 30 min.
12. Swap wash buffer with 100 μL working amplifier mix solution (amplifier mix diluted 1:25 in amplifier diluent buffer) (Fig. 1a, dotted lines). Cover the 96-well plate, and incubate at 40°C for 30 min.
13. Extract the working amplifier mix solution from the wells, and wash $3\times$ with 150 μL wash buffer at room temperature for 2 min each to remove unbound amplifier molecules. IMPORTANT: Do not soak samples in wash buffer longer than 30 min.
14. Protect samples from light from now on, as the label probes contain fluorophores that are photolabile.
15. Replace wash buffer with 100 μL working label probe mix solution (label probe mix diluted 1:25 in label probe diluent buffer, protect from light) (Fig. 1a, colored circles). Cover the 96-well plate, and incubate at 40°C for 30 min.
16. Draw out the working label probe mix solution, and wash $2\times$ with 150 μL wash buffer at room temperature for 2 min each and $1\times$ for 10 min. IMPORTANT: Do not soak samples in wash buffer longer than 30 min.
17. Remove wash buffer. Stain the samples with a 1:100 DAPI solution in $1\times$ PBS at room temperature for 1 min if DNA counterstaining is necessary or if you are not using mounting medium that contains DAPI. Rinse once with $1\times$ PBS at room temperature for 2 min, and store in $1\times$ PBS until mounted (*see Note 10*).

3.5.2 RNAscope (*See Note 13*)

1. Ensure the incubator is set to and maintains 40°C .
2. Bring all reagents to room temperature.
3. Prepare 100 mL of $1\times$ wash buffer from $50\times$ stock solution.
4. Protect samples from light for the entirety of the procedure.
5. If using C2 and/or C3 probes, dilute each probe 1:50 in C1 probe. 50–100 μL of probe solution is sufficient for a 96-well plate well.
6. Transfer the probe solution into as many empty wells as are appropriate for the given experiment. Heat the probes to 40°C for 10 min.
7. In parallel to **step 6**, replace $1\times$ PBS in the specimen-containing wells (from Subheading 3.3, **step 5**) with 3–4 drops of Protease III, and incubate at 40°C for 10 min (*see Note 12*).

8. Carefully remove the Protease III, and immediately rinse samples 3× with 1× PBS at room temperature for 2 min each to eliminate remaining protease (*see* **Note 11**).
9. Aspirate the PBS, and transfer 50 µL of the pre-warmed probe solution to the heart samples (Fig. 1a, ZZ probe pairs). Incubate at 40 °C for 2 h or overnight.
10. Draw off the probe solution, and wash 3× with 1× wash buffer at room temperature for 5 min with gentle agitation to remove unbound probes.
11. Replace wash buffer with 2 drops AMP 1-FL solution (Fig. 1a, dashed lines), and incubate at 40 °C for 30 min.
12. Extract the AMP 1-FL solution, and wash 3× with 1× wash buffer at room temperature for 5 min each with gentle agitation to remove residual AMP 1-FL solution.
13. Replace wash buffer with 2 drops AMP 2-FL solution (Fig. 1a, dotted lines), and incubate at 40 °C for 15 min.
14. Remove the AMP 2-FL solution, and wash 3× with 1× wash buffer at room temperature for 5 min each with gentle agitation to remove remaining AMP 2-FL solution.
15. Replace wash buffer with 2 drops AMP 3-FL solution, and incubate at 40 °C for 30 min.
16. Extract the AMP 3-FL solution, and wash 3× with 1× wash buffer at room temperature for 5 min each with gentle agitation to remove residual AMP 3-FL solution.
17. Replace wash buffer with 2 drops AMP 4-FL solution, and incubate at 40 °C for 15 min. Refer to Table 1 for the desired color combination (*see* **Note 4**).
18. Draw out the AMP 4-FL solution, and wash 3× with 1× wash buffer at room temperature for 5 min each with gentle agitation to remove any lingering AMP 4-FL solution.
19. Replace wash buffer with 2 drops DAPI solution if DNA counterstaining is needed and if the mounting medium does not already contain DAPI.
20. Rinse with 1× PBS and ideally mount the samples immediately in antifade reagent. Alternatively, samples can be stored at 4 °C but for no longer than 8–12 h, as the signal will start to fade in 1× PBS (*see* **Note 10**).

3.6 Mounting the Adult *Drosophila* Heart Tube Preparations

1. A video file that explains a suitable approach for mounting adult *Drosophila* heart preparations is available in [52].
2. Place two small drops (~15 µL) of mounting media centered ~1 cm apart on a glass slide, and place two cover slips on the drops.

3. Place a 10–20 μL drop of mounting medium on a third cover slip.
4. Remove the hybridized heart preparations gently from the 96-well plate using fine-tipped forceps. Handle specimens at the edge of the leftover cuticle. Do not make contact with the heart tube. Place the preparations heart side-down on the drop of mounting medium on the third coverslip. Up to six flies can be placed per drop.
5. Carefully but quickly invert the cover slip, and place it on the top of the glass slide such that the adult heart preparations lie between the two already placed cover slips. This forms a bridge that protects the heart samples from becoming disturbed.
6. Ensure that all hearts are now facing up and are unobscured by residual cuticle.
7. Use mounting medium to fill in any air pockets present between the glass slide and cover slip.
8. Seal the edges with clear nail polish.
9. Keep slides protected from light until ready to image.

3.7 Imaging of Hearts via Confocal Microscopy (See Note 7)

1. Choose appropriate beam splitter, emission filters, laser power, and camera gain, and ideally use the same settings throughout the imaging of samples. The fluorophores conjugated to the label probes used by ViewRNA (DAPI, FITC, Cy3, Cy5, and Cy7) are commonly employed and have preset emission settings available on most microscope software.
2. To faithfully capture all fluorescent dots, take care to acquire a sufficient amount of data in the x , y , and z optical planes. For a typical setup, a $40\times$ immersion objective is sufficient to image close to the Nyquist criterion. However, this must be determined for each instrument used. This is equally important for imaging along the z -axis, in which undersampling can easily cause erroneous data representation due to missed slices.
3. Owing to the rounded and varying morphology of the adult *Drosophila* heart tube between the anterior-most conical chamber and the posterior end, the number of z -sections required to image through the whole tissue varies along the heart's length.
4. Capturing the highest dynamic range is also critical since the intensities can vary between the detected mRNAs. Individual transcripts appear as distinct punctae with mostly uniform intensities, however lower intensity dots should be disregarded (*see* Subheading 3.8). A negative control probe is crucial to establish the amount of background punctae per cell, which should be corrected for in the final analysis.

3.8 Image Analysis and Quantitation (See Note 8)

3.8.1 Basic Image Analysis and Quantitation of mRNA Particles Using ImageJ Particle Counter

1. Open an image that was generated in Subheading 3.7 in the microscope program. Separate emission channels for each label probe and/or DAPI. Stacked images or individual z-sections may be converted to grayscale and exported as TIFFs to ImageJ (Fig. 3A).
2. For a particular cardiac region, open all emission channel files in ImageJ, and convert each image to 8-bit (Image > Type > 8-bit).
3. Change the threshold (Image > Adjust > Threshold) such that the upper and lower limits adequately incorporate punctae that represent transcripts but exclude those that may signify off-target binding or background. Maintain the threshold

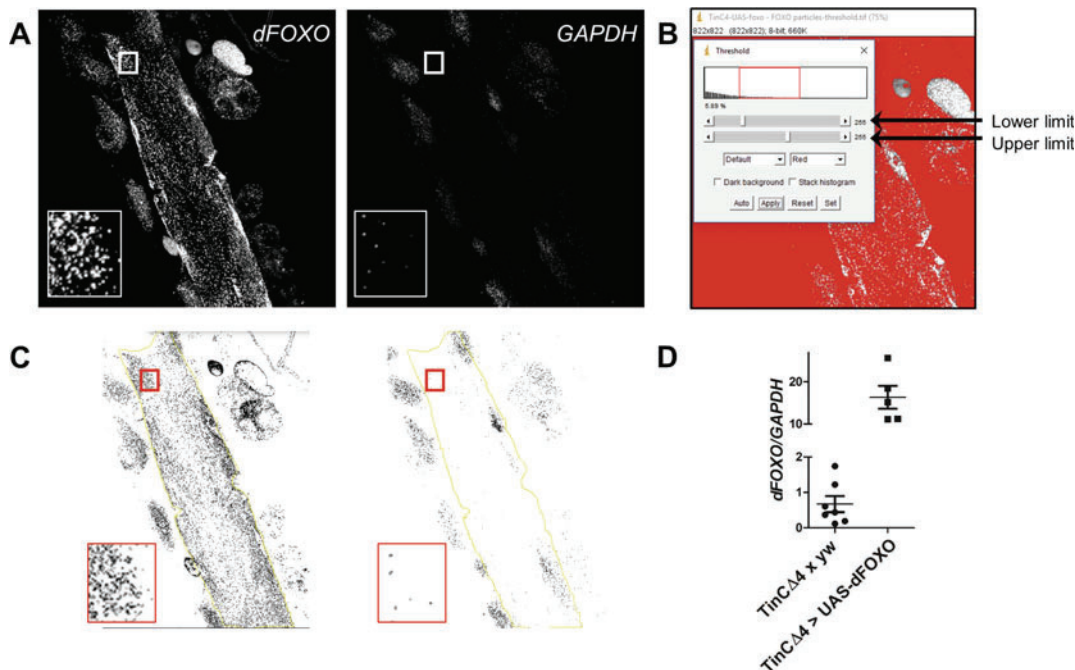


Fig. 3 Quantitation of *dFOXO* mRNA particles from a ViewRNA FISH experiment in a semi-intact *Drosophila* heart tube preparation. (A) Representative images of a *tinCΔ4-GAL4 > UAS-dFOXO* heart, probed for *dFOXO* and *GAPDH* transcripts, converted to grayscale for analysis using ImageJ. Inset displays a region of interest (cardiomyocyte) prior to threshold modification. (B) After conversion to 8-bit, the threshold of each image is arbitrarily changed to a range between 55 and 150 to help eliminate background signals and off-target probe binding from subsequent particle analysis. (C) Representative images, post-threshold adjustment, with the inset displaying the same cardiomyocyte data as in (A). Larger regions of interest, which exclude non-cardiac tissue, can be selected using the free-hand tool in ImageJ (yellow line). (D) Values from *tinCΔ4-GAL4 x yw* control vs. *tinCΔ4-GAL4 > UAS-dFOXO* hearts (Fig. 2C and C') were obtained using the ImageJ particle analysis tool. *dFOXO/GAPDH* was calculated for each heart or region of interest. The graph shows a small representation of the difference calculated in normalized *dFOXO* transcripts between the hearts of the two genotypes. The use of ViewRNA to detect more subtle changes in cardiac *dFOXO* transcript levels was previously published [23]

settings throughout analysis of all other images. Here, we used an arbitrary upper limit of 150 and a lower limit of 55 (Fig. 3B, *see Note 14*) [23].

4. Because the images include signals from tissues other than the heart, use the freehand selection tool to outline a region of interest (ROI) that includes only cardiomyocytes and no adjacent adipose tissue, pericardial cells, or retractors of tergite muscles (Fig. 3C, yellow outline). Copy the ROI tracing to all other channels from the same heart section (Ctrl+Shift+e on PC or Cmd+Shift+e on Mac, *see Note 15*).
5. Count the number of particles in the ROI, which corresponds to the number of transcripts in each channel (Analyze > Analyze particles).
6. For individual specimens in which multiple cardiomyocytes were imaged separately, combine the total number of *dFOXO* particles (or mRNA particles under investigation) and the total number of *GAPDH* particles determined for each channel, from each cell per heart.
7. Normalize the total number of *dFOXO* particles to *GAPDH* mRNA particles per heart (Fig. 3D).

3.8.2 Alternative ImageJ Protocol [53]

1. Install the Bioformats plugin, which allows for seamless import of most scientific file formats into ImageJ.
2. Load the image stack acquired in Subheading 3.7 into ImageJ. Create a Maximum Intensity projection of the stack (Image > Stacks > Z-project > Max.Intensity).
3. Adjust image brightness (Ctrl+Shift+C on PC or Cmd+Shift+C on Mac), and open the ROI Manager (Analyze > Tools > ROI Manager).
4. Create a cell outline with the freehand selection tool (*see Note 15*), and add the ROI to the ROI manager by pressing “t”. Repeat this for all cells you want to analyze.
5. Select all ROIs (check “Show all”). Flatten the image, and save it to document an overview of all ROIs in the open stack.
6. Activate the full stack image, and select the first ROI from the ROI Manager. Duplicate this ROI (Ctrl+Shift+D on PC or Cmd+Shift+D on Mac).
7. Discard image data outside the ROI (Edit > Clear Outside).
8. Scroll through this stack, and select the image range covering all slices of interest.
9. Reduce dimensionality to use only the desired range (e.g., 1–37). To avoid potential double-counting of punctae present in adjacent slices select only even slices, Image > Stack > Tools > Make Substack. Enter the image range followed by -2 to select every other image (e.g., 1-37-2).

10. Split this stack into separate Colors (Image > Color > Split Channels), and continue with thresholding.
11. Run the thresholding command (Ctrl+Shift+T on PC or Cmd+Shift+T on Mac) on the first channel. Use MaxEntropy with Red and Dark background. Calculate threshold for each image, check “dark background,” and apply.
12. Use ImageJ’s built-in “Analyze particles” function to count all punctae of a specific size range automatically. First, determine the typical puncta size by measuring the area of several particles (typically 0.2–2 μm^2). Set circularity to 0.2–1, show “Overlay,” and check “Display results.”
13. Repeat **steps 11–12** for the remaining channels.
14. Repeat **steps 6–13** for the remaining ROIs of the full stack.

4 Notes

1. Protocols for the design and in situ detection of single or multiple RNA probes using both chromogenic and fluorescent labels have been developed and adapted for a majority of model systems. A variety of techniques have also been established to amplify the signal through enzymatic reactions, including alkaline phosphatase or horseradish-peroxidase that cause local precipitation of a specific compound, such as nitro-blue tetrazolium, 3,3' diaminobenzidine or TSA, or more recently by hybridization chain reaction [54]. However, each of these approaches, like early versions of FISH, will be directly affected by nonspecific binding of the probe (Fig. 1B).
2. Gene overexpression or knockdown [55] can be achieved in different tissues in *Drosophila* and in other small model organisms, although the procedure for exposure will differ depending on the tissue being investigated.
3. These procedures were successfully tested with several incubators, including a Robbins scientific model 400, an Eppendorf Thermomixer C for 96-well plates with heated lid, and a Shel-Lab-2350-T water jacketed incubator.
4. The microscopes used in 3.7 were a Leica TCS SPE RGBV confocal microscope with a 40 $\times$ oil immersion lens for View-RNA experiments and a Zeiss Imager.Z1 with Apotome.2 and a 40 $\times$ water immersion lens for RNAscope experiments. Alternatively, an epifluorescence microscope may be used. A limitation of epifluorescence versus confocal microscopy is the inability to scan at a specific focal depth without interfering, out-of-focus signals. However, because bDNA FISH methods produce distinct punctae, or dots, when hybridized to

individual transcripts, background signal from other focal planes should not interfere significantly with interpretation of results after analysis.

5. Additional suitable FISH image acquisition and analysis methods are described in detail in [38].
6. While a target mRNA of 1000 bases is ideal, the target region may be as short as 300 bases; however, signal reduction occurs due to fewer binding probes. Probe design can also be optimized for localization and quantitation of miRNAs, viral RNA, and lncRNA in specific cells in addition to mRNA transcripts. One miRNA can be identified at a time as well as two mRNA targets. miRNA probes are detected using Fast Red fluorescent dye.
7. The following probes were designed by ViewRNA: the *dFOXO* probe set was designed against the longest mRNA product of the *dFOXO* gene, NM_001275628 (type 1, 550 NM excitation, ThermoFisher Scientific, catalog number VF1-18189-VC). The internal control, *GAPDH1*, probe set was designed against the mRNA sequence NM_080369 (type 6, 650 NM excitation, ThermoFisher Scientific, catalog number VF6-18191-VC). Probe set type 4 (488 nm excitation) was not used due to potential autofluorescence of adipose tissue surrounding the heart [56]. A probe set against a nonendogenous, bacterial mRNA sequence, *dapB* (type 1, 550 nm excitation) ThermoFisher Scientific, catalog number VF1-11712-VC), was purchased as a negative control. Up to four different probe types can be combined without overlapping but must be predetermined at the probe design step.
8. The following probes were ordered from RNAscope: *Drosophila melanogaster* *GAPDH1* (NM_080369.3, Advanced Cell Diagnostics, 470801-C1), and yeast *GAL4* (NM_001184062.1, Advanced Cell Diagnostics, 428501-C1). Up to three probe types can be combined, and final fluorophore combinations are determined at the time of the experiment as illustrated in Table 1.

Table 1
Color probe combinations available for use with RNAscope

Probe Channel ID	Amp 4-AltA	Amp 4-AltB	Amp 4-AltC
C1	Alexa 488	Atto 550	Atto 550
C2	Atto 550	Alexa 488	Atto 647
C3	Atto 647	Atto 647	Alexa 488

9. When tissue is fixed for longer than 30 minutes, ssRNA specifically becomes degraded or modified by crosslinking [57, 58].
10. For ViewRNA assays, it was determined that samples should not remain in PBS at 4 °C for more than 16 h before performing in situ hybridization. However, in the case of RNAscope, samples remaining at 4 °C for 1 week after fixation yielded acceptable results. The critical step is to mount hearts immediately after the final color probe step since bDNA probes are not stable long after hybridization. Once mounted, samples are stable for several weeks.
11. Gel-loading tips may be used to ensure no accidental aspiration of the heart tube preparations. Avoid touching the samples, as they tend to stick to the pipette tips. After aspiration, immediately replace buffers so tissues do not dry out.
12. Conditions for protease treatment (e.g., time and concentration) must be empirically determined for different tissues. While 10 min is necessary for the extracellular matrix-rich heart tube, it leads to a noticeable thinning of the abdominal wall muscles, which therefore might require shorter incubation times.
13. While mostly similar to the ViewRNA Cell Assay kit, the RNAscope kit comes with dropper bottles for most reagents, minimizing the handling of the solutions. 1–3 drops per well (25–75 µL) are usually sufficient to keep the samples immersed.
14. The lower limit chosen in ImageJ will minimize punctae that may represent off-target binding of probes from either the ViewRNA or RNAscope assays.
15. Appropriate ROIs are best determined using an overlay of all channels plus DAPI and pasting the ROI onto the images from individual channels. However, this can introduce an element of human error, as the freehand tool is ultimately used to outline the ROI.

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Part III

Omics and Bioinformatic Approaches



Chapter 16

Genome-Wide Analysis for Identifying FOXO Protein-Binding Sites

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Abstract

Forkhead box O (FOXO) proteins comprise a superfamily of transcription factors that play important roles in controlling various biological processes. Transcriptional control constitutes a crucial component in regulating complex biological processes. The identification of cis-regulatory elements is essential to understand the regulatory mechanism of gene expression. Chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) is widely used to identify the cis-regulatory elements of transcription factors and other DNA-binding proteins on a genome-wide level. It is a powerful tool to analyze the regulatory networks underlying the biological processes. Here, we describe a detailed protocol for preparing ChIP-seq samples that are used for sequencing and subsequent data analyses.

