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

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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.

Keywords

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.
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).

¹A 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. 44–48 h after transfection add the proteasome inhibitor MG132 (final concentration: 20 μ M) 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 $^{\circ}$ 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 μ L 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 μ L TALON Metal Affinity Resin (80 μ L 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 $^{\circ}$ 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).

²Octameric ubiquitin is efficiently processed in vivo by cellular ubiquitin-C-terminal hydrolases and thus is more efficiently conjugated to substrates than monomeric ubiquitin [13].

³A 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.

⁴We 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° C.

⁵Only 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.

⁶Detection 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].

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 μ L) 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 μ L 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 μ L 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 μ L 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).

⁷Urea containing buffers need to be prepared fresh to prevent carbamylation of proteins.

⁸Previously 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.

⁹Only 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.

¹⁰Poly-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

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 μ L 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 μ L 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 °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 Histag-specific antibody (or a ubiquitin-specific antibody) reveals all cellular His-tagged ubiquitylated proteins and thus serves as a DUB specificity control.

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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.

¹¹The 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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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 polyubiquitylation 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

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