Characterization of FOXO acetylation

Shang Yao[1], Zimam Mahmud[1], Nikoleta Sachini[1,2], Sathid Aimjongjun[1,3], Paula Saavedra-García[1], Eric W-F Lam[1]

[1]Department of Surgery and Cancer, Imperial College London, Imperial Centre for Translational and Experimental Medicine (ICTEM), Du Cane Road, London W12 0NN, UK

[2]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology - Hellas (FORTH), 70013 Heraklion, Crete, Greece; Department of Biology, University of Crete, 70013 Heraklion, Crete, Greece.

[3]Multidisciplinary Unit, Faculty of Science, Mahidol University, Phayathai, Rajdhevi, Bangkok 10400 Thailand

Correspondence: Eric W.-F. Lam, Department of Surgery and Cancer, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, UK Phone: 44-20-7594-2810; Fax: 44-20-8383-5830; E-mail: eric.lam@imperial.ac.uk

Abstract

FOXO3 is a tumour 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 and thus, causing FOXO3 inactivation at the post-translational level. Therefore, targeting sirtuin proteins renders new avenues for breast cancer treatment. Here, we describe three procedures for studying FOXO3 post-translational modifications controlled by sirtuin proteins in cancer cells.

Keywords: 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 tumour suppressor and inhibits cell growth by inducing many transcription of genes, such as p130 (RB2), Bim, FasL or p27Kip1 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 with oncogenic transformation. FOXO3 can be regulated by different post-translational 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 signalling 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, post-translational modification of FOXO3 by acetylation also controls its transcriptional activity. The FOXO3 acetylation/deacetylation depends on the action of two types of proteins: histone deactylases (HADCs) that include silent information regulator 1 (SIRT1) and SIRT2, and histone acetyl tranferase (HATs), such

as CBP/p300 (5). SIRTs are class I and III HADCs and belong to the Sirtuin family that are NAD-dependent acetylases, these proteins can deacetylate FOXO3. SIRT1, through FOXO3 acetylation, 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 ubiquination-proteosome pathway. Furthermore, SIRT2 regulates FOXO3 activity inducing deacetylation of FOXO3 under oxidative stress increasing the binding of FOXO3 to promoter region of p27Kip1. In addition, CBP/p300 acetylates FOXO3 in three conserved lysine residue (Lys242, Lys245 and Lys262) and this acetylation inhibits DNA binding acitivity, ubiquitination and promotes Akt mediated phospholylation [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 post-translational 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 Americian Type Culture Collection (ATCC, Manassas, VA, USA)**.**
2. Lapatinib (LC laboratories, Woburn, MA, USA), dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C.
3. Dulbecco's modified eagle's medium (DMEM), supplemented with 10% (v/v) foetal calf serum (FCS), 100 unit/ml penicillin/streptomycin and 2 mM glutamine.
4. 25g/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% CO_2.

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 NaCl2, 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. Pre-stained 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).
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 Immunoprecipitation (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× (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 μM) and SIRT2 (IC50= 0.58 μM) 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× 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 inhibitor) 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 µl of Optimem and 5 µl Oligofectamine for each reaction.
3. Incubate at room temperature for 10 min.
4. Prepare 250 µl of Optimem and 7.5 µl of 1× universal siRNA buffer (used as the siRNA vector) containing no siRNA as a control.
5. Prepare 250 µl of Optimem and 7.5 µl (50 nM final concentration) siRNA oligos specific for each gene.
6. Prepare 250 µl of Optimem and 7.5 µl of a non-specific siRNA which does not target any known proteins.
7. Mix Optimem and Oligogectamine from step 2 and Optimem and siRNA from step 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 37ºC and 10% CO_2.
13. Add 2 ml of prepared medium to each well.
14. Incubate in the incubator at 37ºC and 10% CO_2 for further treatment.
15. Treat cells with 1 uM lapatinib after 24 h of incubation.
16. Incubate cells for 8 h in the incubator at 37ºC and 10% CO_2.

17. Trypsinize cells and collect them in falcon tubes. Centrifuge the tubes at 1,200 rpm for 5 min.
18. Dissolve the pellet with DMEM media and collect the cells in eppendorf.
19. Centrifuge again at 2,000 rpm for 5 min.
20. Discard the supernatant and freeze cells at -80ºC until the lysis will be performed.

3.1.2 Western Blotting

A. Preparation of protein lysates and protein quantification

1. Lyse the frozen pellets with NP40 lysis buffer and protease inhibitor cocktail.
2. Keep the lysates on ice for 20 min.
3. Centrifuge the lysate at 13,000 rpm for 10 min at 4°C to remove insoluble lysate material.
4. Transfer the supernatant to clean eppendorf tube.
5. Determine protein concentration by using the Pierce BCA Protein Assay kit according to manufacturer's instructions. Briefly, pipete the samples into microplate wells and add the mix of Reagent A and B. Incubate for 30 minutes at 37 °C and then measure the absorbance at 562 nm in a Tecan Sunrise microplate absorbance reader.
6. Determine protein concentrations by the equation of absorbance x 25 = µg/µl.