Key words Chromatin, Immunoprecipitation, Genomic, Transcription factor, High-throughput sequencing

1 Introduction

Forkhead box O (FOXO) proteins are characterized by the presence of a conserved Forkhead (FKH) DNA-binding domain [1]. The mammalian FOXO family consists of FOXO1, FOXO3, FOXO4, and FOXO6 that function as transcription factors that recognize a core consensus DNA sequence (T/C)(G/A)AAACAA [2, 3], and regulates expression of their target genes involved in a variety of biological processes, including cell cycle arrest, apoptosis, detoxification, energy metabolism, and longevity [4]. The activity of FOXO proteins is regulated at multiple levels by a wide range of physiological stimuli, including insulin, growth factors, nutrients, cytokines, oxidative stress, and micro RNAs [4–7]. In response to stimuli, FOXO proteins undergo posttranslational modifications such as phosphorylation, acetylation, and ubiquitination in association with alterations of protein levels, subcellular localization, and DNA-binding activity. While the conserved FKH DNA-binding domain and the ubiquitous tissue expression of FOXO proteins

suggest functional redundancies, studies using genetically engineered mouse models for FOXO proteins found unique biological functions of each FOXO protein member [4, 8–10]. Thus, it is crucial to better understand how FOXO proteins control their target genes to mediate such a diverse biological function in a well-coordinated fashion. Identifying the DNA-binding sites and regulatory sequence of FOXO proteins is an essential step toward clear understanding of the transcriptional regulatory networks underlying the biological function of FOXO proteins [11].

In recent years, advances in sequencing technologies have been made where chromatin immunoprecipitation (ChIP) was coupled with high-throughput sequencing (ChIP-seq). ChIP-seq is a powerful technology used to identify genomic regions that interact with a specific protein in vivo on a genome-wide level. Unlike ChIP-chip in which the interacted DNA regions with a protein are analyzed by DNA hybridization arrays following ChIP, ChIP-seq provides higher resolution, less noise-to-signal ratio, and broader genome-wide coverage than ChIP-chip [12]. In brief, a ChIP-seq experiment is initiated with covalent cross-links of the interactions between chromatin and proteins by formaldehyde, followed by nuclei isolation and DNA fragmentation by sonication (Fig. 1). The DNA fragments are immunoprecipitated with a specific antibody that recognizes a protein of interest, resulting in selective enrichment of DNA regions associated with the protein. The cross-links are then reversed and the DNA fragments are purified, which in turn is subject to sequencing. The analysis of DNA sequencing provides the binding sites and the precise regulatory sequences occupied by the protein of interest on a genome-wide level. Further data analysis provides regulatory networks and pathways driven by the protein, which would contribute to advance our understanding of the molecular basis of the outcome mediated with the transcriptional regulatory events. While most ChIP-seq experiments were initially performed in cultured cell lines [13, 14], ChIP-seq experiments for various tissues were introduced in recent years [15–17]. In this chapter, we provide an in-depth description of ChIP-seq sample preparation in mouse livers, which could be adapted to other tissues and culture cell lines with minor modifications.

2 Materials

In all steps and preparations of solution, use nuclease-free water.

2.1 Cross-Linking

1. Formaldehyde solution: 37% w/w. Caution: Always handle formaldehyde in a fume hood to minimize inhalation of formaldehyde vapor (*see Note 1*).

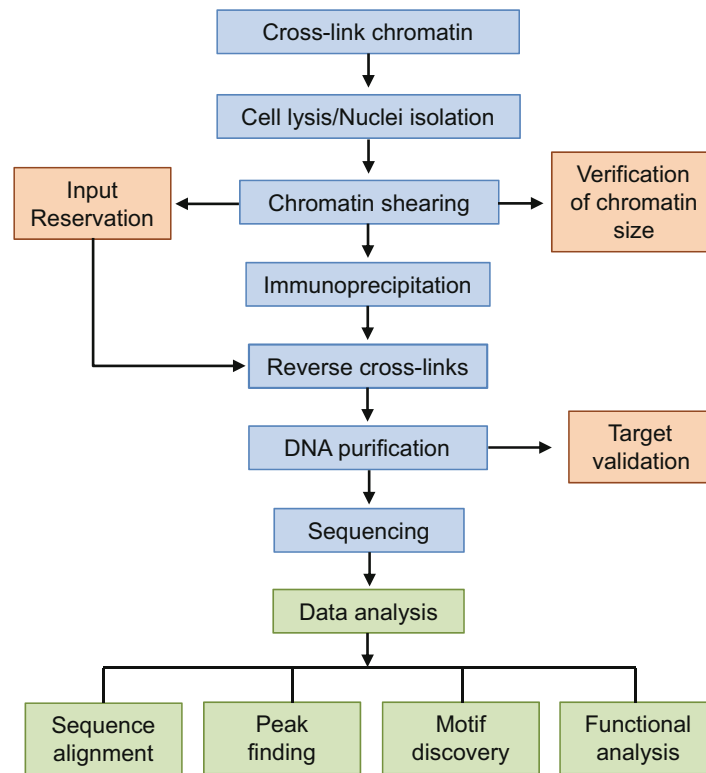


Fig. 1 Schematic view of ChIP-seq procedure. Chromatin is cross-linked with proteins and nuclear proteins are prepared, followed by DNA fragmentation. The DNA fragments associated with a protein of interest are immunoprecipitated with a specific antibody and the cross-links are reversed. The resultant DNA is purified and sequenced. The sequencing data are analyzed to identify the genomic-binding sites of the protein and the regulatory networks

2. Quench solution: 2.5 M glycine.
3. Wash solution: 1× Phosphate-Buffered Saline (PBS), pH 7.4.

2.2 Chromatin Preparation

1. Protease inhibitor cocktail mix: dissolve one cOmplete, Mini, EDTA-free cocktail tablet (Sigma) in 10 ml of cell lysis buffer, homogenization buffer, nuclear lysis buffer, wash solution (1× PBS, pH 7.4), and IP dilution buffer just prior to use. Keep them on ice.
2. Cell lysis buffer: 5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% IGEPAL CA-630 (v/v).
3. Homogenization buffer: 10 mM HEPES, pH 7.6, 25 mM KCl, 1 mM EDTA, pH 8.0, 2 M sucrose, 10% glycerol, 0.15 mM Spermine.
4. Nuclear lysis buffer: 50 mM Tris-Cl, pH 7.6, 10 mM EDTA, pH 8.0, 1% (w/v) SDS.
5. TE buffer: 10 mM Tris-Cl, pH 7.6, 1 mM EDTA, pH 8.0.

6. 5 M NaCl.
7. 10 mg/ml RNase A, DNase and protease-free.
8. 20 mg/ml Proteinase K.
9. IP dilution buffer: 16.7 mM Tris-Cl, pH 7.6, 167 mM NaCl, 1.2 mM EDTA, pH 8.0, 1.1% Triton X-100, 0.01% (w/v) SDS.

2.3 Chromatin Immunoprecipitation

1. Protein A agarose beads, ChIP-grade.
2. 20 mg/ml bovine serum albumin (BSA).
3. Antibodies against protein of interest, ChIP-grade.
4. Normal IgG.
5. Low-salt wash buffer 1: 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0.
6. High-salt wash buffer 2: 20 mM Tris-HCl, pH 7.6, 500 mM NaCl, 0.05% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0.
7. LiCl wash buffer 3: 10 mM Tris-HCl, pH 7.6, 250 mM LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, pH 8.0.

2.4 DNA Elution and Purification

1. DNA elution buffer: 20 mM sodium bicarbonate, 1% (w/v) SDS. Prepare fresh.
2. QIAquick PCR Purification Kit (Qiagen, Cat#28104).

2.5 Bioinformatics Analysis of ChIP-Seq Data

1. Multiple Expectation maximization for Motif Elicitation (MEME) suite: <https://bioconductor.org/packages/release/bioc/html/GOstats.html>.
2. GOstats: <https://bioconductor.org/packages/release/bioc/html/GOstats.html>.
3. KEGG pathway: <http://www.genome.jp/kegg/pathway.html>.

3 Methods

3.1 Tissue Collection and Cross-Linking

1. Dissect tissue and mince immediately with a clean scalpel. Transfer the minced tissue into a 15 ml polypropylene tube containing 7 ml of 1× PBS.
2. Directly add 189 µl of formaldehyde (37%) to a final concentration of 1% formaldehyde. Cross-link the tissue for 10 min on a rotator at room temperature.
3. Quench the cross-linking reaction by adding 350 µl of 2.5 M glycine to a final concentration of 0.125 M glycine. Incubate the tube on a rotator for 5 min at room temperature.

3.2 Chromatin Preparation and Shearing

1. Centrifuge the tube at $2000 \times g$ for 5 min at 4 °C to pellet the tissue. Remove the supernatant.
2. Wash the pellet once with 3 ml of ice-cold 1× PBS wash solution. Centrifuge the tube at $2000 \times g$ for 5 min at 4 °C. Remove the supernatant.
3. Resuspend the pellet in 5 ml of ice-cold cell lysis buffer and transfer it to a prechilled Dounce homogenizer. Use 30 strokes with a tight-fitting pestle (pestle B) to release nuclei.
4. Centrifuge the tube at $2000 \times g$ for 5 min at 4 °C. Remove the supernatant and resuspend the pellet in 6 ml of ice-cold homogenization buffer.
5. Place 3 ml of ice-cold homogenization buffer into a fresh centrifuge tube (Beckman Cat# 331372).
6. Carefully layer 6 ml of nuclear homogenate (from Subheading 3.2, step 4) on the top of the 3 ml of homogenization buffer.
7. To isolate nuclei, centrifuge the tube at $51,000 \times g$ for 1 h at 4 °C in Beckman Coulter SW 41 Ti Swinging-Bucket rotor (*see Note 2*).
8. Remove the supernatant and resuspend the nuclear pellet in 250 μ l of ice-cold nuclear lysis buffer.
9. Transfer the homogenate to a fresh microcentrifuge tube. Rinse the Beckman centrifuge tube with 50 μ l of nuclear lysis buffer and combine to a final volume of 300 μ l.
10. Incubate the lysate on ice for 30 min. At this point, the samples can be stored at –80 °C or proceed to sonication.
11. Sonicate the samples (300 μ l) to shear the chromatin to an average length of 200–500 bp under predetermined conditions. Keep the sample cold during the sonication (*see Note 3*).
12. To assess the chromatin size, take 10 μ l of chromatin and dilute in 10 μ l of TE.
13. Add 1.2 μ l of 5 M of NaCl to a final concentration of 0.3 M of NaCl, and incubate the tube at 65 °C for at least 4 h to reverse crosslinks.
14. Add 10 μ g of RNase and incubate at 37 °C for 30 min.
15. Add 10 μ g of proteinase K and incubate at 45 °C for 30 min.
16. Purify chromatin using QIAquick PCR Purification Kit and run the sample on a 1% agarose gel (Fig. 2). If the agarose gel electrophoresis reveals larger chromatin fragments than desired, perform additional sonication prior to proceeding.
17. Centrifuge the chromatin (from Subheading 3.2, step 11) at $20,000 \times g$ for 10 min at 4 °C to remove cell debris.

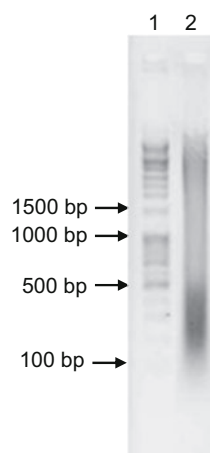


Fig. 2 Verification of chromatin size. Chromatin was sonicated under the conditions of 30 cycles of 10 s on/30 s off using Bioruptor Pico (Diagenode). Sonicated chromatin between 200 and 500 bp in length are shown in lane 2, analyzed by electrophoresis of 1% agarose gel. DNA ladder is shown in lane 1

18. Transfer the supernatant to a fresh tube. If necessary, pool the supernatant and use the same starting material throughout the samples.
19. Dilute the sonicated sample tenfold with IP dilution buffer to reduce SDS concentration below 0.1% to prevent the interference of antibody binding.
20. Aliquot and store the samples at -80°C or proceed to chromatin immunoprecipitation (*see Note 4*).

3.3 Chromatin Immunoprecipitation

1. To prepare blocked protein A (or protein G) agarose beads (*see Note 5*), centrifuge protein A at $1000 \times g$ for 1 min at 4°C and remove the supernatant.
2. Wash the beads twice with 1 ml of IP dilution buffer by centrifuging the beads at $1000 \times g$ for 1 min at 4°C and removing the supernatant.
3. Resuspend the beads with one volume of IP dilution buffer and add 1 mg of BSA per 1 ml of beads.
4. Place the sonicated chromatin (from Subheading 3.2, step 20) into a fresh tube (*see Note 6*) and incubate with $50 \mu\text{l}$ of blocked protein A agarose beads (from Subheading 3.3, step 3) to preclear the chromatin for 2 h on a rotator at 4°C (*see Note 7*).
5. Recover the supernatant by centrifuging at $1000 \times g$ for 1 min at 4°C and transfer to a fresh tube. Save 5–10% of supernatant as an input control.

6. Add 5 μg of antibody and incubate overnight on a rotator at 4 °C (*see Note 8*). For a control, use the same amount of corresponding normal IgG (*see Note 9*).
7. Add 50 μl of blocked protein A agarose beads and incubate for 2 h on a rotator at 4 °C to collect the IP complexes.
8. Centrifuge the tube at $1000 \times g$ for 1 min at 4 °C and carefully remove the supernatant. Avoid direct contact with the beads throughout the washing steps below.
9. Wash the beads twice with 1 ml of ice-cold low-salt wash buffer 1 for 3 min at room temperature.
10. Wash the beads twice with 1 ml of ice-cold high-salt wash buffer 2 for 3 min at room temperature.
11. Wash the beads twice with 1 ml of ice-cold LiCl wash buffer 3 for 3 min at room temperature.
12. Wash the beads once with 1 ml of ice-cold TE for 3 min at room temperature. Centrifuge the tube at $1000 \times g$ for 1 min at 4 °C and discard the supernatant (*see Note 10*).

3.4 DNA Elution and Purification

1. Prepare fresh elution buffer. Elute chromatin with 75 μl of elution buffer by continuous gentle shaking of the tube on a vortexer at setting 2 for 10 min.
2. Centrifuge the tube at $1000 \times g$ for 1 min and transfer the supernatant to a fresh tube.
3. Repeat the elution process three more times and combine the eluates in a total volume of 300 μl .
4. Add 18 μl of 5 M of NaCl to the pooled eluate to a final concentration of 0.3 M of NaCl. For input DNA (from Subheading 3.3, step 5), add 5 M of NaCl to a final concentration of 0.3 M of NaCl.
5. Incubate the sample for 6 h at 65 °C to reverse cross-link.
6. Treat the chromatin with RNase and proteinase K, as described in Subheading 3.2, steps 14–15.
7. Purify chromatin using QIAquick PCR purification kit. Elute the DNA with 50 μl of elution buffer provided in the kit in a low-binding tube (*see Note 11*).
8. Repeat the elution with 50 μl of PCR grade water, followed by additional elution of 100 μl with PCR grade water. Combine the eluates in a total volume of 200 μl .
9. Analyze the enrichment of known targets to verify the efficiency of chromatin IP by qPCR (*see Note 12*).

3.5 Bioinformatics Analysis of ChIP-Seq Data

3.5.1 Alignment and Visualization of ChIP Sequence Reads

Sequence reads obtained in FASTQ format are first aligned to the mouse reference genome mm9 (or later versions) using Bowtie2 [18] to generate BAM (binary SAM) files. BAM files contain information on the exact location of reads in the reference genome, allowing visualization of each read on the UCSC genome browser.

3.5.2 Peak Finding

Peaks are genomic regions that have abundant sequence reads. These peaks are generally interpreted as regions where protein binds to DNA. Model-based analysis of ChIP-seq (MACS or MACS2) [19] is one of the most widely used tools to identify the enriched genomic regions of abundant sequence reads. MACS2 can take in BAM files of two ChIP-seq experiments, for example one file from a pulldown experiment with a specific antibody against a protein of interest, and the second file from a nonspecific pulldown (e.g., IgG) experiment. By comparison of the two files, MACS identifies and generates a list of significantly enriched peaks with their scores. These peak locations are further utilized to run various bioinformatics tools and conduct detailed pathway analyses.

3.5.3 Identification of Transcription Factor Binding Motif

Transcription factors recognize their specific DNA sequences and bind to the motif to regulate their target gene expression. Multiple Expectation maximization for Motif Elicitation (MEME) suite is available at <http://meme-suite.org/>, which allows users to extract motifs in the peak sequences and produce a graphical representation of each nucleotide and the probability of its occurrence in a position-dependent letter-probability matrix [20]. Different likelihood is represented as different sizes of the characters in resulting motifs. MEME also provides the number of occurrences of identified motifs in the list of peak sequences with p -values for statistical significance.

3.5.4 Functional Analysis GOstats

Gene ontology database is a hierarchical annotation of gene products that are assigned to biological terms, called GO terms. Given a set of genes, GOstats (<https://bioconductor.org/packages/release/bioc/html/GOstats.html>) queries the GO database and generates an ordered list of annotated terms that are statistically significant in the given list [21]. Each GO term is assigned with a strength represented as a numerical value called p -value. A GO term with the smallest p -value is the most significant term for the supplied list of genes.

KEGG Pathway

KEGG pathway is a collection of canonical pathways that represent known molecular interactions between gene and gene products working together for a specific biological process. The identified target genes are sprayed on available KEGG pathways (<http://www.genome.jp/kegg/pathway.html>) to produce a list of pathways

along with number of hits in each pathway. This analysis provides better understanding of the biological characteristics of genes that are directly regulated by the transcription factor.

4 Notes

1. *Cross linking conditions*: The duration of cross linking is crucial and an optimal condition should be established depending on the protein of interest [22]. The abundance of protein of interest and the binding affinity of antibody differ from tissue-to-tissue, which can affect the cross linking conditions. However, in general 10 min cross linking with 1% formaldehyde is considered suitable to stabilize transient interactions without significant unspecific cross linking for many cells [23]. It is possible that excess cross linking could mask the epitope recognized by the antibody and influence sonication conditions.
2. *Nuclei isolation*: The presence of cytosolic proteins can interfere with the specific binding of antibody to its targets and reduce the signal-to-noise ratio. Therefore, nuclei isolation is recommended to improve the specificity of antibody binding and enrichment. Additionally, fresh tissues permit efficient isolation of nuclei. When frozen tissues are used, optimization of the nuclei isolation procedure is suggested.
3. *Sonication*: It is essential that an optimal sonication condition needs to be defined empirically to fragment chromatin in size ranging from 200 to 500 bp. The efficiency of chromatin shearing by sonication is dependent on the cell types, amount of chromatin, cross linking time, and sample volume. It is recommended that sonication conditions should be optimized by altering one variable at a time. Keep the samples on ice-cold water during the sonication. We used 30 cycles of 10 s on/30 s off to sonicate 300 μ l of chromatin sample prepared from mouse liver nuclei in an ice-cold water bath using Bioruptor Pico (Diagenode), according to the manufacturer's instructions (Fig. 2).
4. *Fragmented chromatin storage*: Avoid freeze–thaw cycles of sonicated chromatin, as this may reduce the binding of antibody to the chromatin. Store the aliquots of sonicated chromatin at -80°C .
5. *Choice of Protein A or Protein G*: Protein A and Protein G-binding reactivities are dependent on the antibody subtype and the species in which the antibodies have been produced. Refer to informational charts available from manufacturers to select suitable protein-bound beads that capture the antibody.

