REVIEW

Molecular mechanisms of FOXO1 in adipocyte differentiation

Junye Chen^{1,2}, Yi Lu^{3,4}, Mengyuan Tian^{1,2} and Qiren Huang^{3,4}¹Jiangxi Medical College, Nanchang University, Nanchang, Jiangxi Province, People's Republic of China²Nanchang Joint Programme, Queen Mary, University of London, London, UK³Key Provincial Laboratory of Basic Pharmacology, Nanchang University, Nanchang, Jiangxi Province, People's Republic of China⁴Department of Pharmacology, School of Pharmacy, Nanchang University, Nanchang, Jiangxi Province, People's Republic of ChinaCorrespondence should be addressed to Q Huang: qrhuang@ncu.edu.cn

Abstract

Forkhead box-O1 (FOXO1) is a downstream target of AKT and plays crucial roles in cell cycle control, apoptosis, metabolism and adipocyte differentiation. It is thought that FOXO1 affects adipocyte differentiation by regulating lipogenesis and cell cycle. With the deepening in the understanding of this field, it is currently believed that FOXO1 translocation between nuclei and cytoplasm is involved in the regulation of FOXO1 activity, thus affecting adipocyte differentiation. Translocation of FOXO1 depends on its post-translational modifications and interactions with 14-3-3. Based on these modifications and interactions, FOXO1 could regulate lipogenesis through PPAR γ and the adipocyte cell cycle through p21 and p27. In this review, we aim to provide a comprehensive FOXO1 regulation network in adipocyte differentiation by linking together distinct functions mentioned above to explain their effects on adipocyte differentiation and to emphasize the regulatory role of FOXO1. In addition, we also focus on the novel findings such as the use of miRNAs in FOXO1 regulation and highlight the improvable issues, such as RNA modifications, for future research in the field.

Key Words

- ▶ FOXO1
- ▶ adipocyte differentiation
- ▶ 14-3-3
- ▶ microRNA
- ▶ obesity

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Introduction

Obesity is considered to be a syndrome involving chronic and low-level inflammatory response characterized by abnormal production of cytokines and activation of the inflammatory response signalling pathway in adipose tissues (Hotamisligil 2006). Obesity normally means adipocyte hypertrophy and/or hyperplasia, as well as an imbalance between the deposition and expenditure of energy (Wu *et al.* 2017). Adipokines secreted from adipocytes can cause several kinds of obesity-related complications. Indeed, adipose tissue can be divided into white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the most predominant adipose tissue extensively located at the subcutaneous compartment

(referred to as subcutaneous WAT) and the surrounding of internal organs (also known as visceral WAT) (Cinti 2005). WAT is responsible for storage of excess energy in the form of triglycerides and production of adipokines, which readily leads to obesity, inflammation and cancers (Cinti 2005, Qiang *et al.* 2012). Whereas, BAT combusts fat by dissipating heat mediated by uncoupling protein 1 (UCP-1) in mitochondria (Cinti 2005). In 2012, Boström *et al.* found the existence of a third adipocyte type, named brown-fat-like (Boström *et al.* 2012). Currently, scientists term it 'beige' or 'brite' adipocytes, which are generally considered as a middle state between the white and brown adipocytes (Wu *et al.* 2012, Harms & Seale 2013).

The transcription factor FOXO family comprises FOXO1, FOXO3, FOXO4 and FOXO6 (Ogg *et al.* 1997, Tzivion *et al.* 2011). FOXO1 is one of the downstream targets of AKT (Fig. 1) and takes part in cell cycle control, apoptosis, metabolism and adipocyte differentiation (Dowell *et al.* 2003, Battiprolu *et al.* 2012, Song *et al.* 2015). FOXO1 is normally expressed in insulin-responsive tissues and organs (Nakae *et al.* 2002, 2003, Dowell *et al.* 2003), such as the liver, adipose tissue and pancreas. It contains three highly conserved phosphorylation sites in mice, T24, S253 and S316 (corresponding phosphorylation sites in humans are T24, S256 and S319; Asada *et al.* 2007) targeted by phosphorylated AKT (Fig. 1) (Nakae *et al.* 1999). The phosphorylation of FOXO1 at these sites by AKT inhibits the transcriptional activation mediated by FOXO1. Similarly, the acetylation of FOXO1 attenuates its DNA-binding affinity and promotes its shuttling from nuclei to cytoplasm (Yang & Seto 2008). Accordingly, its acetylation could enhance the effect of AKT phosphorylation to further inhibit the transcriptional activation by FOXO1 (Calnan & Brunet 2008). FOXO1 is widely studied in cancer due to its regulation of the cell cycle (Nakae *et al.* 2002, 2008a, Song *et al.* 2015). Given this condition, it is well thought that FOXO1 is involved in the regulation of the cell cycle during adipocyte differentiation (Nakae *et al.* 2002, 2003, 2008a). Numerous studies have shown that PPAR γ is a prerequisite for adipocyte differentiation and activated FOXO1 could inhibit lipogenesis via PPAR γ (Evans *et al.* 2004, Dominy & Puigserver 2010, Song *et al.* 2010, Qiang *et al.* 2012). Based on the accumulated knowledge in this field and our previous studies (Wu *et al.* 2017, Lu *et al.* 2018), this review summarizes the mechanism of FOXO1 in the regulation of adipocyte differentiation in order to shed light on the potential further research.