B. SDS Polyacrylamide Gel Electrophoresis

1. Cast a 6% acrylamide (v/v) resolving gel as mentioned in Table 1.
2. After polymerisation of resolving gel, carefully rinse off all isopropanol using distilled water. Remove any residual water using filter paper.
3. Cast a 5% stacking gel as shown in Table 1.
4. Immediately insert comb due to faster polymerisation reaction. Stacking gel should be polymerised after approximately 10 min.
5. Place the gel in an electrophoresis chamber and ensure comb side is facing inwards.
6. Fill chamber with SDS-running buffer up untill fill line.
7. Remove comb and use a syringe and needle to carefully flush out each well to remove any floating residual polyacrylamide.
8. Using appropriately sized pipettes, load 4 µL of pre-stained protein ladder and the samples into each corresponding well.
9. Connect the chamber after closing into a compatible power pack.
10. Run gel for approximately 2 h at 90 V. Use bromophenol blue line as an indicator of electrophoresis progress.

C. Protein Transfer

1. Place a nitrocellulose membrane in transfer buffer.
2. Soak two sponges and two whatman papers in transfer buffer.

3. 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.
4. Carefully open the glass plate and cut off the stacking gel and place gel over the top of the first whatman paper.
5. Place a labelled membrane on top of the gel.
6. Place second piece of filter paper on top of the membrane followed by second sponge.
7. Use a roller to carefully remove any bubbles in between layers, as this will lead to uneven protein transfer.
8. Prepare wet transfer apparatus filled with transfer buffer and place sandwich cassette into the transfer apparatus with ice.
9. Close lid and plug in transfer chamber. Now run the transfer for 90 min at 90 V.
10. Remove the apparatus and transfer the membrane into a tray with ponceau solution
11. Temporarily stain membrane for 5 to 10 min to check ladder labelling.
12. After labelling ladder, place membrane into TBS-T and wash on a shaker until membrane has no more ponceau.

D. Acetylated FOXO3 Detection

1. Place nitrocellulose membrane in 5% (w/v) BSA for 30 min to inhibit nonspecific binding sites.

2. Incubate membrane with rabbit anti acetylated FOXO3 (Lysine 242/245) antibody diluted at 1:1,000. Keep overnight at 4°C on a shaker.
3. Wash membrane 3 times for 5 min. Each wash with TBS-T in order to remove unbound antibodies.
4. Incubate membrane with anti-rabbit horseradish peroxidase conjugated secondary antibody diluted at 1:2,000 at room temperature for 1 h.
5. Wash membrane 3 times for 5 min each wash with TBS-T to remove unbound secondary antibodies.
6. Prepare membranes for autoradiography signal detection by mixing 1:1 ratio of ECL substrate kit.
7. 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.

3.2 Immunoprecipitation (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.

A. 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 1,200 rpm for 5 min.
5. Dissolve the pellet with DMEM media and collect the cells in eppendorf tube.
6. Centrifuge at 2,000 rpm for 5 min.
7. Remove the supernatant and freeze 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 13,000 rpm 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 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.

B. Beads preparation/Cell lysate Pre-clearing and immunoprecipitation

1. Use 20 µl of Dynabeads A/G per sample. Wash the beads with 500µl 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 hours at 4°C under rotary agitation.
3. When the incubation time is completed remove the beads with the magnetic platform and transfer the pre-cleared 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 pre-cleared 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.
7. Place the tubes on the magnetic separation rack and carefully pipete to remove the supernatant.
8. Gently wash the beads with 500 µl of Lysis Buffer 3 times to remove non-specific binding.
9. After the last wash centrifuge the beads gently (1,000-2,000 rpm for 1 min at 4°C) to remove the excess of Lysis Buffer.
10. Resuspend the bead pellets in 2× 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.

3.3 Duolink *in situ* Proximity Ligation Assay (PLA)

This assay is used to detect, visualize and quantify protein expression, protein interactions, and specific post-translational 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 a 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 solution consists of nucleotides, polymerase as well as fluorescently labelled oligonucleotides, the replicated product can be easily visible as distinct fluorescent spot when viewed with a fluorescence microscopy.

A. Fixation and Permeabilization

1. Seed 15,000 cells per well in 8-well chamber slide.
1. Remove media from the 8-well chamber slide.
2. Rinse each well with 200 µl 1× PBS 3 times for 5 min.
3. Fix in 80 µl 4% paraformaldehyde/PBS pH 7.4 at room temperature for 10 min.
4. Rinse each well with 200 µl 1× 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× PBS 3 times for 5 min.