6. *Quantification of starting materials*: It is important that the amount of starting materials should be similar in each sample. The reading of sonicated samples at OD260 can be used as a reference to roughly estimate DNA concentration, allowing an allocation of a similar amount of starting materials throughout the samples. Make up the volume of each sample equal with IP dilution buffer.
7. *Chromatin Preclear*: Preclear of chromatin can reduce nonspecific binding of antibody.
8. *Antibody*: It is critical to use a high-quality ChIP-grade antibody. The optimal amount of antibody varies considerably based on the sensitivity and specificity of the antibody and the sample conditions such as cell types, the amount of chromatin, and cross-linking time. Therefore, the optimal conditions need to be tested empirically. Excess antibody could increase nonspecific chromatin pulldown and result in increased background signals, whereas insufficient antibody could lead to reduced sensitivity of the antibody [24]. In our previous FoxO1 ChIP-seq study, we used 5 µg of antibody [17].
9. *Controls*: Prepare chromatin immunoprecipitated with the equivalent amount of IgG instead of an antibody and use as a negative control. Additionally, prepare an “input” control that is generally expressed as a percentage.
10. *Background reduction*: If there is high background observed, nonspecific binding of chromatin with antibody/protein A (or protein G) beads can be reduced by additional washes.
11. *Low-binding tubes*: The amount of DNA recovered from chromatin immunoprecipitation is low and the use of low-binding tubes is recommended.
12. *Verification of ChIP efficiency*: Before proceeding to sequencing, verify the enrichment of target sites by quantitative PCR. Design primers that amplify the genomic region that is known to be bound by the protein of interest and perform qPCR with the purified ChIP DNA sample. Results can be analyzed relative to those of IgG control.

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Chapter 17

Mathematical Modeling of Nuclear Trafficking of FOXO Transcription Factors

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Abstract

Nuclear cytoplasmic flux of Foxo transcription factors is paramount in cellular gene regulation. For example, excluding Foxo from skeletal muscle nuclei is necessary to avoid muscle wasting through elevated protein breakdown. Constructing a mathematical model of the signaling process leading to alteration of Foxo nuclear cytoplasm ratio is useful in predicting and interpreting such ratio changes. In this chapter we derive a general mathematical model for nuclear cytoplasmic flux. We apply this model to Foxo flux and take advantage of rapid phosphorylation approximation and conservation conditions to reduce the Foxo flux model. We constrain our model with data from mouse skeletal muscle with applied IGF. This procedure provides an example of what might be called the central approach of mathematical modeling: The cycling of a biological question through mathematical formulation and back to biological interpretation.

Key words Mathematical modeling, Nuclear cytoplasmic flux, Foxo transcription factor, Skeletal muscle

1 Introduction

Mathematical modeling provides a way to translate biological old pathways to results that, upon conversion back in to the biological context, may enlighten that which might otherwise remain hidden. Here, we consider mathematical modeling as applied to nucleo/cytoplasmic movements of Foxo1, a member of the DNA-binding forkhead domain class of transcription factors, in living cells.

The general process follows a cycle: (1) Formulation of a biological question; (2) Articulation of the related biological process as elementary steps; (3) Translation of elementary steps into a mathematical formulation; (4) Analysis and Simulation of the formulated model possibly including time course, parameter variation mimicking experiment, parameter estimation based on experiment, sensitivity to these parameters; (5) Interpretation of mathematical results in the biological context; (6) Repeat, but once the core

model is established going from questions (1) to analysis (4) is often much more efficient.

In the present chapter we detail steps applied to study nucleocytoplasmic translocation of Foxo-1. Details of Foxo background as needed to motivate the question(s) and set up the steps can be found in other chapters.

Specifically for Foxo we can ask, “How does Foxo translocate into and out of the nucleus in response to external signals? “In general the full system of Foxo posttranslational modifications (PTMs; e.g., phosphorylation, methylation, acetylation, ubiquitination) is complex. However, individual modifications can be selectively examined by modulating individual PTM pathways using specific biological or pharmacological ligands, while leaving pathways to other PTMs relatively unperturbed [1–4]. So the focus we take is on a critical component, phosphorylation by Akt at a single effectively lumped site. This works well with the experimental data of nuclear and cytoplasmic fluorescence of the fusion protein of Foxo1 with green fluorescent protein (Foxo1-GFP).

We construct a mathematical model using several applied mathematics techniques such as mass balance, law of mass action, conservation, and volume ratios, which helps to simplify systems [5]. Specifically, we observe reductions due to large cytoplasmic-nuclear volume ratio and the assumption that de/phosphorylation is relatively fast compared to nuclear flux. These reductions are implemented in a reduced model. Furthermore, assumptions are made of negligible protein synthesis or breakdown over a 1–2 h experiment. We can then analyze the system solving it analytically, where possible, or numerically, observe the time course of the nucleocytoplasmic ratio in control and various experimental conditions (e.g., applications of pathway modifiers AKT-I, PI3K-I, IGF-1, etc.), and test the parameters and their sensitivities. Critically the analysis must be interpreted in the biological context and compared to data. Inevitably shortcomings are revealed or new ideas are formed that must be addressed.

We develop these techniques in terms of a general case in Subheading 2.1, and then apply them specifically to Foxo1 in Subheading 2.2.

1.1 Modeling Introduction

Modeling transcription factor action can have multiple facets from posttranslational modifications to transfer between cytosolic and nuclear spaces to DNA-binding affinities. However, the components used to model such a system should be chosen to accommodate comparisons with data that may be available. Model predictions may then dictate other key data that should be obtained in subsequent experiments. A schematic of fluxes and interactions for transcription factors is shown in Fig. 1.

We describe in general three mathematical modeling techniques that apply to the Foxo system with current measurement

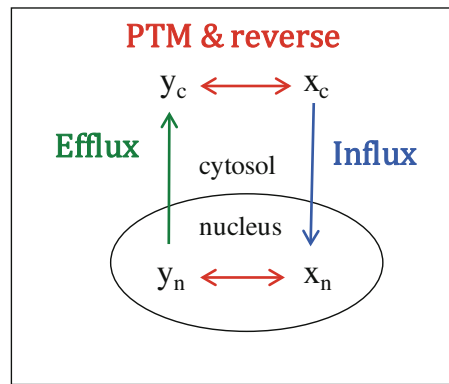


Fig. 1 Core processes in posttranslational modification (PTM)-dependent nucleo-cytoplasmic translocation in a cell

methods. (1) The Law of Mass Action provides a way to track the rate of change of a reacting chemical population including flux balance as a system of ordinary differential equations (ODEs). (2) Using that the relative volume fraction of the nucleus is quite small compared to the cytosolic volume in muscle cells, the system of ODEs can be reduced using a small perturbation approximation. (3) Parameter estimation in comparison with related data generates a verified model.

With a verified model system, we test the system by predicting outcomes of novel experiments. The results of these experiments then indicate the validity or shortcomings of the model, which may then be revised.

1.2 Introduction

Using the general approach, we will create a mathematical model that investigates the nucleo cytoplasmic movements of Foxo1 inside the skeletal muscle fiber when external stimuli are applied.

The transcription factor Foxo1 has specific action in skeletal muscle for regulation of muscle mass. Foxo1 translocation from the cytosol into the nucleus initiates transcription of factors that upregulate proteins that lead to muscle protein degradation and consequent muscle atrophy. The wider scope of this project is to gain insight into which, if any, external stimuli or pharmacological agents could potentially reduce the rate of skeletal muscle atrophy. This insight would help patients who are on bed rest because of aging or a medical ailment because their recovery process would become easier if their muscles were not as weak as they become when immobile.

2 Methods

2.1 Mathematical Modeling

We begin by describing the mathematical methods in the general context of Fig. 1.

2.1.1 Reaction

We use the Michaelis-Menten formalism to write the enzymatic conversion, $x \rightarrow y$, of one molecule (concentration = x) into another molecule (concentration = y), such as that in phosphorylation, by $dx/dt = -k_{\text{cat}} \times E \times x/(x + K_m) = -dy/dt$, where k_{cat} is the catalysis step rate, E is the concentration of enzyme, and K_m is the backward enzyme binding plus catalysis rates divided by the forward enzyme-binding rate, $K_m = (k_b + k_{\text{cat}})/k_f$. If the enzymatic process is well below saturation then we can assume that x is much less than K_m and so $dx/dt \sim -k_{\text{cat}} \times E/K_m \times x = -kp \times x$. And the loss of x leads to the gain in y , so $dy/dt = kp \times x$. Similarly, in the reverse reaction, $y \rightarrow x$, can result in the loss of y , $dy/dt = -ku \times y$ and the gain of x , $dx/dt = ku \times y$. Combining both of these processes yields for x , $dx/dt = -kp \times x + ku \times y$. This reaction can occur both in the cytoplasm (involving xc and yc), and in the nuclei (involving xn and yn) (see **Note 1**).

2.1.2 Translocation

Furthermore, we can think of the movement from one space into another as either diffusion (i.e., without active transport) or actively transported. The rate of change of say xn under diffusion would just be proportional to the difference in concentrations across the boundary, $dxn/dt = D(xc - xn)$. However, an active process assuming sufficient energy would only depend upon the driving side concentration much like the enzymatic process, i.e., $dxn/dt = kI \times xc$ where kI is the influx rate constant.

For molecules larger than about 40 kD nuclear influx and efflux both occur via corresponding nuclear import or nuclear export machinery that requires concentration gradients of co-transported molecules to be present. We assume that the concentrations of these co-transported molecules remain constant during an experiment. We further assume that the transported molecules (xc or yn) are at sufficiently low concentration that their unidirectional flux is proportional to their concentration (i.e., the transport systems are far from saturation by x or y , and are thus operating in their linear range in terms of x or y).

In response to transport, the molecules in the original space are decremented, $dx_c/dt = -kI \times xc$. Similarly for efflux, $dy_c/dt = kE \times yn$ and $dyn/dt = -kE \times yn$. Now this is true if the two spaces are equal in size. However, if they are not of equal size, molecular balance must be taken into account.

2.1.3 Impact of Disparate Volume Fractions

To account for volumes, say V , that might be different we write concentrations x and y as number of molecules divided by volume, $x = X/V$ and $y = Y/V$. For reactions in the same space, using molecules or concentrations is equivalent but for reactions crossing spaces, such as the influx and efflux, we need to write the balance in terms of molecules $dXn/dt = kI \times Xc$. We would like to have the quantities in terms of concentrations however, so we multiply and

divide by (constant nuclear and cytosolic, for example) (*see* **Note 2**) volumes, V_n and V_c , to get.

$$V_n \times d(X_n/V_n)/dt = V_c \times kI \times X_c/V_c$$

or $dxn/dt = kI \times V_c/V_n \times xc$. From this we can see an important point that the rate of change of the number of molecules in one space can be greatly affected by the volume ratio of the spaces. Similarly, the efflux can be represented as $dyc/dt = kE \times V_n/V_c \times yn$.

2.1.4 Combining Processes

Combining the reactions within each space and fluxes between spaces as drawn in Fig. 1 we get

$$dxn/dt = -kpn*xn + kun*yn + kl*Vc/Vn*xc$$

$$dyn/dt = kpn*xn - kun*yn - kE*yn$$

$$dxc/dt = -kpn*xc + kun*yc - kl*xc$$

$$dyc/dt = kpn*xc + kun*yc + kE*Vn/Vc*yn,$$

where **influx**, **efflux**, or **reaction** terms are color coded as in Fig. 1.

2.2 Application of Modeling to Foxo

We now apply the mathematical techniques described in the previous section to Foxo flux between cytosol and nucleus.

2.2.1 Phosphorylation-Dependent Foxo Nucleo-Cytoplasmic Fluxes

To enter the nucleus Foxo1 must be unphosphorylated. Foxo is phosphorylated in response to any of a number of upstream factors that activate Akt [6, 7], and is dephosphorylated by protein phosphatase 2A [8].

Akt is traditionally located in the cytosol. However, phosphorylated Foxo1 is extruded from the nucleus after entering unphosphorylated indicating a phosphorylation mechanism within the nucleus as well as the cytosol. Measuring effective influx and efflux rates of unphosphorylated and phosphorylated Foxo, respectively informs the mechanisms of phosphorylation and flux, but these confounding processes cannot be isolated using current measurement methods. Mathematical methods may prove useful for quantifying these processes if not assisting to disaggregate them. Specifically, what is the change in influx and efflux in the presence of external stimuli?

Figure 2a shows a cartoon of the known processes that occur within the skeletal muscle cell. The application of an external stimulus, such as insulin growth factor 1 (IGF-1), sets off a domino series of events leading up to the phosphorylation of the Forkhead Box transcription factor1 (Foxo-1) in the nucleus and in the

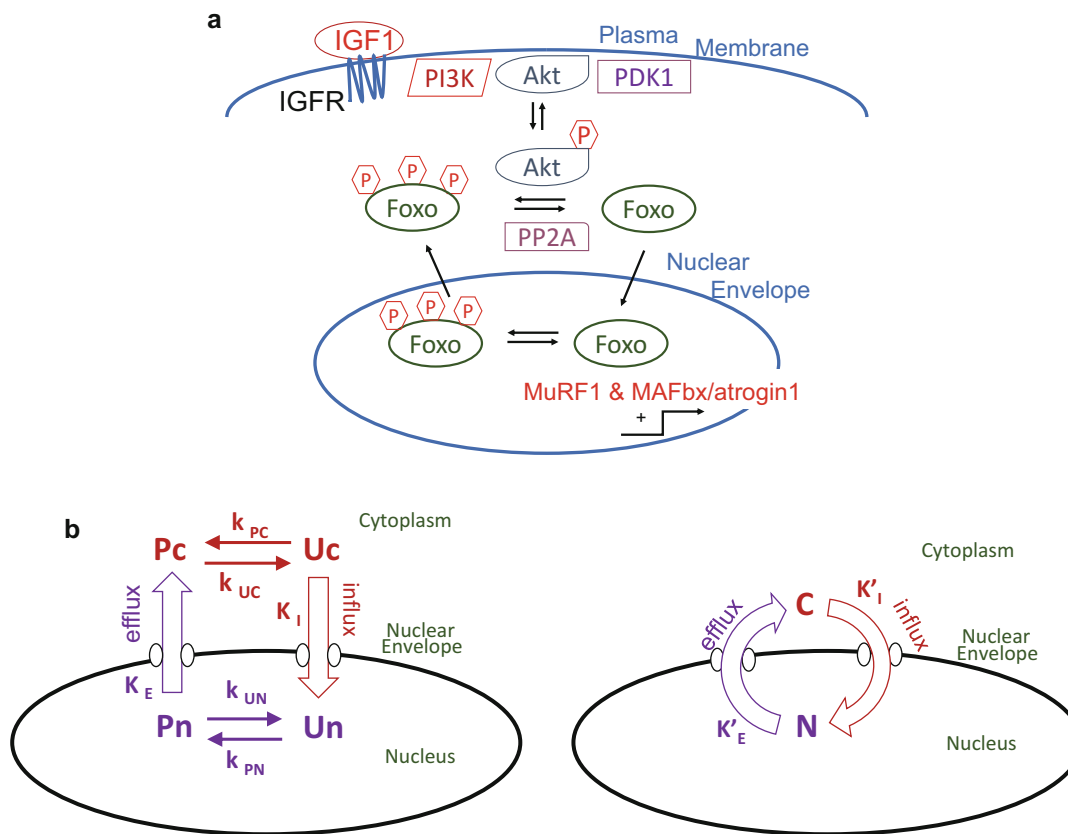


Fig. 2 (a) From Wimmer et al. [10], the cascade of events that occur within the skeletal muscle cell when an external stimuli is injected. Upon IGF-1 being injected, P13K is activated, which in turn activates Akt to phosphorylate Foxo-1 in the cytoplasm beginning the translocation flow of Foxo-1 into and out of the nucleus. A similar pathway to Akt phosphorylates Foxo-1 in the nucleus, but it is not yet known what pathway this is. **(b)** (A) Schematic representing the four-state model of the atrophy process within the skeletal muscle cell. This model describes the flow of Foxo-1 through phosphorylation in the cytoplasm, unphosphorylation in the cytoplasm, phosphorylation in the nucleus, unphosphorylation in the nucleus, and influx and efflux into and out of the nucleus, respectively. **(b)** Effective influx and efflux of Foxo-1 after compressing the four-state model into a two-state model where the influx and efflux rates absorb the phosphorylation rates

cytoplasm. It is important to recognize that only unphosphorylated Foxo-1 can translocate into the nucleus and phosphorylated Foxo-1 can translocate into the cytoplasm. A higher proportion of Foxo-1 in the nucleus leads to a faster rate of skeletal muscle protein degradation due to Foxo activation of transcription of ubiquitin ligases [9].

Because of this fact, we aim to examine the nuclear to cytosolic ratio of Foxo-1 in order to determine if various external stimuli will cause muscle degradation or prevent it.

2.2.2 Four State Model

We create a four-state model, as shown in Fig. 2b(A), to describe the flow between phosphorylated and unphosphorylated states in both the nucleus and cytosol as well as between them. Using the laws of mass action, we translate the diagram into a set of equations to describe this flow, shown in equation set Eq. (1). Looking specifically at the creation of the rate of change for dephosphorylation in the cytoplasm, $\frac{dU_c}{dt}$. Figure 2b(A) shows that dephosphorylated Foxo-1 is gained through the rate of dephosphorylation of Foxo-1 in the cytoplasm from phosphorylated Foxo-1 in the cytoplasm ($k_{Uc}P_c$), phosphorylated Foxo-1 is lost through the rate of phosphorylation in the cytoplasm from dephosphorylated Foxo-1 in the cytoplasm ($-k_{Pc}U_c$), and dephosphorylated Foxo-1 is lost through the rate of influx into the nucleus from dephosphorylated Foxo-1 in the cytoplasm ($-k_I U_c$). Summing these three components results in $\frac{dU_c}{dt}$ in equation set (1). The same method can be applied for the remaining three equations in equation set (1).

$$\begin{aligned}
 \frac{dU_c}{dt} &= -k_{Pc}U_c + k_{Uc}P_c - k_I U_c \\
 \frac{dP_c}{dt} &= k_{Pc}U_c - k_{Uc}P_c + \frac{V_n}{V_c}k_E P_n \\
 \frac{dU_n}{dt} &= -k_{Pn}U_n + k_{Un}P_n + \frac{V_c}{V_n}k_I U_c \\
 \frac{dP_n}{dt} &= k_{Pn}U_n - k_{Un}P_n - k_E P_n
 \end{aligned} \tag{1}$$

where k_{Pn} is the rate constant of nuclear phosphorylation, k_{Un} is the rate constant of nuclear dephosphorylation, k_{Pc} is the rate constant of cytosolic phosphorylation, k_{Uc} is the rate constant of cytosolic dephosphorylation, k_I is the rate constant of Foxo-1 influx into the nucleus, k_E is the rate constant of efflux from the nucleus, V_c is the cytosolic volume, and V_n is the nuclear volume [10].