Figure 1

Schematic of human FOXO1 primary structure. FOXO1 contains the forkhead DBD, NLS, NES and TAD domains. Acetylation sites (K245, K248 and K262) by CBP/P300, phosphorylation sites (T24, S256 and S319) by AKT and 14-3-3 binding sites (T24 and S256) are shown. DBD, DNA-binding domain; NES, nuclear export sequence; NLS, nuclear localization signal; TAD, transactivation domain. A full colour version of this figure is available at <https://doi.org/10.1530/JME-18-0178>.

Research history of FOXO1

Researches on FOXO1 has been going on for more than two decades. In 1993, FOXO1 gene was cloned for the first time by Galili *et al.* in the study of the t(2; 13) chromosomal translocation in rhabdomyosarcoma (Galili *et al.* 1993). It was found that a gene encoding the transcription factor paired box 3 (PAX3) fused with another gene encoding a homology protein containing the forkhead DNA-binding domain (DBD). X-ray crystallography (Clark *et al.* 1993) and nuclear magnetic resonance analyses (Jin *et al.* 1998) then showed that FOXO1 has a butterfly-like structure. Moreover, Clark *et al.* further characterized the three-dimensional structure of the DBD of HNF-3 γ /forkhead complexed with DNA at 2.5 Å resolution (Clark *et al.* 1993). The secondary structure of the forkhead domain contains four α -helices (H1, H2, H4 and H3), three β -strands (S1–S3) and two winged loops (W1 and W2) (Jin *et al.* 1998, Obsil & Obsilova 2011). H1 and H2 present H3 to the major groove of double-stranded DNA, where H3 directly interacts with the target DNA sequence (Clark *et al.* 1993, Obsil & Obsilova 2011). The dynamic data strongly supported the presence of H4 (Marsden *et al.* 1997) and the fourth helix was further proved to be inserted between H2 and H3 (Marsden *et al.* 1998). These findings fuelled further studies on FOXO1.

In 1996, Kohn *et al.* reported that overexpression of constitutively activated AKT could promote adipocyte differentiation (Kohn *et al.* 1996). Thereafter, Sakaue *et al.* found that adipocyte differentiation is related to PI3K (Sakaue *et al.* 1998). Until 1999, Datta *et al.* found that AKT could inhibit the transcriptional activity of FOXOs (Datta *et al.* 1999). A reduction in the transcriptional activity of FOXO1 caused by AKT phosphorylation was detected (Kaestner *et al.* 2000). In 2001, Nakae *et al.* confirmed that FOXO1 was involved in the regulation of G6P and PEPCK (Nakae *et al.* 2001). In 2003, Hribal *et al.* found that FOXO1 was regulated by PI3K in myoblast differentiation (Hribal *et al.* 2003). Meanwhile, Nakae *et al.* also found that FOXO1 was regulated by PI3K in adipocytes (Nakae *et al.* 2003). They reported that haploinsufficient *Foxo1*^{+/-} could restore the number and size of adipocytes in mice fed with high-fat diet (Nakae *et al.* 2003). Moreover, constitutively active non-phosphorylated nuclear FOXO1 inhibited the differentiation of preadipocytes (Nakae *et al.* 2003). Dominant-negative *Foxo1* with deletion of transcriptional activation domain (TAD) restored the differentiation of embryonic fibroblasts isolated from insulin receptor knockout mice and promoted adipocyte differentiation *in vitro* (Nakae *et al.* 2008a). Knockdown of *Foxo1* was