B. Blocking and Incubation with Primary Antibodies

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.

C. 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 gentle in 1× 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.

D. Ligation

1. Tap off the PLA probe solution and wash the slides gentle in 1× 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× ligation stock and 64 µl of high purity water.
3. Add ligase to the ligation solution from 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.

E. Amplification

1. Tap off the ligation-ligase solution and wash gentle with 1× 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 last step at 1:80 solution and vortex e.g. add 1 µl polymerase to 80 µl amplification solution.
4. Add 80 µl amplification-polymerase solution to each well.
5. Incubator the slides for 100 min in incubator at 37 °C.

F. Preparation for Imaging

1. Tap off the amplification-polymerase solution and wash the slides in 1× Wash buffer B 2 times for 10 min in gentle shaker.
2. Wash the slides in 0.01× Wash buffer B for 1 min.
3. Air dry the slides at room temperature in the dark.
4. Carefully remove the chamber from 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 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. For the protein preparation, lysate the cells as quickly as possible to avoid protein damage.
3. In order to avoid protein degradation, the samples should be on ice in most of the steps described above.
4. Formation of polyacrylamide is based on a polymerisation process. Addition of APS and TEMED at the last moment, is highly recommended.
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.

References

[1] S.S. Myatt, E.W.F. Lam, The emerging roles of forkhead box (Fox) proteins in cancer, Nature Reviews Cancer 7(11) (2007) 847-859.

[2] M. Schmidt, S.F. de Mattos, A. van der Horst, R. Klompmaker, G.J.P.L. Kops, E.W.F. Lam, B.M.T. Burgering, R.H. Medema, Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D, Molecular and Cellular Biology 22(22) (2002) 7842-7852.

[3] R.H. Medema, G.J.P.L. Kops, J.L. Bos, B.M.T. Burgering, AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27(kip1), Nature 404(6779) (2000) 782-787.

[4] F. Zhao, E.W. Lam, Role of the forkhead transcription factor FOXO-FOXM1 axis in cancer and drug resistance, Front Med 6(4) (2012) 376-80.

[5] H. Daitoku, J. Sakamaki, A. Fukamizu, Regulation of FoxO transcription factors by acetylation and protein-protein interactions, Biochim Biophys Acta 1813(11) (2011) 1954-60.

[6] U.B. McGovern, R.E. Francis, B. Peck, S.K. Guest, J. Wang, S.S. Myatt, J. Krol, J.M.M. Kwok, A. Polychronis, R.C. Coombes, E.W.F. Lam, Gefitinib (Iressa) represses FOXM1 expression via FOXO3a in breast cancer, Molecular Cancer Therapeutics 8(3) (2009) 582-591.

[7] B. Peck, C.Y. Chen, K.K. Ho, P. Di Fruscia, S.S. Myatt, R.C. Coombes, M.J. Fuchter, C.D. Hsiao, E.W.F. Lam, SIRT Inhibitors Induce Cell Death and p53 Acetylation through Targeting Both SIRT1 and SIRT2, Molecular Cancer Therapeutics 9(4) (2010) 844-855.

[8] F.J. Alcain, J.M. Villalba, Sirtuin inhibitors, Expert Opin Ther Pat 19(3) (2009) 283-94.

[9] E.W. Lam, J.J. Brosens, A.R. Gomes, C.Y. Koo, Forkhead box proteins: tuning forks for transcriptional harmony, Nat Rev Cancer 13(7) (2013) 482-95.

[10] M. Shiota, A. Yokomizo, E. Kashiwagi, Y. Tada, J. Inokuchi, K. Tatsugami, K. Kuroiwa, T. Uchiumi, N. Seki, S. Naito, Foxo3a expression and acetylation regulate cancer cell growth and sensitivity to cisplatin, Cancer science 101(5) (2010) 1177-85.

[11] G. Zhao, J. Cui, J.G. Zhang, Q. Qin, Q. Chen, T. Yin, S.C. Deng, Y. Liu, L. Liu, B. Wang, K. Tian, G.B. Wang, C.Y. Wang, SIRT1 RNAi knockdown induces apoptosis and senescence, inhibits invasion and enhances chemosensitivity in pancreatic cancer cells, Gene therapy 18(9) (2011) 920-8.

[12] C.M. Grozinger, E.D. Chao, H.E. Blackwell, D. Moazed, S.L. Schreiber, Identification of a Class of Small Molecule Inhibitors of the Sirtuin Family of NAD-dependent Deacetylases by Phenotypic Screening, J. Biol. Chem. 276(42) (2001) 38837-38843.