We can sum concentration of phosphorylated and unphosphorylated Foxo-1 in the cytoplasm and in the nucleus, respectively,

$$\begin{aligned}
 C &= U_c + P_c, \\
 N &= U_n + P_n,
 \end{aligned}$$

where C is the total concentration (phosphorylated plus unphosphorylated) of Foxo-1 in the cytoplasm and N is the total concentration (phosphorylated plus unphosphorylated) of Foxo-1 in the nucleus. Similarly, we can take the time derivatives of C and N to get:

$$\begin{aligned}\frac{dC}{dt} &= \frac{dU_c}{dt} + \frac{dP_c}{dt} = \widehat{k}_E P_n - k_I U_c \\ \frac{dN}{dt} &= \frac{dU_n}{dt} + \frac{dP_n}{dt} = -\frac{V_c}{V_n} \widehat{k}_E P_n + \frac{V_c}{V_n} k_I U_c\end{aligned}$$

where $\widehat{k}_E = \frac{V_n}{V_c} k_E$ is now the fixed scaled efflux.

We assume that the total number of molecules of Foxo1 in a cell (phosphorylated and dephosphorylated; in the nucleus and in the cytoplasm) to be conserved over the time of the experiments (i.e., there is negligible net synthesis or degradation of Foxo1 during an experiment). This can be seen as summing the two previous equations while scaling the nuclear equation by $\varepsilon = \frac{V_n}{V_c}$ to get $\frac{dC}{dt} + \varepsilon \frac{dN}{dt} = 0$ (see **Note 3**).

2.2.3 Rapid De/Phosphorylation Assumption Reduces Four- State Model

Using a rapid phosphorylation and dephosphorylation approximation we can reduce a system like that in Section A4 to a reduced system that is appropriate and more simply analyzed.

Assuming that the phosphorylation and dephosphorylation processes occur much faster than the rates of influx and efflux, we can then consider the rapid equilibration situation [10]. Accordingly, $k_{Pc} U_c = k_{Uc} P_c$ in the cytoplasm, and $k_{Pn} U_n = k_{Un} P_n$ in the nucleus. With these relations and the conservation equations, it is possible to produce the effective efflux (k'_E) and effective influx (k'_I) rate constants shown in Fig. 2b(B). These new rate constants are the previous influx and efflux rate constants, seen in Fig. 2b(A), scaled by rate constant fractions to yield equation set (2).

$$\begin{aligned}\frac{dC}{dt} &= \varepsilon(k'_E N - k'_I C) \\ \frac{dN}{dt} &= -k'_E N + Dk'_I C\end{aligned}\tag{2}$$

Equation set (2) has modified rate constants in order to absorb the phosphorylation and dephosphorylation rate constants in the four-state model. These modified rate constants are described in equation set (3).

$$\begin{aligned}k'_I &= \frac{k_{Uc}}{k_{Uc} + k_{Pc}} k_I \\ k'_E &= \frac{k_{Pn}}{k_{Pn} + k_{Un}} \widehat{k}_E\end{aligned}\tag{3}$$

Equations (3) in represent the effective fluxes in the two-state model in Fig. 2b(B). If we rewrite these equations we can see these as the fraction of dephosphorylation in the cytoplasm or the fraction of phosphorylation in the nucleus, respectively

$$\frac{k'_I}{k_I} = \frac{k_{Uc}}{k_{Uc} + k_{Pc}}$$

$$\frac{k'_E}{\widehat{k}_E} = \frac{k_{Pn}}{k_{Pn} + k_{Un}}$$

When $k'_I \rightarrow k_I$ then the cell is approaching full cytoplasmic dephosphorylation and, similarly, when $k'_E \rightarrow \widehat{k}_E$ the cell is approaching full nuclear phosphorylation.

2.2.4 Solution to the Reduced System

Part of the value to reducing the four-variable system is the ability to readily solve the system and interpret the terms in a biophysical way. Conservation of Foxo-1 in the entire system (nucleus and cytosol) gives us the relationship $C_T = C + \varepsilon N$ where C_T is the initial total amount of Foxo-1 in the Cytosol. We can use the integrating factor method of ordinary differential equations to solve for $N(t)$ from equation set (2).

$$N(t) = C_T \left[\left(\frac{N_0}{C_T} - \frac{k'_I}{k'_E + \varepsilon k'_I} \right) e^{-(k'_E + \varepsilon k'_I)t} + \frac{k'_I}{k'_E + \varepsilon k'_I} \right]$$

Now we can divide $N(t)$ by $C \sim C_T$ and using this conservation equation as well as taking the limit as epsilon goes to 0, both for the condition of negligible nuclear compared to cytoplasmic volume in muscle fibers [8] we obtain the following nuclear to cytosolic ratio of Foxo-1 as a function of time:

$$\frac{N}{C}(t) = \left[\left(\frac{N_0}{C_T} - \frac{k'_I}{k'_E} \right) e^{-(k'_E)t} + \frac{k'_I}{k'_E} \right] \quad (4)$$

where N_0/C_T is the initial nuclear/cytoplasmic ratio of Foxo-1, k'_I/k'_E is the final value of N/C , and k'_E is the rate constant for the change from the initial to final N/C (see **Note 4**).

An important aspect of this more or less straightforward exponential equation is that this model contains all of the constant rates of phosphorylation, influx, and efflux imbedded within composite parameters k'_E and k'_I , which are fitted using the experimental data and can be manipulated to accommodate and even predict results of new experiments.

2.2.5 Obtaining the Experimental Data

To achieve experimental monitoring of Foxo nucleo/cytoplasmic distribution adult mouse skeletal muscle fibers in culture serve as our primary model system for live cell time course confocal imaging. As in numerous previous studies from our lab, we will use a computer controlled high-performance confocal fluorescence microscope system to obtain fluorescence images of multiple cultured muscle fibers in parallel over a time period of 1–2 h, from which we determine the time course of the nuclear/cytoplasmic distribution of Foxo1-GFP. This model muscle fiber system

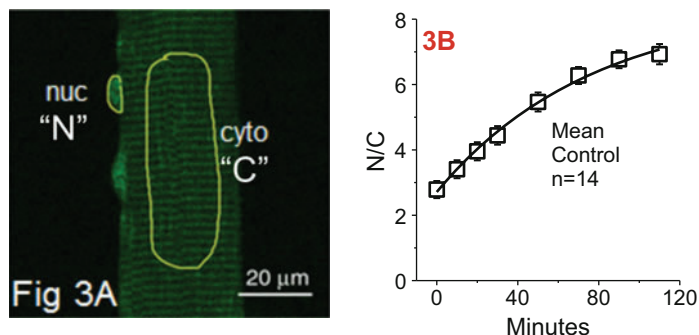


Fig. 3 (a) Fluorescence in skeletal muscle showing Foxo-GFP in the nuclei and cytoplasm. (b) Data and model fit to control N/C time course. Error bars are ± 1 SEM. For symbols lacking error bars, error is less than symbol size. Bar plot bars without error bars are from fits to the average time course

provides an excellent experimental tool for studying molecular mechanisms for transcription factor signaling in live skeletal muscle fibers, as we do here with Foxo1-GFP.

In the image acquisition and analysis of our system, the time course of Foxo1-GFP can be tracked simultaneously in nuclei and cytoplasm in successive images of multiple fibers by using a computer controlled stage on a laser scanning confocal fluorescence microscope. Using a single four-compartment commercial cover glass bottom culture dish, fibers can be monitored in parallel under four different experimental conditions during the same experiment. Following the experiment, mean pixel fluorescence, proportional to mean Foxo1-GFP concentration, is determined in user-defined nuclear (N) and cytoplasmic (C) areas of interest (Fig. 3a) using image analysis software (e.g., Image J).

2.2.6 Model Fit to Experimental Data

Foxo1 is *not* at steady state at the start of the experimental period, but approaches steady state under control conditions. “Control” muscle fibers that were not exposed to any agent exhibit a relatively slow gradual rise in nuclear Foxo1-GFP (Fig. 3b), when the bathing solution is changed from culture medium to fresh growth factor-free recording solution about 30 to 45 min prior to the start of recording. Application of fresh medium causes the nuclear to cytoplasmic concentration ratio (N/C) of Foxo1-GFP in the fibers to increase toward a new quasi-steady state level characteristic of the growth factor-free experimental condition. N/C approaches its new steady state along a single-exponential time course (curve fit to data in Fig. 3b), as predicted by the reduced model. Using the reduced model (above), the single-exponential function fit to the time course of N/C under control conditions (Fig. 3b) yields values for the apparent unidirectional rate constants for Foxo1 nuclear influx and efflux, k'_I and k'_E respectively, under the control conditions.

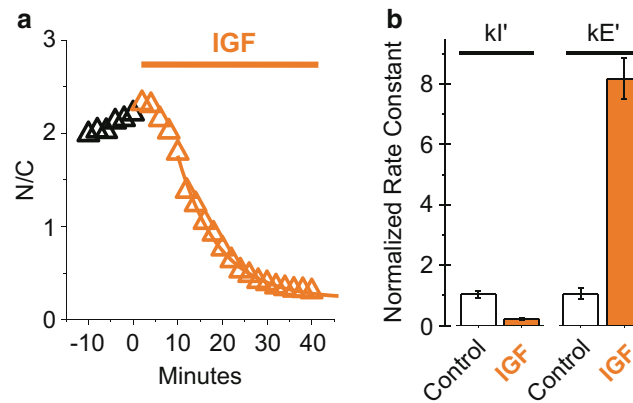


Fig. 4 (a) Application of IGF rapidly decreases N/C (data and model fit). (b) Effective flux parameters estimated from the data. In the presence of IGF the influx decreases while the efflux is significantly increased

IGF1 induces a marked *decrease* in the apparent unidirectional rate constant for Foxo1-GFP nuclear influx. Using Foxo1-GFP expressed in adult FDB muscle fibers, we first showed that IGF1 (100 ng/mL), which activates the PI3K/Akt pathway, caused a marked and rapid decline in N/C (Fig. 4a; recorded using 2 min sampling intervals between images in order to capture the rapid time course of decline of N/C, and presented on an expanded time scale compared to Fig. 3b). The observed rapid and pronounced decrease in nuclear Foxo1-GFP after IGF1 addition (Fig. 4a) could in principal be due to an increased unidirectional nuclear efflux, a decreased unidirectional influx, or some combination of the two. Without a model it is not apparent what the underlying change may be, but by applying the 2-state reduced mathematical model presented here to the measured Foxo1 nuclear cytoplasmic redistribution we first found that the model could closely reproduce the observed N/C time course under control conditions (curve fit to data in Fig. 3b) and during activation of Akt activity by IGF1 (curve fit to declining phase in Fig. 4a). The values for the apparent rate constant k_I' for unidirectional nuclear influx of Foxo1-GFP obtained from the fits were decreased by about an order of magnitude in IGF1 (Fig. 4b, k_I' , left filled bar compared to open bar control), indicative of a similarly *decreased* fractional *de*phosphorylation (i.e., increased phosphorylation) of Foxo1 in the cytoplasm, as expected for IGF1 activation of the IGF1/PI3K/Akt pathway in the cytoplasm.

IGF1 application also dramatically *increases* the apparent rate constant for FOXO1-GFP unidirectional nuclear efflux by over 8 times (Fig. 4b, k_E' , right filled bar compared to right open bar control), indicating an over eightfold increase in fractional phosphorylation of Foxo1 within the nuclei. This novel observation

indicates that IGF1 strongly activates Akt (or some other Foxo1 kinase that promotes Foxo1 nuclear efflux) within the muscle fiber nuclei. This implies that a signaling pathway must be present to link the plasma membrane IGF1 receptor to the intra nuclear activity of Akt (or of some other intra nuclear Foxo phosphorylating enzyme). The decrease in k'_I and the increase in k'_E in response to IGF1 application produce a physiologically coordinated response to IGF1 since both changes have the effect of decreasing the nuclear content of Foxo1, thus decreasing Foxo1 transcriptional effectiveness. Prior to our studies there was little prior information regarding the time courses of Foxo1 nuclear movements, and almost no consideration of the relative roles of nuclear vs. cytoplasmic signaling pathways and mechanisms regulating the nuclear vs. cytoplasmic changes.

3 Notes

1. If enzyme behavior, instead of near its sensitivity (K_m), is near saturating ($x \gg K_m$), then.

$$dx/dt = -k_{cat} \times E,$$

a constant degradation rate. But this approximation can become problematic if x does decay and $x \gg K_m$ becomes inappropriate.

2. If we are concerned about volumes changing in time, then we should write X as $x \times V$, so that.

$$dX/dt = d(x \times V)/dt = dx/dt \times V + x \times dV/dt$$

The additional term, $x \times dV/dt$ scaled by the volume, would have to be subtracted from the right hand side of the equation for dx/dt showing that if the volume is increasing for any level of x this effectively decreases the concentration.

3. $\frac{dC}{dt} + \epsilon \frac{dN}{dt} = 0$ is conservation of Foxo in the cell seen by integrating up the equation to get $C + \epsilon N = \text{Constant}$ Recalling $\epsilon = \frac{V_n}{V_c}$, then $V_c \times C + V_n \times N = \# \text{ Foxo molecules in the cytoplasm and nucleus.}$
4. Notice that in Eq. (2) we could have used that C is not changing very rapidly to hold C constant, then dividing the N equation by C we get the scalar ordinary differential Eq. (4) $\frac{d(N/C)}{dt} = -k'_E(N/C) + k'_I$, which, of course, leads to the same solution in (4).

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Chapter 18

Following Transcriptome to Uncover FOXO Biological Functions

Raymond Liang, Vijay Menon, and Saghi Ghaffari

Abstract

Two and half million red blood cells (RBC) are generated every second in a healthy adult. The process of RBC production known as erythropoiesis requires a meticulous synchrony between signaling processes and the activity of many transcription factor complexes. FOXO3 is a transcription factor that is responsive to signaling processes and essential for the erythroid proliferation and maturation, RBC formation, and lifespan. Here, we discuss how using an integrated computational and experimental systems biology approach new and unanticipated FOXO3 functions in terminal erythropoiesis were uncovered. These combinatory approaches identified FOXO3 as a key regulator of terminal erythropoiesis. As a result, a new mode of FOXO3 participation in erythroid transcription complex formation has been proposed.

Key words Erythropoiesis, Red blood cell (RBC), Enucleation, Mitochondria, Mitophagy, FOXO, FOXO3, Transcriptome, RNA sequencing

1 Introduction

Erythropoiesis is the complex process of RBC generation through stepwise differentiation and lineage restriction of hematopoietic stem cells (HSC). This process begins with HSC differentiation to multipotent progenitor cells, which in turn generate erythroid lineage-committed progenitors. Primitive erythroid progenitors called burst-forming unit erythroid cells (BFU-Es) then further differentiate into colony-forming unit erythroid cells (CFU-Es). Finally, the last stages of RBC maturation involved four progressively differentiated erythroblast stages culminating in reticulocyte formation and maturation into an RBC. Terminal erythroblast maturation is characterized by reduced cell size, chromatin condensation, and synthesis and accumulation of hemoglobin. To completely mature, erythroblasts also need to expulse their nuclei through a process called enucleation. The ensuing reticulocytes remodel their membrane and clear first mitochondria—through autophagy—and then the remaining organelles before transitioning

into fully mature erythrocytes. These processes are coordinated by signals generated by the erythropoietin receptor (EpoR) and other growth factor/cytokine receptors in crosstalk with erythroid transcription factors including GATA-1, KLF-1, TAL-1, and their cofactors as well as FOXO3 [1, 2]. While the function of these transcription factors in early stages of erythroblast differentiation has been known, their participation in terminal erythroblast maturation has just begun to be elucidated. Impaired maturation of erythroblasts and subsequent alterations in kinetics of RBC production (decreased or increased) are associated with different blood disorders ranging from anemia to leukemia [3, 4].

FOXO3 belongs to the FOXO (four-related mammalian FOXO1, 3, 4, 6) family of transcription factors [5, 6]. FOXO proteins are involved in various cellular processes ranging from DNA damage response to metabolism. FOXO3 is regulated by erythropoietin receptor (EpoR) signaling [7–10] and is the main active FOXO in primary erythroid cells [11]. During erythropoiesis the expression and activity of FOXO3 increase with erythroblast maturation in mice and humans supporting FOXO3 involvement in terminal erythropoiesis. In agreement with this, FOXO3 has crucial functions in homeostatic and stress-induced erythropoiesis [11–15]. In primary erythroid cells FOXO3 regulates oxidative stress and metabolic processes and is in cross talk with mTOR signaling [11, 16].

To elucidate the exact function FOXO3 carries out during terminal erythroid maturation, the transcriptome of adult bone marrow WT and *Foxo3*^{−/−} mouse erythroblasts at three distinct and increasing stages of maturation that precede erythroid enucleation were compared using deep sequencing of isolated RNA. Hierarchical clustering of gene expression patterns identified a cluster of genes that are normally upregulated as erythroblasts mature, but fail to become induced in *Foxo3*^{−/−} erythroblasts. Pathway enrichment analysis of these clusters revealed multiple cellular processes linked to enucleation and autophagy. In parallel, functional assays involving imaging flow cytometry, confocal immunofluorescence imaging, and ex vivo maturation also revealed defects in autophagic flux. Similar approaches exposed defective enucleation in *Foxo3*^{−/−} erythroblasts. Subsequently, many genes involved in enucleation and autophagy pathways were validated to be FOXO3 direct targets by qRT-PCR and chromatin immunoprecipitation. In addition, restoration of FOXO3 through viral transduction rescued aberrant expression of autophagy and enucleation genes and rescued the maturation defects of *Foxo3*^{−/−} erythroblasts. Together, these analyses enabled the generation of a testable transcription complex model that suggests FOXO3 interacts with other erythroid transcription factors (i.e., GATA-1, TAL1, and LDB1) to activate erythroid-specific processes during the terminal stages of erythroid maturation.

In this protocol, we describe the methods for RNA-seq processing and subsequent *in silico* analyses that uncovered new and unanticipated functions of FOXO3 that are critical for terminal erythroid maturation.

2 Materials

2.1 Bone Marrow Isolation and FACS Sorting

1. Wild type and *Foxo3*^{-/-} mice aged 9–12 weeks.
2. Sorting Media—IMDM + 2% FBS.
3. Staining Media—PBS + 2% FBS.
4. Cell Counting Media—Water + 3% Acetic Acid.
5. TER119, CD44 antibodies.
6. DAPI.
7. FACS machine.
8. 5 mL FACS tubes, 15 mL Falcons, microcentrifuge tubes, 1 mL syringe, 26 ½ gauge needles.

2.2 RNA Extraction and RNA-seq Processing

1. RNAeasy Micro Plus Kit (Qiagen).
2. Illumina True Seq RNA prep Kit.
3. Hi Seq 2000 platform (Illumina).

2.3 Chromatin Immunoprecipitation

1. Tris–HCl pH 8.0.
2. 0.4% formaldehyde in PBS.
3. NaCl.
4. NP-40.
5. SDS.
6. EDTA.
7. FOXO3a antibody.
8. Magna ChIPTM Protein A + G magnetic beads.
9. Lysis Buffer (10 mM Tris–HCl pH 8.0, 10 mM NaCl, 0.2% NP40).
10. ChIP dilution buffer (20 mM Tris–HCl, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton, 0.01% SDS).
11. Wash Buffer (20 mM Tris–HCl, pH 8.0, 2 mM EDTA, 50 mM NaCl, 1% Triton, 0.1% SDS).
12. DNA isolation kit.

3 Methods

3.1 Bone Marrow Isolation

1. Sacrifice mouse and separate both hind legs from skin of mouse.
2. Clear off muscle from tibia and femur and proceed to flushing.
3. Fill syringe with sorting media and insert into ends of tibia or femur and dispense media to flush out bone marrow cells into 15 mL falcon tube containing 3 mL of sorting media. Repeat until bones appear clear of all cells.
4. Pool bone marrow cells from up to 3 mice in one tube.