found to significantly suppress adipocyte differentiation as well (Munekata & Sakamoto 2009). Moreover, Nakae *et al.* further found that FCoR, a murine FOXO1 inhibitor, could directly acetylate FOXO1 through its intrinsic acetyltransferase activity. It can also disturb the interaction between SIRT1 and FOXO1 to inhibit the deacetylation of FOXO1 since SIRT1 is a deacetylase for FOXO1. Based on these two mechanisms, FCoR could curtail FOXO1 activity and promote adipocyte differentiation (Nakae *et al.* 2012). Of note, *Fcor* has no human analogue, which requires further researches for humans. Matsumoto *et al.* confirmed that FOXO1 is required for the function of PGC1 that promotes translation of *Ucp1* during brown adipocyte differentiation (Matsumoto *et al.* 2007). Ortega-Molina *et al.* reported that BAT has lower levels of phosphorylated AKT and phosphorylated FOXO1 as well. Then they verified that *Pten*^{tg}, carrying additional copies of *Pten*, could induce the binding of FOXO1 to the promoters of *Ucp1* and *Pgc1*, thus ameliorating brown adipocyte differentiation (Ortega-Molina *et al.* 2012). Recently, Nagashima *et al.* identified numerous compounds bound to purified FOXO1 by mass spectrometry affinity screening. One of them is 5-amino-7-cyclohexyl-aminoacyl-1-ethyl-6-fluoro-4-oxo-1-dihydroquinoline-3-carboxylic acid (AS1842856) (Nagashima *et al.* 2010). It has been shown that AS1842856 is a FOXO1 inhibitor that inhibits the transcriptional activity of FOXO1 by blocking the phosphorylation of FOXO1 and repressing interaction with DNA-binding sites (Zou *et al.* 2014). By means of such a mechanism, AS1842856 can further restrain autophagy, lipid accumulation (Liu *et al.* 2016) and adipocyte differentiation (Zou *et al.* 2014). At present, a large number of studies on FOXO1 in adipocyte differentiation are still continuing.

Dynamics of FOXO1 activation during adipocyte differentiation

Based on *in vitro* studies, the process of adipocyte differentiation contains several stages, including multipotent mesenchymal precursors, committed preadipocytes, growth arrest, mitotic clonal expansion, terminal differentiation and maturation of adipocytes (Tang *et al.* 2003, Gerin *et al.* 2009). When the preadipocyte cells grow to the 90% confluence, the growth inhibition resultantly occurs. At the clonal expansion stage, cells re-enter the cell cycle and carry out mitosis at least two times under the stimulation of lipogenic hormones and mitogenic compounds (Cornelius *et al.* 1994, Shamina & Rangwala 2000). During the terminal differentiation

stage, cells return to the growth arrest stage and result in the transformation from immature adipocytes to mature adipocytes (Cornelius *et al.* 1994, Shamina & Rangwala 2000). Zou *et al.* studied the activation and inactivation kinetics of FOXO1 through the phosphorylation and dephosphorylation of FOXO1 during adipocyte differentiation under natural conditions. The findings indicated that FOXO1 presented a sigmoid activation pattern during adipocyte differentiation (Zou *et al.* 2014). FOXO1 in 3T3-L1 preadipocytes was the least active on day 1 presented as inactivation peak 1 (IP1), which represented that cells re-entered the cell cycle and clonal expansion. Subsequently, FOXO1 reached the first activation peak (AP1) on day 3 when the cells were right in the growth arrest period after mitosis, which potentially supports the fact that FOXO1 regulates the cell cycle of preadipocytes (Nakae *et al.* 2003, Cheng & White 2011). IP2 occurred on day 5 accompanied by the expression upregulation of PPAR γ and adiponectin, which was consistent with the results of Liu *et al.* (2014). IP3 appeared on day 9 between AP2 on day 7 and AP3 on day 10 (Zou *et al.* 2014). The 'terminal differentiation' phase (day 4–12) exhibits multiple switches of the activation-inactivation, implying that FOXO1 has a complex regulatory role. Since the activation of FOXO1 during adipocyte differentiation is a periodic dynamic fluctuation, this may explain the paradox occurred in some experiments. For example, Kim *et al.* reported an increase of p-FOXO1 and p-AKT in 3T3-L1 cells under the treatment with PI3K inhibitor analogues, such as epigallocatechin-3-gallate (EGCG) (Kim *et al.* 2010, Kim & Sakamoto 2012).