[13] T.F. Outeiro, E. Kontopoulos, S.M. Altmann, I. Kufareva, K.E. Strathearn, A.M. Amore, C.B. Volk, M.M. Maxwell, J.C. Rochet, P.J. McLean, A.B. Young, R. Abagyan, M.B. Feany, B.T. Hyman, A.G. Kazantsev, Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease, Science 317(5837) (2007) 516-519.

	Resolving gel				Stacking gel
	7%	10%	12%	14%	**5%**
dH_20-ml	5.02	4.02	3.35	2.68	**3.67**
1.5M Tris pH 8.8	2.5	2.5	2.5	2.5	**-**
1.5M Tris pH 6.8	-	-	-	-	**0.42**
30%Acrylamide	2.33	3.33	4.00	4.67	**0.83**
10% SDS -µl	100	100	100	100	**50**
25% APS -µl	40	40	40	40	**20**
TEMED-µl	10	10	10	10	**10**
Total-ml	**10**	**10**	**10**	**10**	**5**

Table 1. Composition of Resolving and Stacking Gel

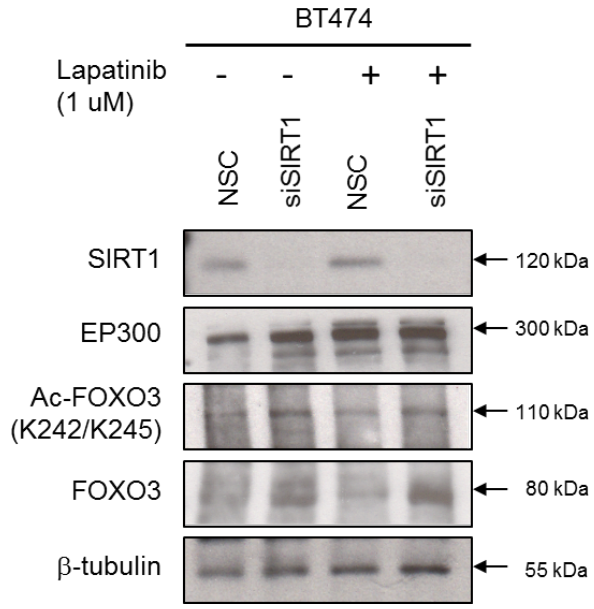

Figure 1: BT474 cells were transiently transfected with SIRT1 siRNA and a non-specific control (NSC) siRNA . The BT474 cells were treated with 1 µM lapatinib at 48 h post-transfection. Proteins obtained from whole cell extracts after 8 h of lapatinib treatment. Western blotting was performed using the 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.

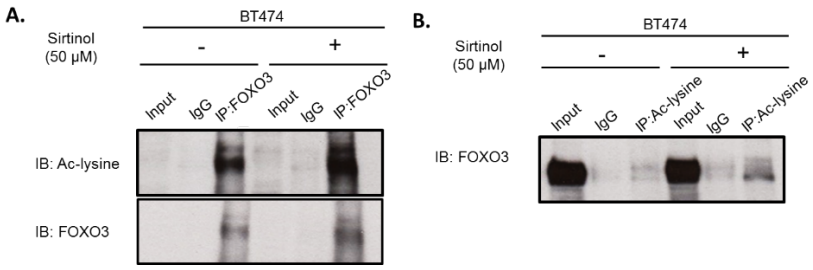

Figure 2: Protein lysates of BT474 cells were obtained following treatment with or without 50 uM sirtinol for 24 h. (A) Co-immunoprecipitation (Co-IP) was accessed with anti-FOXO3. Subsequent immunoblotting was performed using antibodies against Ac-Lysine and FOXO3. (B) A reverse Co-IP was performed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

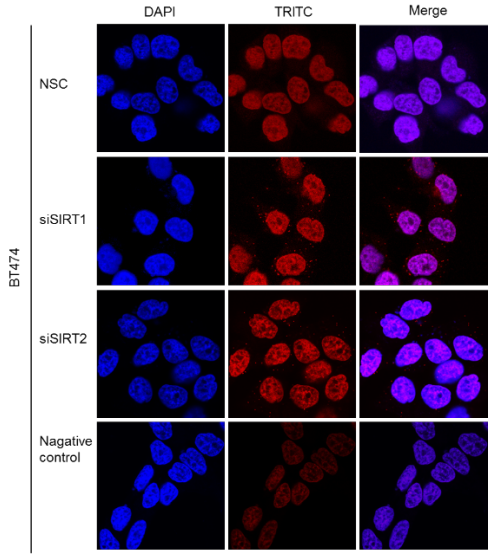

Figure 3. Duolink proximity ligation assay (PLA) for detection of FOXO3 and its post-translational modification of acetylated-lysine in BT474 cells. Cells were transiently transfected with SIRT1 and SIRT2 siRNA and fixed at 48 h post-transfection. Cells were transfected with non-specific siRNA used as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