3.2 FACS Sorting (see Note 1)

1. Filter isolated bone marrow cells through 35 μ m nylon mesh cap FACS tube.
2. Pellet cells by centrifuging at $200 \times g$ and resuspend in 1 mL of cell staining media.
3. Dilute 10 μ L of BM cells into 990 μ L of cell counting media to lyse RBCs and count on hemocytometer.
4. Pellet cells again and resuspend to 150×10^6 cells/mL—subsequent step will consider antibody staining in 1 mL of staining buffer with 150×10^6 nucleated cells.
5. Add 40 μ L of TER119 and CD44 fluorescence-conjugated antibodies and mix thoroughly by pipetting. (Additionally, prepare unstained and single stained controls using 0.5×10^6 cells in 100 μ L of staining buffer).
6. Incubate at 4 °C in the dark for 15 min.
7. Wash off excess antibody by adding an additional 1 mL of staining buffer and pellet cells at 200 g for 5 min.
8. Aspirate the supernatant and resuspend in 1 mL of sorting media containing 1 μ g/mL of DAPI to distinguish viable cells (DAPI negative).
9. Further dilute cells to a concentration of 15×10^6 cells/mL (nucleated) and filter into the required number of 35 μ m nylon mesh cap FACS tubes.
10. Prepare 3 FACS tubes containing 1 mL of sorting media each as collection tubes for the three erythroblast populations to be sorted.
11. Bring cells to FACS machine and run control samples to determine proper gating of subsequent positive and negative populations.
12. Gate out debris, non-singlet, and dead cells based on forward scatter, side scatter, and DAPI fluorescence.
13. Mouse erythroblasts are specifically detected by the TER119 antibody. The progressive stages of erythroblast maturation

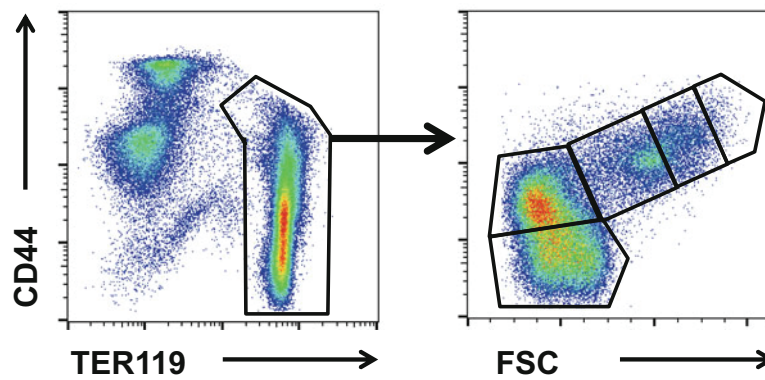


Fig. 1 Gating strategy for flow cytometry: pro-, basophilic, polychromatophilic and orthochromatic erythroblasts from WT and *Foxo3*^{-/-} BM were FACS-sorted according to their forward scatter and cell surface expression of CD44 and TER119

can be resolved by CD44 expression and FSC parameters that decrease with maturation (Fig. 1).

14. Sort proerythroblasts (Gate 1), basophilic erythroblasts, polychromatic erythroblasts (Gate 2), and orthochromatic erythroblasts (Gate 3) into collection tubes until all BM cells are sorted (*see Note 2*).

3.3 RNA Extraction

1. Transfer cells to 5 mL conical tubes or multiple microcentrifuge tubes and pellet at $200 \times g$ for 5 min (*see Note 3*).
2. Wash cells once with 1 mL of staining buffer (consolidate cells into a single tube if multiple tubes were used), pellet cells again at 200 g for 5 min, and carefully aspirate all supernatant.
3. Add 300 μ L RLT buffer from the RNeasy kit with 2-mercaptoethanol added at the concentration noted to the cell pellet.
4. Fully lyse cells by pipetting and mixing further by vortexing for up to a minute.
5. Lysed cells in RLT buffer can be stored at -80°C for extended periods of time (our lab has tested samples stored up to 6 months with no RNA degradation).
6. From here, follow the manufacturer's instructions to isolate RNA.
7. Analyze RNA quality and quantity using bioanalyzer to determine if RNA quality and quantity is sufficient for cDNA synthesis, library prep, and subsequent sequencing, dependent on type of next-gen sequencer being used.
8. Following RNA extraction, cDNA synthesis is carried out using the SuperScript IITM Reverse Transcriptase from Invitrogen.

qRT-PCR is carried out using SYBR Premix Ex Taq™ II (Tli RNase H Plus).

For mRNAseq libraries, RNA is extracted as mentioned above and mRNAseq libraries are prepared using the Illumina True Seq RNA prep kit. A Hi Seq™ 2000 platform (Illumina) is used to sequence the samples in parallel lanes in order to obtain around 10^7 single end 100 bp reads per sample.

3.4 RNA-Seq Processing

1. Map RNA-seq reads to mouse genome with TopHat or preferred method.
2. Process mapped reads to calculate fragments per kilo-base per million reads (FPKM) or other method of normalization to allow comparison between transcriptome profiles of WT and *Foxo3*^{-/-} cells.
3. Test for differentially expressed genes between WT and *Foxo3*^{-/-} populations using Cufflinks or preferred method.
4. For downstream routes of analysis only genes with a FPKM >1 and at least 1.8 fold difference between WT and *Foxo3*^{-/-} samples are good starting points.
5. The total number of reads would be in the range of 30–50 million for the WT and around 10–50 million for the *Foxo3*^{-/-} samples.

3.5 Identifying FOXO3 Regulated Cell Processes with RNAseq Data

1. If distinct populations of WT and *Foxo3*^{-/-} cells are used as with erythroblast populations, the gene expression data can be clustered (Otherwise genes will either cluster as upregulated or downregulated when comparing just one population of WT and *Foxo3*^{-/-} cells). Using programs such as Cluster 3.0 or GENE-E, perform k-means clustering by gene to reveal how gene expression patterns change from population to population and how it differs in *Foxo3*^{-/-} populations.
2. Perform pathway enrichment analysis of differentially expressed genes or clusters of genes with programs such as FuncAssociate, ENRICH, or others. Such analysis should reveal multiple statistically significant pathways.
3. Additionally, chromatin immunoprecipitation enrichment analysis (ChEA) can reveal other transcription factors that regulate similar sets of genes based on published ChIP-seq datasets [17]. The known function of these transcription factors can be correlated with pathways identified from the previous step as a starting point for functional analysis.

3.6 Chromatin Immunoprecipitation

1. Chromatin immunoprecipitation or ChIP is carried out in TER119+ cells derived from WT total bone marrow cells. Following FACS, the cells are fixed in 0.4% formaldehyde/1XPBS and subsequently lysed (*see Note 4*).

2. The lysate is then sonicated using a diagenode bioruptor sonicator for 30 cycles of 30 s on/30 s off at 4 °C (to be optimized according to the sonicator available). This is then further diluted in ChIP dilution buffer. The diluted cell lysate is incubated overnight at 4 °C in anti-FOXO3a antibody and Magna ChIPTM Protein A + G magnetic beads.
3. The beads are washed in and the bound complexes are reverse cross-linked after which DNA isolation and qPCR analysis is carried out.
4. As a negative control, TER119+ cells derived from *Foxo3*^{-/-} bone marrow are used.
5. The putative binding sites are identified using MatInspector from Genomatix.

3.7 Validating FOXO3 Regulated Cell Processes with Functional Analysis

1. Validate differential expression of key genes within pathway by qRT-PCR comparing WT and *Foxo3*^{-/-} cells of interest.
2. Dependent on the pathway being deregulated in the absence of FOXO3, specific functional assays should be performed to validate defects in the identified pathways (e.g., we identified a failure to upregulate autophagy genes in *Foxo3*^{-/-} erythroblasts and determined defective autophagy with multiple methods of measuring autophagic flux).
3. Ectopic expression of FOXO3 in *Foxo3*^{-/-} cell type should restore expression of deregulated genes to WT levels and rescue functional defects of *Foxo3*^{-/-} cells. To confirm if various pathways are also directly contributing to defect seen in *Foxo3*^{-/-} cells, aberrant expression of key genes in the defective pathway should also be ectopically expressed and analyzed for rescue. Figure 2 illustrates the strategy for identifying FOXO3 functions in terminal erythropoiesis (see Note 5).

4 Notes

1. This protocol can apply to other cell populations of blood and would require different combinations of cell surface markers to sort for the populations of interest.
2. Due to the nature of sorted erythroblasts being sequential in maturation subsequent analysis did not require biological replicates. However for comparisons between WT and *Foxo3*^{-/-} cells of other populations at least three replicates are most likely required to obtain statistical significance in finding differential expression.
3. At least 300,000 cells per population are required to comfortably produce enough RNA for subsequent steps. If cells will be used for other types of sequencing (e.g., single-cell RNAseq) or

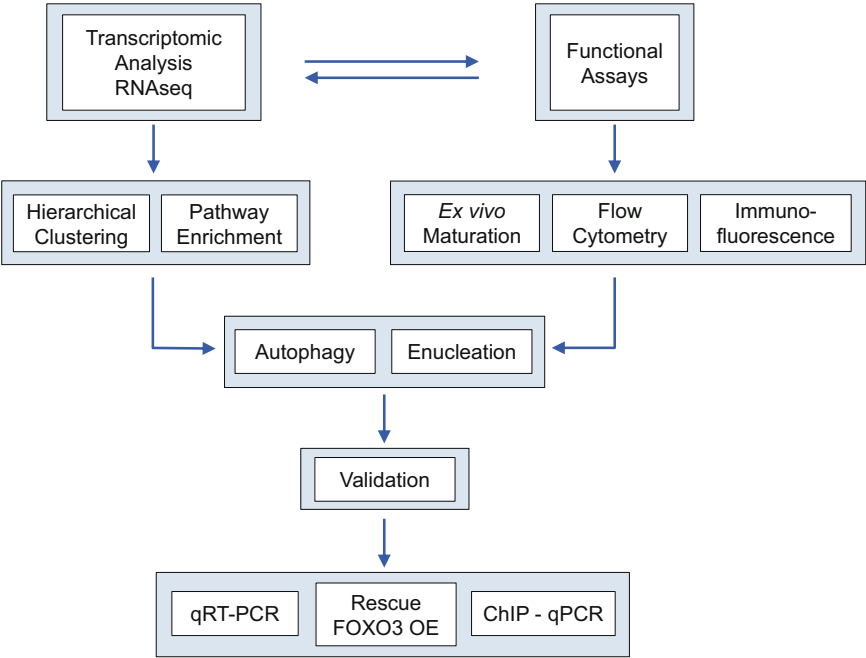


Fig. 2 Strategy for identifying FOXO3 functions in terminal erythropoiesis

validation by qRT-PCR much fewer amounts of cells are required to produce usable amounts of cDNA. Our lab has been able to robustly perform qPCR with 5000–10,000 cells.

4. To confirm direct regulation of specific target genes, chromatin immunoprecipitation (ChIP) should be performed with FOXO3-specific antibody (see protocol).
5. As each individual study can be highly specific and different from each other, this protocol provides just a guideline to how to analyze and identify FOXO3 regulated pathways in blood cells. Different types of studies most likely will involve specialized toolsets and would require additional types of analysis to adapt to the specific study.

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Part IV

Animal Models



Chapter 19

Hydra as Model to Determine the Role of FOXO in Longevity

Thomas C. G. Bosch

Abstract

In non-senescent *Hydra*, continuously high activity of transcription factor FOXO contributes to continuous stem cell proliferation. Here, we describe how genetic manipulation of Hydra polyps using embryo-microinjection allows uncovering the role of FOXO in coordinating both stem cell proliferation and antimicrobial peptide0073, effector molecules of the innate immune system, and regulators of the microbiome.

Key words Hydra, Stem cells, Aging, Microbiome, FOXO, Embryo microinjection, Reporter gene constructs, Antimicrobial peptides (AMPs)

1 Introduction

Numerous studies show that ageing is not a series of stochastic defects but a directed biological program that has a conserved genetic background [1] and is dependent on both genetic and environmental factors [1, 2]. While most ageing research has been done in short-living models, some animals show no ageing phenotype and are considered non-senescent. The freshwater polyp Hydra has an estimated life span that exceeds 1400 years [3]. Due to three continuously proliferating stem cell lineages that give rise to about 20 cell types [4], the animals are able to continuously reproduce asexually via budding. A single homolog of the longevity gene *foxO* is expressed in all three stem cell lineages in Hydra [5]. Functional analyses uncovered FOXO's role in both stem cell regulation and interaction with the microbiome [6]. In *Hydra*, the microbiome is selectively assembled by a species-specific combination of AMPs which are predominantly expressed in epithelial cells [7, 8]. FOXO-deficient *Hydra* polyps show in addition to defects in stem cell maintenance a severe change of the immune status and drastically altered expression of AMPs [6]. Remarkably, deficiency in FOXO signaling in *Hydra* not only leads to dysregulation of antimicrobial peptide expression. FOXO loss-of-function polyps

also are more susceptible to colonization of foreign bacteria and impaired in selection for bacteria resembling the native microbiome [9, 10]. These findings reveal a key role of FOXO signaling in the communication between host and microbiota and embed the evolutionary conserved longevity factor FOXO into the holobiont concept.

2 Materials

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|-------------------------------------|---|
| 2.1 Organisms and Cell Lines | <i>Hydra</i> strains: <i>Hydra vulgaris</i> strain AEP.
Feeding strain: <i>Artemia salina</i> . |
| 2.2 Media | <i>Artemia</i> medium.
31.8 g sea salt/l Millipore H ₂ O.
<i>Hydra</i> medium.
0.29 mM CaCl × 2H ₂ O, 0.33 mM MgSO ₄ × 7 H ₂ O, 0.5 mM NaHCO ₃ ,
0.08 mM K ₂ CO ₃ . |
| 2.3 Expression Vector | The expression construct used is plasmid pUC19, which contains 1386 bp of the <i>H. vulgaris</i> actin 5 flanking region, transcription start site, native initiator codon, and the first 10 amino acids of <i>H. vulgaris</i> actin. EGFP cDNA is inserted into this plasmid (see ref. 12). In addition, the EGFP reporter gene is flanked by 3 genomic region of the <i>H. vulgaris</i> actin gene, including the termination polyadenylation signal. |
| 2.4 Imaging Technologies | In vivo observations are documented using standard digital camera. |

3 Methods

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| 3.1 Cultivation of <i>Artemia salina</i> | <i>Artemia salina</i> serve as food for all <i>Hydra</i> species. For hatching, eggs are incubated overnight at 32 °C in <i>Artemia</i> medium under permanent air supply. For feeding of <i>Hydra</i> polyps, nauplii are collected, washed with water to remove salts, and resuspended in hydra medium. |
| 3.2 Cultivation of <i>Hydra</i> | All <i>Hydra</i> strains are cultured at 18 °C in <i>Hydra</i> medium with a 12 h day night cycle according to standard methods [11]. Animals are fed three to five times per week. |
| 3.3 Generation of Transgenic <i>Hydra vulgaris</i> Strain AEP | Stable knockdown lines for FOXO can be achieved by generating transgenic polyps expressing a FOXO hairpin construct fused to eGFP under control of an actin promoter. The vectors are injected |

into *Hydra vulgaris* (AEP) embryos as described [12]. By selecting for eGFP-expression, mass cultures with epithelial expression of the according construct can be generated. Clonal animals without any eGFP-expressing cells serve as control for the corresponding line [13].

3.3.1 Phylogenetic Analyses of FOXO in Hydra for use in Reporter Gene Constructs

Phylogenetic analysis of FOXO is based on the predicted amino acid sequence of the forkhead domain. The analysis involves 31 amino acid sequences aligned using ClustalW [14] with the following multiple alignment parameters: Gap Opening Penalty: 10.00, Gap Extension Penalty: 0.20. The most parsimonious tree is found when using MEGA 4 [15] and Close-Neighbor-Interchange algorithm in which the initial trees are acquired with random addition of sequences (10 replicates). All ambiguous positions must be removed for each sequence pair giving a total of 104 positions in the final dataset. The branch lengths are calculated using the average pathway method and are in the units of changes over the whole sequence. Maximum Parsimony and Maximum Likelihood bootstrap values are calculated based on 1000 replicates. Maximum Likelihood bootstrap is implemented using MEGA 4 [15] (with JTT model of amino acid change). Bayesian posterior probabilities are calculated using MrBayes version 3.1.2 [16, 17] with two independent runs of 3,500,000 generations each, sampled every 1000 generations with four chains.

3.3.2 Generation of Expression Constructs for Overexpression of foxO

To achieve an overexpression of *foxO*, a construct containing the coding region of *foxO* cloned into LigAF expression vector (Subheading 2.3) in front of *eGFP* is generated. The *foxO* sequence is flanked by a *SbfI* and *PacI* cutting site for site-directed mutagenesis. The *foxO* coding part is amplified from cDNA with primers containing the *SbfI* and *PacI* cutting site, digested with the corresponding enzymes and ligated into LigAF vector prior to electroporation into *E. coli*. Afterward, the uptake of the plasmid by bacteria has to be checked by colony check PCR. A selected clone is propagated in an LB liquid culture and submitted to plasmid mini-preparation. Subsequently, the correctness of the construct must be proven by sequencing. Prior to the injection into *Hydra* embryos, a plasmid midi-preparation has to be performed.

3.3.3 Generation of Expression Construct for Downregulation of foxO

For stable knockdown of *foxO*, a hairpin construct containing a *sense* and *antisense* sequence from *foxO*, separated by a linker sequence, cloned into LigAF expression vector behind *eGFP*, is generated (Subheading 2.3). The *foxO* hairpin is flanked by an *AsiSI* and *EcoRI* cutting site for site-directed mutagenesis. After transcription, this sequence will form a hairpin structure that binds

to the *foxO* transcript, leading to degradation of the hybrid by the RNAi machinery.

3.3.4 Selecting the Antisense Sequence

The 500 bp *foxO antisense* sequence from a highly variable part of the gene (Subheading 3.3.1) is amplified from cDNA together with a 300 bp linker using primers containing the *AsiSI* and *SpeI* cutting site. The corresponding *sense* sequence is amplified using primers containing the *SpeI* and *EcoRI* cutting site. Afterward, both products are digested with *SpeI* and ligated.

3.3.5 Ligation

The ligation product is subcloned into pGEM[®]-T vector, digested with *AsiSI* and *EcoRI*, and ligated into LigAF vector prior to electroporation into *E. coli*.

3.3.6 Quality Check

Afterward, the uptake of the plasmid by bacteria must be checked by colony check PCR. A selected clone is propagated in an LB liquid culture and submitted to plasmid miniprep. Subsequently, the correctness of the construct must be proven by sequencing. Prior to the injection into *Hydra* embryos, a plasmid midi-preparation has to be performed.

3.3.7 Hydra Embryo Microinjection

Transgenic *H. vulgaris* strain AEP are generated at the University of Kiel Transgenic *Hydra* Facility (<http://www.unikiel.de/zoologie/bosch/transgenic.htm>) as described by Wittlieb et al. [12]. A plasmid concentration of 1 µg/µL is used and 1% Rhodamine B Isothiocyanate-Dextran R9379 should be added as a tracer. The solution is injected into *Hydra* embryos at the 2 to 4 cell developmental stage.