FOXO1 shuttling between nucleus and cytoplasm

FOXO1 normally stays in the nucleus and keeps its transcriptional activity, thereby it could serve as a downstream protein to control the cell cycle. Residues within the basic region of the FOXO1 DBD (Fig. 1) are essential for nuclear targeting (Rena *et al.* 2001). The shuttling of FOXO1 needs the interaction between importins and exportins (VanDerHeide *et al.* 2004). Exportin 1, also termed as chromosomal region maintenance 1 protein (CRM1), recognizes nuclear export signals (NESs) presented in FOXO1 including leptomycin-sensitive NES (Vogt *et al.* 2005). NES contains a leucine-rich sequence downstream of the C-terminal AKT phosphorylation site, for instance, sequence MENLLDNLNLL of FOXO1 (Obsil & Obsilova 2008). Nuclear localization signals (NLSs) do not correspond to a particular consensus sequence but instead

form two different classes. One is called monopartite NLS, consisting of a single basic cluster of amino acid residues, and bipartite NLS comprising two basic clusters (Obsil & Obsilova 2008). Pan *et al.* reported that FOXO1-NESm (mutated) and FOXO1-NLSm primarily remained in the nucleus and the cytoplasm, respectively (Pan *et al.* 2017). In general, both NLS and NES interact with post-translational modifications of FOXO1 to regulate the translocation of FOXO1. However, there have been only a few studies on NLS and NES of FOXO1, and the mechanism of their interactions still needs further exploration.

FOXO1 phosphorylation

PI3K is a heterodimer comprising a catalytic subunit (110kDa) and a regulatory or adaptor subunit (Vanhaesebroeck & Waterfield 1999, Vanhaesebroeck & Alessi 2000). One of the main downstream mediators of PI3K signal is AKT, also known as PKB. The activated AKT is separated from plasma membrane and transported to cytoplasm and nucleus where it phosphorylates serine or threonine residues within AKT phosphorylation motifs of target proteins (Lawlor & Alessi 2001).

The transcriptional activation of FOXO1 is mainly mediated by binding to the conservative recognition motif TTGTTTAC of target genes, and its activity is inhibited by insulin and insulin-like growth factor-1 (IGF-1) signalling pathways (Li *et al.* 2017). FOXO1 binds to the DNA target in nuclei via sequence-specific interactions mediated by H3 and basic regions in the C-terminal of DBD (Obsil & Obsilova 2011). The interaction is expeditious and reversible, reflecting a dynamic process. Phosphorylation of FOXO1 at S256 by AKT decreases the binding affinity of FOXO1 to target DNA and increases the phosphorylation of other sites, such as T24 and S319 by AKT and other kinases, including serum- and glucocorticoid-induced kinase (SGK). The activity of FOXO proteins can be regulated by their cellular localization. Phosphorylation of FOXO1 causes it to be excluded from the nucleus (Zhang *et al.* 2002). Protein phosphatase 2 (PP2A) has been shown to be a FOXO1 phosphatase, which dephosphorylates FOXO1 either in the cytoplasm or in the nucleus (Yan *et al.* 2008).

Lu *et al.* demonstrated that EGCG or PI3K inhibitor LY294002 could repress the levels of p-FOXO1 and PI3K in 3T3-L1 cells and inhibit the adipocyte differentiation (Lu *et al.* 2018). Tang *et al.* found that the phosphorylation of all three AKT sites, or some of them, reduced the transcriptional activity of FOXO1 (Tang *et al.* 1999). They

first demonstrated the mutations of three phosphorylation sites into alanine residues enhanced the transcriptional activity of FOXO1 and made it resistant to the inhibition by AKT. S256 is located in a basic region of the FOXO1 DBD (Fig. 1), and phosphorylation at S256 may directly affect FOXO1-DNA binding and nuclear targeting. Thus, Guo *et al.* found that insulin and insulin-like growth factors could induce S256 phosphorylation of FOXO1 and further inhibit its transactivation (Guo *et al.* 1999). The mutation of serine to alanine (S256A) in the forkhead domain of FOXO1 could completely abolish the increase in total FOXO1 phosphorylation induced by insulin, but mutations of N-terminal and C-terminal phosphorylation sites did not display such an effect (Nakae *et al.* 2000). Rena *et al.* found that the S256A FOXO1 mutant was a dominant-negative inhibitor of AKT, which prevented conformational changes due to phosphorylation and inhibited phosphorylation of T24 and S319 *in vitro*. At the same time, T24A and S319A mutations prevented them from being phosphorylated, but they did not inhibit phosphorylation at other sites (Rena *et al.* 2001). Therefore, the so-called 'gatekeeper' S256 phosphorylation is a prerequisite for phosphorylation of T24 and S319.