3.4 Maceration of Hydra Cells for Measuring Tissue Growth

Transgenic and control polyps are placed in a tube and the medium is replaced by 50 µL maceration solution per polyp as described [18]. Tubes are incubated in a water bath at 32 °C for 30 min. The cells are dissociated by flipping the tube and fixed by the addition of an equal volume of an 8% paraformaldehyde solution. 50 µL of the suspension are placed onto gelatine-covered glass-slides with a droplet of a 1% Tween20 solution. Slides are air-dried overnight and embedded with Glycerol/PBS [1, 9] for microscopic analysis.

3.5 Transcriptome Analyses for Discovering Changes in Gene Expression

For transcriptome sequencing, transgenic lines are cocultured in shared HM with according controls for at least 4 weeks in five independent replicates. After sampling, animals are frozen in TRIzol at −20 °C until RNA-extraction with the PureLink RNA Mini Kit according to the manufacturer's protocol. Additionally, an optional on-column DNA digestion can be performed. The RNA is eluted in 30 µL and checked for sufficient quality. If necessary, the RNA is purified using 1-butanol and diethyl ether [19] and frozen at −80 °C until further use. Total RNA sequencing with previous ribosomal depletion is performed for 20 libraries on the Illumina HiSeq2500 v4 platform, with 125 bp paired-end sequencing of

12 libraries per lane. This usually results in 30–40 million reads per sample after quality control. Adapters are trimmed using PRINSEQ-lite 0.20.4 [20] and Cutadapt 1.13 [21]. Subsequently, mapping against the *Hydra vulgaris* (AEP) transcriptome [5] is performed using Bowtie2 2.2.9 [22]. Differentially expressed contigs are identified with the DESeq2 1.16.1 package [23].

3.6 Generation of Germ-Free Animals

Germ-free (GF) polyps can be obtained by treating animals for 2 weeks with an antibiotic cocktail containing rifampicin, ampicillin, streptomycin, and neomycin in final concentrations of 50 µg/mL each and spectinomycin at 60 µg/mL, as previously described [24]. Since rifampicin stock is dissolved in DMSO, control polyps are incubated in the corresponding final DMSO concentration (0.1%) for the same period of time. Antibiotic solution and control polyp medium are replaced every 48 h. After 17 days in the antibiotic cocktail, the animals are transferred to sterile hydra medium that is further replaced every 48 hours until they are used. The GF status of the polyps should be tested twice per week, starting at day 19–20, by plating an antibiotic-treated macerated polyp from every replicate produced on R2A agar, which supports growth of hydra microbiota. Absence of colonies following 3 days of incubation at 18 °C shows the GF status. To further confirm the absence of microbes in the tissue, macerates of GF polyp (*see* Subheading 3.5) are analyzed by 16S rDNA amplification, using universal 16S rRNA primers Eub-27F and Eub-1492R.

3.7 Microbial Recolonization Experiments of Germ-Free and Transgenic Polyps

GF animals can be monocolonized by incubating the polyps with a pure culture of each of the main colonizers *Curvibacter* AEP 1.3, *Duganella* C 1.2, *Undibacterium* C 1.1, *Acidovorax* AEP 1.4, and *Pelomonas* AEP 2.2. These bacteria are cultured from existing isolate stocks in R2A medium at 18 °C for 3 days. Approximately 5×10^5 cells (based on OD600 quantification) of each bacterial strain are added separately to 50 mL sterile hydra medium containing 20–30 GF polyps on day 20. Likewise, GF polyps are recolonized with a mixture of the five main colonizers in equal proportions (5×10^5 cells /strain). After 3 days of incubation, the polyps are washed with and transferred to sterile hydra medium. Conventionalized animals can be obtained by incubating GF polyps with tissue homogenates of control animals (one control polyp per one GF polyp) in 50 mL sterile hydra medium. The conventionalized polyps are washed and transferred to sterile hydra medium 24 h. after colonization. This medium is replaced every 48 h. until the animals are used. Recolonizations can be verified by plating tissue homogenates on R2A agar and by 16S rDNA amplification and sequencing.

3.8 DNA Extraction and 16S rRNA Profiling to Study the Microbiome

Genomic DNA was extracted from individual polyps with the DNeasy Blood & Tissue Kit (Qiagen) as described in the manufacturer's protocol. Elution was performed in 50 μ L. Extracted DNA was stored at -20°C until sequencing. Prior to sequencing the variable regions 1 and 2 (V1 V2) of the bacterial 16S rRNA genes are amplified according to the previously established protocol using the primer 27F and 338R [25]. For bacterial 16S rRNA profiling paired-end sequencing of 2×300 bp is performed on the Illumina MiSeq platform. Chimeric sequences are identified with ChimeraSlayer [26] and verified manually before removal from the data set.

3.8.1 Analysis of Bacterial Communities

OTU picking is performed using the pick_open_reference_otus.py protocol with at least 97% identity per OTU and classification was conducted using the ribosomal database project (RDP) classifier. OTUs with less than 50 reads are removed from the data set to avoid false positive OTUs that may originate from sequencing errors [27]. The number of reads is normalized to the lowest number of reads in the dataset. Alpha diversity is calculated using the Chao1 metric implemented in Qiime using ten replicates of rarefaction per sample. Beta diversity is determined in a PCoA by 100 jackknifed replicates using binary Pearson distances. Bacterial groups specifically associated with control or FOXO[−] polyps are identified by LEfSe [28]. LEfSe couples robust tests for measuring statistical significance (Kruskal–Wallis test) with quantitative tests for biological consistency (Wilcoxon–rank sum test). The differentially abundant and biologically relevant bacterial groups are ranked by effect size after undergoing linear discriminant analysis. All P values were corrected for multiple hypotheses testing using Benjamini and Hochberg's false discovery rate correction (*q*-value). A *q*-value of 0.25, an effect size threshold of 3.0 (on a log₁₀ scale), and a mean abundance of at least 0.1% in one of the treatments were used for all bacterial groups discussed in this study.

3.9 Statistics

Statistical analysis is performed using two-tailed Student's-*t*-test or Mann-Whitney-*U*-test if applicable. If multiple testing was performed, *p*-values were adjusted using the Benjamini-Hochberg correction [29].

4 Notes

One important and often underestimated issue in making transgenic polyps (*see* Subheading 3.3.4) is the fact that it needs a mass culture of several thousand female *Hydra vulgaris* AEP before starting with embryo microinjection. We inject usually 60–100 fertilized eggs per day to get statistically significant results. For having such a large number of mature eggs available, a careful culture system including the removal of unwanted male polyps

must be established. Within EGFP-positive cuticle stage embryos, expression usually is intense and appears nonuniform within patches of blastomeres. These “patchy” animals can be used directly for tracing cells during development or regeneration. To obtain fully transgenic tissue, mosaic founder polyps showing stable eGFP expression in a group of stem cells are expanded further by clonal propagation for up to 3–4 weeks until buds are developing which contain a fully transgenic cell lineage. In our experience, upon hatching, about 10% polyps stably express EGFP in either the endodermal or ectodermal epithelial stem cells. Moreover, 3% polyps express EGFP permanently in the interstitial cell lineage.

Acknowledgment

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Chapter 20

Genetic Ablation of FOXO in Mice to Investigate Its Physiological Role

Hwanhee Oh and Jihye Paik

Abstract

Recent reports emphasized the role of FOXO family of transcription factors in nervous system homeostasis. Most studies employed primary neuronal cultures, established animal models for neuropathology, or invertebrate models. Demonstration of the normal and pathophysiological function of mammalian FOXO under complex in vivo conditions requires genetic study. Therefore, the conditional knockout mouse is an invaluable platform. Here, we describe the methods of establishing and analyzing nervous system-specific ablation of FOXO isoforms in mice. This chapter offers a detailed method to validate the deletion of *Foxo* genes in vivo and to study its role in the nervous system. Investigation of FOXO function by using the mouse system may advance our understanding of nervous system aging as well as neurodegenerative diseases.

Key words FOXO, Nervous system, Brain, Neurodegeneration, Aging, Immunohistochemistry, Mouse model

1 Introduction

Mammalian FOXO transcription factors have become critical regulators of cell fate and function in the nervous system. All four mammalian homologs FOXO1, 3, 4, and 6 are implicated in neural function [1, 2]. In addition, there have been studies on DAF-16, the *Caenorhabditis elegans* ortholog of mammalian FOXO in neuronal function, such as learning and memory [3, 4], axonal degeneration and regeneration, and neurite outgrowth [5]. Collectively, these raised the question of whether the regulatory function of DAF-16 is conserved in mammalian nervous system as well.

Recently, we investigated the function of FOXO using nervous system specific conditional knockout mice and uncovered that FOXO protects against axonal degeneration during chronological aging [6]. Our study further demonstrated that depletion of neuronal FOXO 1, 3, and 4 is sufficient to initiate axonal degeneration and associated reactive gliosis. Most importantly, FOXO deficiency

advanced common mammalian aging phenotypes in the brain. To date there have been no genetic proof of concept studies addressing the functional role of FOXO in neurodegenerative conditions. Therefore, it remains to be determined whether FOXO-dependent neuroprotective effect has any role in neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis. Investigation of FOXO function by using the mouse system may advance our understanding of nervous system aging as well as neurodegenerative diseases.

In this chapter, we describe the materials and methods for how to analyze conditional deletion of *Foxo* in mice by genotyping, measuring the expression of specific FOXO isoforms at the mRNA and protein levels, and histological assessment, focusing on the brain. Furthermore, targeting the FOXO recombination by means of breeding to *Nestin-cre* or *hGFAP-cre* drivers effectively ablate the expression in the spinal cord as well. Animals with spinal cord deletion of FOXO expressions exhibit motor dysfunction due to extensive thoracic cord axonal degeneration [6]. We describe an easy and reliable method to determine the progression of axonal degeneration due to FOXO loss by simple hind leg clasping behavior.

2 Materials

2.1 Animal and Genotyping

1. Foxo1, 3, and 4 (Foxo1/3/4^{L/L}) mice have been described [1, 7]. Foxo1^{L/L} and Foxo3^{L/L} mice are now available from The Jackson Laboratory (STOCK 024756-Foxo1^{tm1Rdp/J}, 024668-Foxo3^{tm1Rdp/J}, and 024757-Foxo4^{tm1Rdp/J}). Nestin-cre (#003771), hGFAP-cre (#004600), and Synapsin1-cre (#003966) are also available.
2. Tail biopsies (~1 mm) from mice.
3. Alkaline tail lysis buffer: 17.5 μ L of 10 N NaOH and 14 μ L of 0.5 M EDTA (pH 8.0) in 10 mL dH₂O.
4. Neutralization buffer: 40 mM Tris-HCl (pH 4.5).
5. Genotyping PCR mixture: 0.5 μ L of 10 μ M primer, 0.5 μ L of 10 mM each dNTP, 1 μ L of 25 mM MgCl₂, 0.2 μ L of GoTaq DNA polymerase (Promega), 3 μ L of 5 $\times$ green GoTaq reaction buffer (Promega) in total 15 μ L mixture.
6. Primers for genotyping of Foxo1, 3, 4, and cre alleles (see Table 1).
7. DNA running buffer: Tris-borate-EDTA buffer, 89 mM Tris base, 89 mM Boric acid and 2 mM EDTA (TBE).
8. Agarose gel: 3% of agarose gel for Foxo1 genotyping, and 1.5% of agarose gel for others.

Table 1
Primers for genotyping

Gene	Primers	Sequences
Foxo1	oFK1ckA oFK1ckB oFK1ckD	5'-GCTTAGAGCAGAGATGTTCTCACATT-3' 5'-CCAGAGTCTTTGTATCAGGCAAATAA-3' 5'-CAAGTCCATTAAATTCAGCACATTGA-3'
Foxo3	Nhe1 F3 Nhe1 F4 LSL F4	5'-TGTC AAGATGGCAGCAGCGG-3' 5'-GGCAACTCCCGTCTTTTCCTCT-3' 5'-AGCGCATCGC _s CTTCTATCGCCTTC-3'
Foxo4	oAFXckD oAFXckE oAFXckM	5'-CTTCTCTGTGGGAATAAATGTTTGG-3' 5'-CTACTTCAAGGACAAGGGTGACAG-3' 5'-TGAGAAGCCATTGAAGATCAGA-3'
Cre	cre26 cre36 Gabra 12 Gabra 70	5'-CCTGGAAAATGCTTCTGTCCG-3' 5'-CAGGGTGTTATAAGCAATCCC-3' 5'-CAATGGTAGGCTCACTCTGGGAGATGATA-3' 5'-AACACACACTGGCAGGACTGGCTAGG-3'

2.2 Brain and Spinal Cord Isolation

1. Surgical scissors and forceps for tissue dissection.
2. Adult mouse brain matrix (Kent Scientific Corporation) and razor blades.
3. 18-gauge needle and 20 mL syringe for flushing out the spinal cord.

2.3 Western Blot Analysis

1. Tissue lysis buffer: 150 mM NaCl, 50 mM Tris pH 7.4, 0.5% Sodium deoxycholate, 0.1% SDS, 1% NP40.
2. BCA Protein Assay Reagent.
3. 6× SDS-PAGE sample buffer: 40% Glycerol, 12 mM EDTA, 6% beta-mercaptoethanol, 300 mM Tris (pH 6.8), 0.006% bromophenol blue, 12% SDS.
4. Running buffer: 25 mM Tris Base, 192 mM glycine, 0.1% SDS, pH 8.3 (no need to adjust pH).
5. Transfer buffer: 25 mM Tris Base, 192 mM glycine, 20% methanol.
6. PVDF membrane.
7. Wash buffer: 50 mM Tris, 150 mM NaCl, pH 7.6, 0.05% Tween20 (TBST).
8. Blocking and secondary antibody dilution buffer: 5% non fat dry milk (NFDM) in TBST.
9. Primary antibody dilution buffer: 3% bovine serum albumin (BSA) in TBS.

Table 2
Primers for qRT-PCR

Gene	Primers	Sequences
m18 s	F	5'-CCGATAACGAACGAGACTCTGG-3'
	R	5'-AGGGTAGGCACACGCTGAGCC-3'
mFoxo1	F	5'-CTACGAGTGGATGGTGAAGAGC-3'
	R	5'-CCAGTTCCTTCATTCTGCACTCG-3'
mFoxo3	F	5'-CCTACTTCAAGGATAAGGGCGAC-3'
	R	5'-GCCTTCATTCTGAACGCGCATG-3'

10. Antibody: FOXO1 (#2880, rabbit monoclonal Cell Signaling), FOXO3 (NB100-614, rabbit polyclonal, Novus Biologicals).
11. ECL Pico Chemiluminescent peroxidase substrate.
12. X-ray films and a film processing system.

2.4 qRT-PCR Analysis

1. TRIzol™ Reagent.
2. cDNA synthesis kit.
3. DNase I Amplification Grade.
4. Power SYBR™ Green PCR Master Mix.
5. Primers for m18s, mFoxo1, and mFoxo3 (*see* Table 2).

2.5 Perfusion Fixation

1. Ketamin/Zylazine cocktail for anesthetization.
2. 4% paraformaldehyde in PBS.
3. 23-G winged butterfly needle and 20 mL syringe.
4. Cal-Ex decalcifier.

2.6 Immunohistochemistry

1. Xylene for deparaffinization and 100%, 95%, 70% ethanol for rehydration and dehydration.
2. Antigen retrieval sodium citrate buffer: 10 mM sodium citrate, 0.05% Tween 20, pH 6.0.
3. 3% hydrogen peroxide.
4. VECTASTAIN® Elite ABC-Peroxidase staining kit (Rabbit IgG) and DAB peroxidase (HRP) substrate.
5. Antibody: FOXO1 (rabbit monoclonal, #2880, Cell Signaling), FOXO3 (rabbit monoclonal 1, #12829 FOXO3a (D19A7), Cell Signaling), IBA-1 (rabbit polyclonal, #019-19,741, Wako), and GFAP (rabbit polyclonal, # Z0334, DAKO).

3 Methods

3.1 Generation of Specific Neural Cell-Targeted Foxo Knockout Mice

1. Generate mice with isoform-specific *Foxo* null neural cells. Cross homozygous floxed *Foxo* (*Foxo^{L/L}*) mice to desired cre transgenic lines through two successive generations: *Nestin-cre* for global neural deletion of target genes in both central and peripheral nervous systems beginning embryonic day 11, human GFAP promoter (*hGfap*) for targeting the dorsal and medial regions of the telencephalon beginning embryonic day 13.5 [8], or for targeting mature neurons *Synapsin1-cre* mice [9] (see Note 1).
2. Genomic DNA preparation for PCR: lyse tissue biopsies by heating to 95 °C for 30 min with 100 µL alkaline tail lysis buffer, followed by cooling down to 4 °C for 15 min. Immediately add 100 µL neutralization buffer and mix well.
3. Set up PCR reaction by adding 1 µL of genomic DNA to genotyping PCR mixture. Use primers oFK1ckA, oFK1ckB, and oFK1ckD to detect the wild-type allele (115 bp), the lox allele (149 bp), and deleted allele (190 bp) for *Foxo1* genotyping (see Fig. 1a). PCR cycling can be performed by the following conditions: step 1: 94 °C 3 min; step 2: 94 °C 30 s; step 3: 63 °C 1 min; step 4: 72 °C 1 min; repeat steps 2–4 for 34 cycles; step 5: 72 °C 5 min; step 6: 10 °C hold. Run the whole PCR reaction products on agarose gel with DNA running buffer.
4. Use primers Nhe1 F3, Nhe1 F4, and LSL F4 to detect the wild-type allele (300 bp) and the lox allele (550 bp) for *Foxo3* genotyping (see Fig. 1b). PCR condition is the same as *Foxo1* PCR.
5. Use primers oAFXckD, oAFXckE, and oAFXckM to detect the wild-type allele (313 bp), the lox allele (555 bp), and the deleted allele (471 bp) for *Foxo4* genotyping (see Fig. 1c) (see Note 2). PCR cycling can be performed by the following conditions: step 1: 94 °C 5 min; step 2: 94 °C 30 s; step 3:

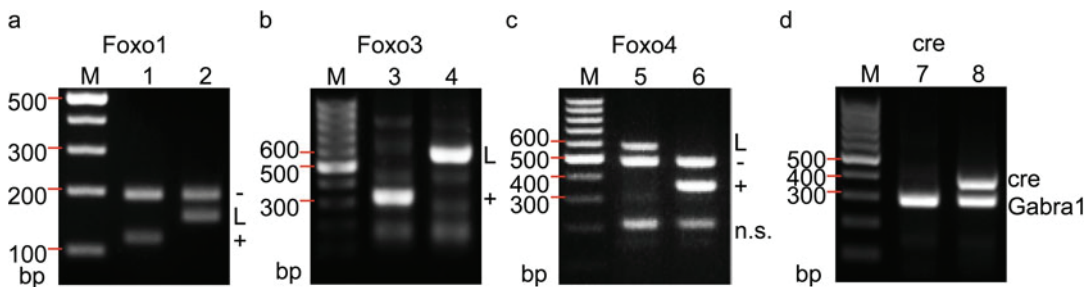


Fig. 1 Genotyping of (a) *Foxo1*, (b) *Foxo3*, (c) *Foxo4*, and (d) *cre*. M, 100 bp DNA ladder; 1, *Foxo*±; 2, *Foxo1*L/-; 3, *Foxo3*+/+; 4, *Foxo3*L/L; 5, *Foxo4*L/-; 6, *Foxo4*±; 7, *cre*-; 8, *cre*+. *Gabra1* is an internal positive control for PCR reaction. n.s., nonspecific band

55.5 °C 1 min; step 4: 72 °C 1.5 min; repeat step 2–4 for 32 cycles; step 5: 72 °C 5 min; step 6: 10 °C hold.

6. Primers to genotype cre transgene, cre26, cre36, Gabra12, and Gabra70 were used to detect the internal control (300 bp) and cre transgene (400 bp) (*see* Fig. 1d). PCR condition is the same as Foxo1 PCR.

3.2 Brain and Spinal Cord Isolation

1. For immunohistochemistry, 4% PFA fixation by perfusion should be done prior to following steps.
2. For brain isolation, remove the scalp, and scrape away muscle from the dorsal and posterior part of the skull. Carefully remove skull bone, and take out brain. For molecular analysis, the brain can be further dissected into cerebellum, cortex, hypothalamus, rostral ventrolateral medulla, mid brain. For histological analysis, the whole brain can be cut into 1–2 mm thickness series by using a brain matrix, orderly placed facing down in a histology cassette, and processed for paraffin embedding.
3. For spinal cord isolation, remove the skin, and tease away muscle. Disarticulate vertebral column just below skull and at a level even with the pelvis. And cut parallel to the spine separating the rib cage from the column. For molecular analysis, insert a needle into anterior to flush out the spinal cord with PBS. For histological analysis, the trimmed column needs to be further fixed and decalcified as described below (Subheading 3.6).

3.3 Western Blot Analysis for FOXO 1 or 3

1. Isolated brain and spinal cords are homogenized in tissue lysis buffer (100 mg in 500 µL) by triturating through p200 pipette tips.
2. After centrifugation for 10 min at 14,000 × *g* at 4 °C in a micro-centrifuge, transfer the supernatant to a new tube.
3. Next, determine protein concentration by a BCA assay. After adjusting the protein concentration and adding 6× SDS-PAGE sample buffer, samples are heated to 95 °C for 5 min.
4. Lysates are subjected to SDS-PAGE (10 µg total protein/lane) and run with running buffer and transferred to a PVDF membrane using transfer buffer (100 v, 2 h).
5. Blots are incubated with blocking buffer (1 h at room temperature), and then further with desired antibodies (FOXO1, 1:1000; FOXO3, 1:3000 diluted in primary antibody dilution buffer, overnight in the refrigerator). Rinse off primary antibodies by 3 times 5 min washes with TBST. Incubate blots with secondary antibodies (goat anti rabbit-HRP 1:10,000 in secondary antibody dilution buffer for 1 h at room temperature) and rinse off by 3 washes with TBST. Treat blots with ECL peroxidase substrate for detection of desired bands on x-ray films (*see* Fig. 2).

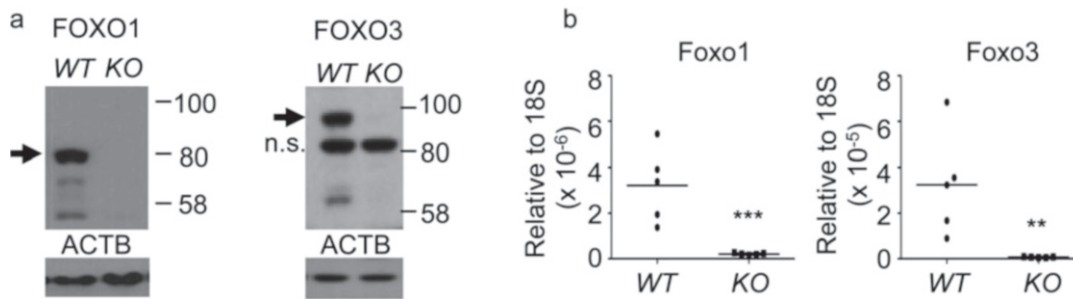


Fig. 2 Western blots and qRT-PCR of 10 month-old Foxo1/3/4 WT and KO cerebellum. (a) Arrows point to correct target bands of FOXO1 or 3. n.s., nonspecific band. (b) Statistical differences were determined by Student *t*-test. ****P* < 0.005, ***P* < 0.01

3.4 qRT-PCR Analysis for Foxo mRNA

1. Isolate total RNA with TRIzol following the manufacturer's instruction, and treat RNase-free DNase I for 30 min at 37 °C, and add 5 mM EDTA followed by heat inactivation of the enzyme at 65 °C for 10 min.
2. Prepare cDNA samples from 1 µg of total RNA by cDNA synthesis kit.
3. Set up qRT-PCR reaction using cDNA samples and the SYBR Green Master Mix.
4. The relative mRNA level can be calculated as unit values of $2^{[Ct(18\text{ s}) - Ct(\text{gene of interest})]}$. See Fig. 2 for representative results.

3.5 Perfusion and Immunohisto- chemistry

1. Anesthetize the mouse by i.p. injection of 150 mg/kg Ketamin and 15 mg/kg Xylazine.
2. Open the abdomen and cut the diaphragm and ribs. Next, insert needle directly into left ventricle and make incision of right atrium.
3. Following the first run of 10 mL of PBS, perfuse with 20 mL of 4% paraformaldehyde.
4. Dissect out brain and spinal cord tissues and post-fix in 4% paraformaldehyde overnight.
5. For decalcification of spinal bone, treat the whole tissue with Cal-Ex overnight followed by extensive wash with water.
6. Perform deparaffinization of 5 µm paraffin embedded sections by two changes of xylene and rehydrate through 100%, 95%, 70% ethanol, and H₂O.
7. Treat slides by heating to 95 °C in antigen unmasking buffer for 45 min.
8. For immunohistochemistry using DAB, treat sections with 0.3% hydrogen peroxide for 10 min in order to block endogenous peroxidase activity.

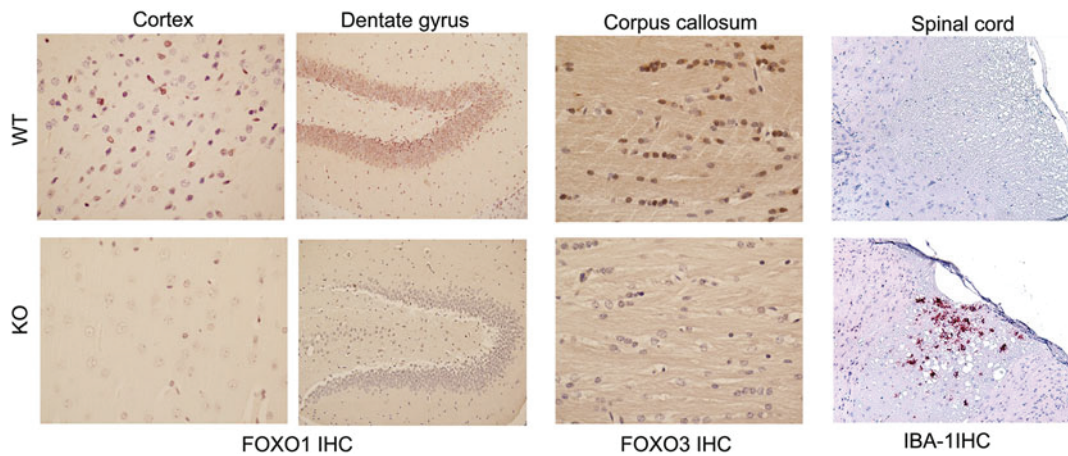


Fig. 3 IHC analysis of *WT* and *Foxo1/3/4 KO* mice. FOXO1 IHC from cortex (60 $\times$) and dentate gyrus (20 $\times$). FOXO3 IHC from corpus callosum (60 $\times$). Microgliosis in thoracic spinal cord of *KO* animal is shown by IBA-1 IHC (20 $\times$)

9. Stain sections with the following antibodies. FOXO1 (1:100 dilution) and FOXO3 (1:1000 dilution) for confirming deletion (*see Note 3*). IBA-1 (1:500 dilution,) for microglia, and GFAP (1:400 dilution) for astroglia (*see Fig. 3*) (*see Note 4*). Perform antibody and substrate reactions per the manufacturer's instruction for staining kits.

3.6 Assessing the Progression of Axonal Degeneration

1. Quantitate progression of degeneration by scoring hind leg clasping by suspending animals by grabbing their tails. Assess hindlimb clasping according to Guyenet et al. [10]: assign a score of 0 if the hindlimbs are splayed outward, away from the midline; assign a score of 1 if one hindlimb is retracted to the abdomen for more than 50% of the time; assign a score of 2 if both hindlimbs are retracted toward the abdomen for more than 50% of the time; assign a score of 3 if its hindlimbs are entirely retracted toward the abdomen for more than 50% of the time (*see Fig. 4*).

4 Notes

1. These three different strains have been generated and characterized by our group [6]. There are no overt developmental defects.
2. *Foxo4* is located on the X chromosome. Males are hemizygous for *Foxo4*.
3. The expression of FOXO1 is most obvious in the dentate gyrus. FOXO3 is expressed all throughout the brain. FOXO4



Fig. 4 Leg clamping behavior and scoring. The hindlimbs are splayed outward (left); one hindlimb is retracted (middle); both hindlimbs are retracted (right)

is expressed at low levels. FOXO6 expression is high during development and decreases in mature brains.

4. It is common to have reactive gliosis in *Foxo1/3/4*^{L/L}:cre+ brain sections [6]. Checking for white matter vacuolization by H&E staining or reactive gliosis by IBA-1 or GFAP IHC analysis could serve as one of functional confirmations.

Acknowledgments

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Part V

Human Genetic and Clinical Studies



Chapter 21

Analysis of *FOXO3* Gene Polymorphisms Associated with Human Longevity

Timothy A. Donlon, Philip M. C. Davy, and Bradley J. Willcox

Abstract

Next-generation DNA sequencing has ushered in a new era of genotype-phenotype comparisons that have the potential to elucidate the genetic nature of complex traits. Since such methods rely on short sequence reads and since the human genome is composed largely of repetitive DNA elements larger than these read lengths many results cannot be mapped and are discarded, thus eliminating a large portion of the genome from analysis. Discerning associations in complex traits, such as longevity, will require either longer read lengths or methods to address these sequence complexities. Whole genome analysis, such as Genome Wide Association Studies (GWAS), also suffers from the repetitive nature of the human genome, as there exist many gaps in the availability of useable genetic markers, often in interesting regulatory regions. Methods are described here whereby some of these problems have been addressed by targeted DNA sequencing, full exploitation of available public databases, and a careful evaluation of genomic features where we use the *FOXO3* gene as an example to identify functional variations and how they may relate to longevity.

Key words Long-range DNA sequencing, Long-range PCR, Genome complexity, Repetitive DNA

1 Introduction

About half of the human genome is comprised of repeats; composed of transposable elements, satellite DNAs, gene families, duplicated chromosomal regions, and pseudogenes [1, 2]. The simple repeat family, “Alu element,” is ~300 bp in length and occupies about 10% of the genome. The problem of aligning these repeats is a recurrent difficulty in sequence mapping algorithms so that the automated detection of such elements is no trivial task. DNA repeats are filtered out by most DNA alignment programs (i.e., RepeatMasker and Censor). To date, the most commonly used NGS platforms provide 200–300 bp sequence reads that must be integrated into a reference genome. For de novo assembly, repeats that are longer than the read length create gaps in the assembly and are ignored. This fact, coupled with the short length of NGS (next-generation sequencing) output, results in a

more fragmented assembly than Sanger sequencing, in which read lengths are ≥ 700 bp. In addition to creating gaps, repeats can be erroneously collapsed on top of one another and can cause complex, misassembled rearrangements. Almost 30 million base pairs (Mbp) of euchromatic genome sequence are either absent from, or have no assigned locations in, current assemblies of the human genome, presumably due to the limitations in mapping repetitive elements [3]. This problem has led to an abundance of single-nucleotide polymorphisms (SNPs) in public databases that are from non-allelic, homologous sequences, such as pseudogenes and gene families. Take *FOXO3* and its pseudogene, *FOXO3B*, for example. As of August 8, 2011, there were 38 reported sequence variants in the *FOXO3* coding region that included 21 synonymous, 16 non-synonymous (including our newly found variant), and 2 frame-shift variants (GRCh37, NCBI dbSNP Build 134). After chromosome 6-specific, long-range PCR amplification, sequencing, and genomic mapping these SNPs were found either to reside in the *FOXO3B* pseudogene on chromosome 17 or were misidentified by *FOXO3-FOXO3B* alignment [4]. We were able to sequence a 120 Kb region of the *FOXO3* gene in order to filter out all the erroneous SNPs so that we could develop a better understanding of the potential functional variants associated with longevity [5].

In this chapter, we describe the methods used to discriminate between true DNA sequence variants and the plethora of unfiltered data present in the public domain in order to provide guidelines for a more successful campaign of enumerating genotype-phenotype correlations for complex traits.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 M Ω -cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. All equipment should be nuclease-free to reduce DNA degradation.

2.1 Region-Specific Amplification

1. For long-range DNA amplification, primers should have a T_m ranging from 68 to 70 °C. Check all primers against BLAST to make sure there are no two forward and reverse primers near one another and be sure to inactivate the “mask repeats” option when performing a BLAST screen so that a complete analysis of sequence homologies can be assessed.
2. Long-range DNA amplification may be performed using either a mixture of rTth and VENT[®] polymerases or a recombinant enzyme kit.

3. PCR product clean-up columns or 96-well filter plates can be used for isolating product from primers.
4. A 384-well format is also available for a larger number of samples.
5. Vacuum pump for filter plates used cleaning up PCR product or centrifuge that will hold a sufficient number of samples.
6. Vacuum manifold for attaching plate.
7. Microtiter plate shaker for mixing.

3 Methods

3.1 Long-Range DNA Amplification

A method of long-range PCR [6] can be used to generate locus-specific products of 16–18 kb in order to avoid complications from repetitive DNA (*see Note 1*).

3.1.1 PCR Purification, Using a 96 Ultra-Filtration (UF) Plate on a Vacuum Manifold (*See Note 2*)

1. Prepare the vacuum manifold according to the instructions. Place a waste tray inside the base of the manifold.
2. Place the appropriate UF Plate (selected for your PCR product) on top of the vacuum manifold.
3. Pipet the PCR samples onto the UF Plate (*see Note 3*).
4. Apply a vacuum and maintain at -800 mbar for 10 min or until the wells are completely dry. Slowly release pressure and switch off vacuum source (*see Note 4*).
5. (Optional Wash Step): Add 50 μ L deionized water to each well, apply a vacuum, and maintain at -800 mbar for 10 min or until the wells are completely dry. Switch off vacuum source.
6. Carefully remove the UF Plate from the vacuum manifold.
7. Carefully tap the 96 UF Plate on a stack of clean absorbent paper to remove any liquid that might remain on the bottom of the plate.
8. Add 20 μ L deionized water to each well (*see Note 5*).
9. Shake the UF Plate on a microplate shaker for 2 min at medium speed.

Note: The purity of the DNA obtained after elution is sufficient for most applications without this wash step being required. If a higher purity is needed for a specific application, this step should be carried out.

Note: Ensure that the UF Plate is fixed securely on the top of the shaker.

- (a) Alternatively, purified DNA may be dissolved by pipetting samples up and down ~ 20 times.

10. Recover the purified PCR product by pipetting the eluate out of each well. For easier recovery of the eluates, the plate can be held at a slight angle.

3.1.2 PCR Purification, Using the 96 UF Plate: Centrifugation Method

1. Pre-wet membrane for maximum sample recovery.
2. Place a 96 filter plate over a solid-bottom receiver plate. Make certain that the receiver plate and the filter plate wells correspond to fit. Use a plastic tape to keep the two plates together.
3. Add 50 μL of PCR dH_2O into the well of the UF filter plate.
4. Place filter and receiver plates together into a standard swinging bucket microtiter plate rotor assembly.
5. Centrifuge at $2000 \times g$ for 15 min.
6. After all fluid has evacuated from the well(s), discard receiver plate.
7. Place the pre-wetted filter plate over a new solid-bottom receiver plate. Use a plastic tape to keep the two plates together.
8. Add 25 μL of PCR sample(s) into the pre-wetted filter plate well(s).
9. Place filter and receiver plates together into a standard swinging bucket microtiter plate rotor assembly.
10. Centrifuge at 2000 to $3000 \times g$ for 10 min. (Centrifuge time may vary, depending upon concentration of sample (*see* **Note 6**))
11. After the centrifugation, add 20 μL of buffer (TE^{-4}) to each well. Pipette the buffer (TE^{-4}) up and down several times (10 to 20 time). Allow the plate to sit for 10 min, then aliquot the liquid into a new PCR plate.
12. Determine DNA concentration for DNA sequencing reactions.

3.1.3 DNA Sequencing

Primers can be designed using a wide variety of free online tools. It is imperative to remove unincorporated primers prior to sequencing. Amplified products are purified using a number of products, such as columns noted above or ethanol precipitation, and sequenced by either capillary electrophoresis or next-generation sequencing (*see* **Note 7**).

3.1.4 DNA Sequence Alignment

There's a plethora of DNA sequence alignment software, the input of which will depend on the sequencing methods output files. Large structural variation may be assessed by digesting the long-range PCR products with *EcoRI* and/or *HindIII* and comparing digests on a 0.7% agarose gel.

Table 1
Relative number of SNPs in public databases and the KHHP cohort

dbSNP (total)	dbSNA (MAF ≥ 0.01)	HaploReg	RegulomeDB	KHHP cohort
12,456	1182	1617	476	146

The above information shows the number of single-nucleotide polymorphisms (SNPs) from the online database “dbSNP” (total), including copy number variants with minor allele frequencies ≥ 0.01 , HaploReg, RegulomeDB, and the Kuakini Honolulu Heart Program cohort of 95 individuals ≥ 95 years of age

3.1.5 Identification of Functional Variants

There is a huge list of putative variants present in public databases, such as dbSNP. Many of these variants are merely sequencing errors and need to be filtered out before searching for functional variants. The 1000 genomes project has reduced the number of error-derived variants. Actual variants can be identified by including those present in two or more subjects (Table 1 for example). For our FOXO3 project, all variants were screened on the RegulomeDB site, which includes known and predicted regulatory elements in the intergenic regions, as well as regions of DNAase I hypersensitivity, binding sites for transcription factors, and promoter regions that have been shown to regulate transcription. The strength of this site is that it highlights the location of the SNP relative to sites of chromatin modification and transcription factor binding and highlights the SNP relative to the TFBS canonical sequence so that it can be easily determined if the SNP would be expected to modify binding. One of the shortcomings of RegulomeDB is that it does not evaluate variants for their effects on chromatin modification and binding efficiencies, something that can be quite challenging. Sources of these data included public datasets from GEO, the ENCODE project, and published literature [7]. Unless otherwise indicated, all locations on chromosome 6 use the GRCh37.p13 genome build (<http://www.gencodegenes.org/releases/19.html>).

We also screened the FOXO3 variants using HaploReg (<http://archive.broadinstitute.org/mammals/haploreg/haploreg.php>), which is a tool for exploring annotations of the noncoding genome at variants on haplotype blocks, such as candidate regulatory SNPs at disease-associated loci [8]. Using LD information from the 1000 Genomes Project, linked SNPs, and small indels can be visualized along with chromatin state and protein-binding annotation from the Roadmap Epigenomics and ENCODE projects, sequence conservation across mammals, the effect of SNPs on regulatory motifs (Fig. 1), and the effect of SNPs on expression from QTL studies. We searched HaploReg version 4.1 for the region chr6:108866973–109011102 using the Nov 5, 2015 build, hg38. By selecting an individual SNP, one can obtain information on the tissue types that demonstrate differential chromatin

Position Weight Matrix ID (Library from Kheradpour and Kellis, 2013)	Strand	Ref	Alt	Match on:
				Ref: GGGAGGACTGTGTGGCTGGAGGCAGCTGCAGTAGAAGTTGGCAGTGCCACTGTGGACTT Alt: GGGAGGACTGTGTGGCTGGAGGCAGCTGCGGTAGAAGTTGGCAGTGCCACTGTGGACTT
Ascl2	-	11.1	11.6	NBYVVCASCTGCBVHNK
HEN1_1	+	7.9	11.6	DDGGNMKCACTGCKBCCYHD
HEN1_2	+	8.9	13.1	NMKCAGCTGCKB
Myf_1	-	17.1	6.6	MRRCARCWGSWG
Myf_3	+	13.5	12.5	MNSVRVCAGGTGNHGDNR
Rad21_disc8	+	11.4	11	SSHGSSMNSNSNS
Znf143_disc3	+	12.3	11.4	GSVBBSBGGGVVNBGERGB

Fig. 1 Example of Regulatory Motifs Altered by SNP (rs2802288) in HaploReg. The online-database, HaploReg, was searched for SNP rs2802288. In addition to this SNP 12 other neighbors that are in linkage disequilibrium (LD) are shown on a new page and include information as the degree of LD, the population allele frequencies, histone marks, eQTL, proteins bound, and transcription factor/enhancer motifs that are changed by the SNP (not shown here). Selection of rs2802288 results in a list including histone marks and the cell types evaluated as well as a table of motifs that are altered by the SNP (shown above). This table shows the transcription factors, the strand $\pm$, reference sequence, SNP (Alt) sequence, and the canonical sequence for the motif (shown as a nucleotide ambiguity code). Selection of the following link: **Position Weight Matrix ID (Library from Kheradpour and Kellis 2013)** will bring up a page that includes all the known canonical sequences for that transcription factor/enhancer so that they may be evaluated further. For example, RAD21_disc8 leads to a column whereby RAD21 may be selected to show various experiments that discovered RAD21 binding and their associated heatmap scores. Note that not all enhancers are present in the Weight Matrix ID and may require searching other databases, such as JASPAR (<http://jaspar.genereg.net>)

modification, DNase I sensitivity, and/or expression level. Select a SNP that has a changed motif and the page will reveal additional information. For example, at the bottom of the page is an alignment of the genomic sequence surrounding the SNP and the canonical sequences to the altered putative motifs. A score indicates the binding efficiencies (position weight matrix) of the reference and variant sequences [9] (see **Note 8**).

4 Notes

1. When validating PCR primers via BLAST it is important to turn off the “mask repeats” function in order to determine the correct complementary sites available in the genome. Otherwise, a primer containing a repeat may incorrectly be interpreted to be “unique.” There will be many similar sequences over a long region of the large amplicon. Using primers with a higher annealing temperature will help to reduce the number of primer-complementary sequences in the large amplicon and provide cleaner results. Be aware of which enzyme you’re using for PCR and whether it can survive an extended denaturation step, otherwise it will be necessary to perform a manual “hot start.” The length of the target sequence will affect the required starting extension time; generally, allow 30–60 s per kb of target.

2. A number of products are available for purifying PCR product from primers and are available in tube or plate format. The important parameters are a cutoff of about 50 bp. Be prepared to lose as much as half of the product in the clean-up phase.
3. Processing PCR sample volumes larger than 150 μ L may lead to increased processing time and incomplete primer removal.
4. If the PCR volume exceeds 50 μ L, a longer vacuum time is needed. Apply the vacuum until all wells are dry. Approximately 10 min of vacuum application are needed for each 50 μ L PCR volume. DO NOT OVERDRY THE SAMPLES as they will be difficult to remove from the filter.
5. DMSO (50% v/v), 3 $\times$ SSC, and EB (10 mM Tris-Cl, pH 8.5) or similar buffers can be used instead of water for elution.
6. Don't spin the plate too hard or the DNA may become dislodged and lost. Spin just hard and long enough to remove the excess liquid.
7. Again, select primers and screen them using BLAST (turn off "mask repeats"). DNA sequencing technologies are improving all the time. The important issue to keep in mind is that the genome is quite complex and any methods that can be used to reduce this complexity will result in a more complete evaluation of variation. As sequencing reads become longer (i.e., 500 bp) the need for locus-specific amplification is reduced since it will be more accurate to accurately map the most common repeats, such as Alu, which are $\sim$ 300 bp in length.
8. Be aware of which genome assembly (i.e., build) for the SNPs you are searching or HaploReg will not return the correct region.

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Analysis of *FOXO3a* Gene Polymorphism Associated with Asthma

Tejas Shah and Kalpana Joshi

Abstract

Single-nucleotide polymorphisms (SNP) represent genetic variation in a human population; 99.9% of the DNA sequence is identical and remaining 0.1% of DNA contains sequence variants. Around ten million SNPs are identified post human genome project. Identification of SNPs associated with disease phenotype has diagnostic potential and may predict drug response. Several technologies genotype thousands of SNPs simultaneously, for instance, genotyping arrays, gene-chip arrays, bead array technology, pyrosequencing, TaqMan approach, etc., however Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) remains a simplest, reliable, and cost effective method. In PCR-RFLP, a fragment that is to be analyzed for a mutation is amplified from genomic DNA using PCR, digested using appropriate restriction enzyme and then separated by gel electrophoresis. Sets of primer are used to amplify a fragment of interest and are designed in such a way that it flanks the polymorphic site and is located in such a way as to generate uneven sized fragments upon restriction endonuclease cleavage of the amplified PCR products. *FOXO3a* plays a key role in maintaining immunoregulation and is associated with several inflammatory diseases. Here, we report the PCR-RFLP protocol developed for detection of polymorphism in *FOXO3a* gene (rs13217795) by amplifying a 667 bp fragment followed by restriction endonuclease analysis using *PagI* to obtain RFLP patterns.

Key words *FOXO3a*, Asthma, rs13217795, SNP, Polymorphism, PCR-RFLP

1 Introduction

FOXO3a, a member of the forkhead class “O” (FOXO) transcription factor family which controls a broad range of biological processes such as DNA damage repair, apoptosis, and cell cycle regulation. Recent articles have established involvement of *FOXO* gene in development, growth, stress resistance, apoptosis, cell cycle, immunity, metabolism, and aging [1–3]. We for the first time investigated an association between single-nucleotide polymorphism (SNP) of the *FOXO3a* (rs13217795, C > T transition) gene and asthma and found that rs13217795 possibly contributed to T cells hyperactivity in Indians [4]. Also meta-analysis revealed significant association between the *FOXO3a*; rs13217795

polymorphism and longevity in terms of the C vs. the T allele (OR = 1.27, 95% CI = 1.10–1.46, $P = 0.001$) and was gender specific [4]. Polymorphisms are inherited differences found among the individuals in more than 1% of normal population. Hence further exploration of the role of the *FOXO3a* gene in human longevity and other aging phenotypes is required in different populations. We have previously used Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique to detect SNP and insertion or deletion (Indels) of bases in the human genome [5, 6]. PCR-RFLP-based analysis is a prominent technique for genotyping of species-specific variations. Important advantages of the PCR-RFLP technique include low cost and lack of requirement for advanced instruments. In addition, the design of PCR-RFLP analyses generally is simple and can be achieved using publicly available programs specially to identify appropriate restriction enzymes and designing of primers. This chapter describes the approach used to detect rs13217795 polymorphism in human *FOXO3a* gene using the PCR-RFLP technique.

2 Materials

Prepare all solutions using ultrapure water (ultrapure water is prepared by purifying deionized water, having sensitivity of 18 M Ω at 27 °C) and molecular grade reagents. Prepare all reagents and store at room temperature, unless indicated otherwise.

2.1 Equipment

1. Thermocycler.
2. Hot water bath with temperature controller.
3. Electrophoresis unit with power pack.
4. UV cabinet/ gel documentation system.

2.2 PCR-RFLP

1. Purified human DNA [7].
2. Primers.
3. *Taq* Polymerase (3 U/ μ L).
4. *Taq* Polymerase buffer A (Tris with 15 mM MgCl₂).
5. dNTP mix (10 mM, 2.5 mM each dNTPs).
6. DNA ladder (100 bp).

Store above reagents at –20 °C

7. Agarose.
8. Ethidium bromide (EtBr) (10 mg/mL) (*see Note 1*).
9. Gel-loading dye.
10. Tris—borate—EDTA buffer (5 $\times$ -TBE) (1.1 M Tris; 900 mM Borate; 25 mM EDTA; pH -8.3) (*see Note 2*).

11. Restriction enzyme (*see* **Note 3**).
12. Sterile deionized water.

3 Methods

3.1 Identification of Restriction Enzyme and Primer Design

1. The single-nucleotide polymorphism (SNP); rs13217795 in *FOXO3a* gene is selected target in this method (*see* **Note 4**).
2. Obtain available sequence of selected SNP through NCBI using Reference SNP (refSNP) number, in this protocol it is *rs132177995*.
3. Save the latest sequence in FASTA format.
4. Go to restriction enzyme site.
(Examples: 1. Webcutter 2.0: <http://bio.lundberg.gu.se/cutter2/>
2. NEBcutter V2.0: <http://www.labtools.us/nebcutter-v2-0/>).
5. Enter the sequence or upload the FASTA format and make restriction enzyme analysis in database.
6. Choose the best fitted restriction enzyme (*see* **Note 5**).
7. Once the best enzyme is chosen, design a primer pair using software tools such as Gene Runner, Primer3, etc. In the present method the designed primer amplifies the 667 bp region sequence in *FOXO3a* gene (rs132177995, C > T transition) Fig. 1.
8. Analyze the specificity of primers using BLAST search and PCR (*see* **Note 6**).

3.2 PCR RFLP

To optimize the PCR reaction and condition for RFLP, set up the PCR using 50–150 ng DNA, 5–15 pmol of primers, 30–45 PCR cycles, and 58–62 °C of annealing temperature. The PCR reaction and conditions optimized by us are mentioned in Tables 1 and 2.

1. Perform the PCR reaction for isolated DNA using designed primer pairs (Table 1) [3].
2. Carry on the PCR reaction as mentioned in Table 2. The total volume of PCR reaction was 20 µL.
3. Thermal cycling parameters for PCR reaction is mentioned in Table 3.
4. The amplified products are examined in 2% w/v agarose gel (Fig. 2).
5. Prepare the restriction mixture on ice for digesting 10 µL of PCR product as indicated in Table 4 (*see* **Note 7**).

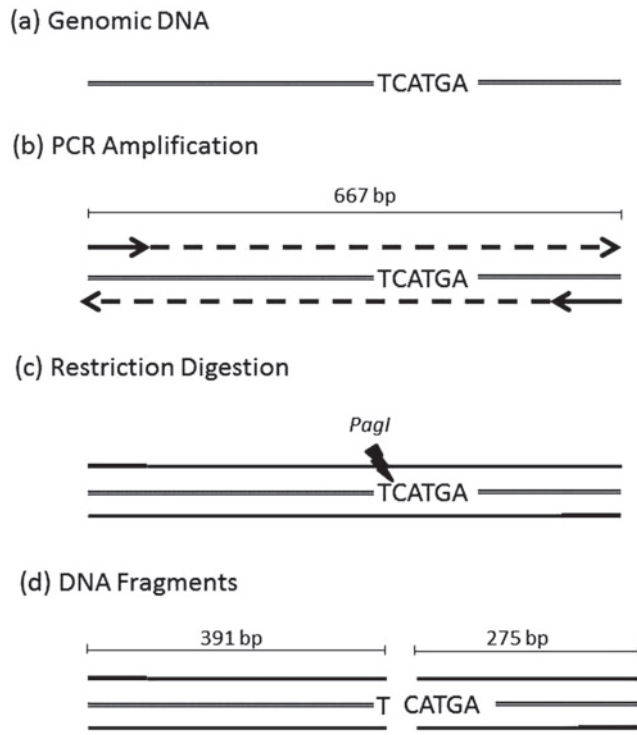


Fig. 1 Schematic representation of restriction site for *PstI* intergenic region sequence (667 bp) to detect rs13217795 polymorphism (not to scale)

Table 1
Technical data for SNP analysis in human FOXO3a gene

Gene name	SNP	Primers	GC content (%)	Annealing temperature (°C)	Restriction enzyme	Amplicon size (bp)
FOXO3a	rs13217795	F: 5'- CTCCTTGGTTCAGTTTGGTG - 3' R: 5'- ATGAGTGAAGATGGAAGTAAGC - 3'	52.6% 40.9%	62 °C	<i>PstI</i>	667 bp

6. Incubate at 37 °C for 15 min using Thermalcycler or water bath.
7. Prepare 2% w/v agarose gel for analysis of digested samples.
8. After incubation, mix 20 µL of the digested samples with loading dye. Slowly load the sample mixture into the wells of the submerged gel under TBE using a disposable micropipette.
9. Run the agarose gel at 90 V for 2 h 30 min. Examine the gel using UV cabinet/gel documentation system and photograph the gel showing the RFLP patterns (Fig. 3).
10. Analyze the restriction profiles as mentioned in Table 5.

Table 2
PCR reaction

Reaction components	Volume (effective concentration)
DNA template (100 ng)	0.5 μ L
Taq Polymerase buffer A	3 μ L
dNTPs (10 mM)	1 μ L
Primers (10 pmol)	0.3 μ L each
Taq DNA Polymerase (3 U/ μ L)	0.3 μ L
Deionized water	14.6 μ L
Total	20 μ L

Table 3
PCR condition

Steps	Temperature	Time
1. Initial denaturation.	95 °C	5 min
2. Denaturation.	95 °C	30 s
3. Annealing.	62 °C	30 s
4. Extension.	72 °C	1 min
Go to step 2 and repeat 35 times		
5. Final Extension	72 °C	5 min
Hold at 25 °C		

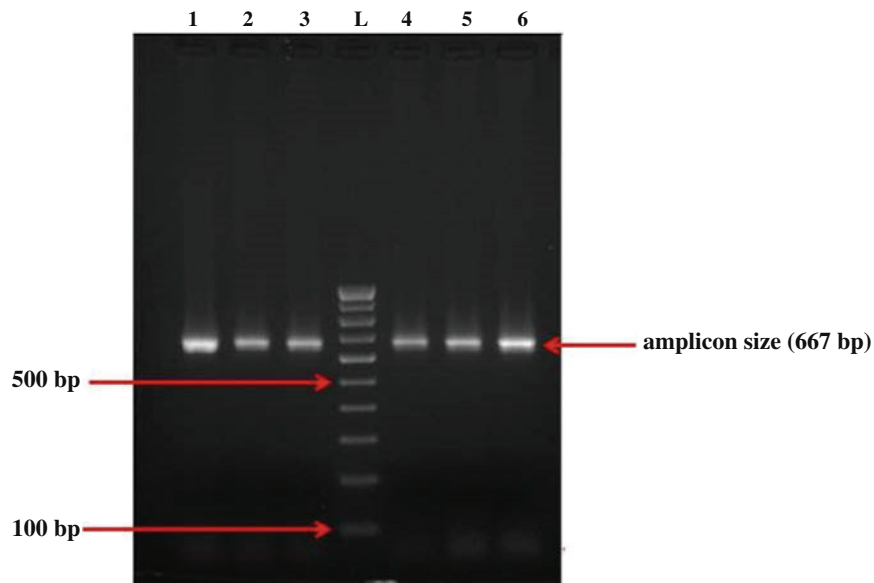
**Fig. 2** Agarose gel picture of desired amplicon

Table 4
RFLP reaction mixture

Reaction components	Volume
PCR reaction mixture	10 µL
Nuclease-free water	14 µL
10× Buffer	5 µL
<i>PagI</i> enzyme	1 µL
Total	30 µL

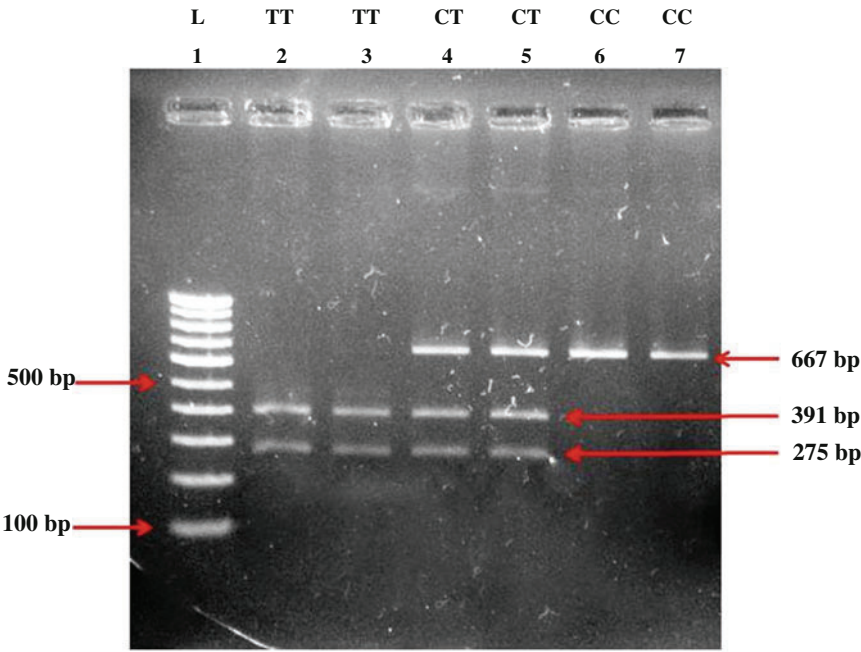


Fig. 3 Electrophoretic analysis showing the restriction profiles

Table 5
Restriction profiles

Band profile	Genotype
667 bp	Homozygous wild type (C/C)
667 bp, 391 bp, 275 bp	Heterozygous wild type (C/T)
391 bp, 275 bp	Mutant type (T/T)

4 Notes

1. Take precautions while handling EtBr, it is highly toxic as it is a carcinogen. Allow gel to cool to 60–70 °C. Add EtBr to 0.5 µg/mL final concentration.
2. Prepare 5× TBE buffer in stock. Use 0.5× TBE buffer for the preparation of agarose gel and electrophoresis buffer, i.e., add 100 mL of 5× TBE buffer in 900 mL of sterile deionized water.
3. It should be selected on the basis of map restriction site analysis using software (refer **step 4** in Subheading 3.1). In this method *PagI* was used as restriction enzyme (New England Biolabs Inc., USA).
4. There are several softwares which help to design PCR-RFLP in which selection of primers and restriction enzymes are incorporated.
5. Restriction fragments smaller than 100 bp should be avoided as they are not clearly resolved on agarose gel. The restriction enzyme should be reliable and cheap.
6. The designed primer pairs should be highly specific and should amplify only the studied species and desired amplicon size should be obtained [in this protocol 667 bp]. No additional or nonspecific bands should be detected.
7. Details like restriction digestion reaction mixture and incubation time is provided by manufacturers in package insert. Make all additions of reaction components on ice. After addition vortex mixture gently and spin the mixture shortly to settle down all droplets on the walls.

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