Phosphorylation of S256 is also involved in the process of nuclear exclusion. Phosphorylated FOXO1 enters the cytoplasm via nuclear exclusion and loses its transcriptional activity (Brunet *et al.* 1999). A transient transfection study showed that S256 phosphorylation could inhibit the function of FOXO1 independently and proved its prerequisite position to other phosphorylation (Rena *et al.* 2001). However, a GFP fusion protein assay showed that T24/S319 phosphorylation was a prerequisite for FOXO1 nuclear exclusion (Zhang *et al.* 2002). Heide *et al.* reported that phosphorylation of serine residues in AKT phosphorylation motifs of the FOXO1 forkhead domain introduces a negative charge, which may affect NLS (Van Der Heide *et al.* 2004). By replacing S256 with aspartate (S256D) and introducing negative charge at this site, the nuclear exclusion of FOXO1 can be induced, which is similar to phosphorylation at S256. Notably, the T24A and S319A mutants could lead to the nuclear-targeting effect of FOXO1 and abrogate the effect of S256D on FOXO1 transport, thus abolishing nuclear exclusion (Zhang *et al.* 2002). Apparently, further research is needed to elucidate the relationship between these three residues.

Murine FOXO1 contains three highly conserved AKT recognition motifs, RXRXXS/T, in which 'X' denotes any residue (Alessi *et al.* 1996). The AKT phosphorylation sites located in both the N-terminus and the forkhead domain

are responsible for the nuclear exclusion because they affect the function of NLS and the association with 14-3-3 proteins (Brunet *et al.* 1999, Brownawell *et al.* 2001). The human S256 AKT conservative phosphorylation motif on target protein is Arg-Arg-Arg-Ala-Ala-Ser, while FOXO1's residues 251–253 are exactly Arg-Arg-Arg, which conforms to this motif. Furthermore, since residues 251–253 and 256 are also located within the DBD of FOXO1, the nuclear targeting of FOXO1 was completely abolished in unstimulated cells when these three arginine residues were replaced by neutral amino acids (Rena *et al.* 2001). Therefore, the three arginine residues are thought to be related to nuclear targeting. Altogether, phosphorylation of FOXO1 by AKT regulates the nuclear exclusion and nuclear targeting of FOXO1, thus affecting the transcriptional activity mediated by FOXO1.

FOXO1 and 14-3-3

The molecular weight of the 14-3-3 is approximately 30 kDa. It has a U-shaped structure. With the 'U-structure', 14-3-3 can recognize and bind phosphorylated serine or threonine residues. Meanwhile, this protein can form homodimers and heterodimers with other family members (Van Der Heide *et al.* 2004). Scientists have shown that phosphorylated FOXO1 (p-FOXO1) can bind to 14-3-3 and become inactivated, which is caused by FOXO1 translocation (Fig. 2) (Brunet *et al.* 1999, 2002, Zhao *et al.* 2004, Tzivion *et al.* 2011).

Binding of 14-3-3 to p-FOXO1 promotes nuclear exclusion

Nagashima *et al.* identified that purified FOXO1 bound to 14-3-3 by mass spectrometry (Nagashima *et al.* 2010). Brunet *et al.* further confirmed that after FOXO3 phosphorylation by AKT, 14-3-3 enhances the nuclear exclusion of FOXO3 by interacting with two nuclear export sequences located at the C-terminal of FOXO3. Moreover, 14-3-3 inhibits the re-entry of FOXO3 into nuclei by masking two NLSs, ²⁴⁸RRR₂₅₀ and ²⁶⁹KKK₂₇₁, which neighbour the binding sites of 14-3-3 and human FOXO3 phosphorylation site S253. Thus, the sequestration of FOXO3 in cytoplasm is promoted (Brunet *et al.* 2002). They also demonstrated that the bindings of 14-3-3 to phosphorylated FOXO1 in nuclei induces a conformational change of FOXO1, which exposes NES to exportin/CRM1 and promotes nuclear exclusion of the 14-3-3/FOXO1 complex (Brunet *et al.* 2002). Although the N-terminal-binding motif of 14-3-3 is far from NES, 14-3-3 can still affect the conformational

Figure 2

Schematic model for the regulation of FOXO1 by reversible phosphorylation and acetylation. FOXO1 recruits CBP/P300 to initiate an acetylation. After acetylation of FOXO1, the FOXO1 phosphorylation by p-AKT is induced, and then 14-3-3 binds to the phosphorylated sites to trigger the nuclear exclusion. SIRT1 and SIRT2 deacetylated FOXO1 in the nucleus and cytoplasm, respectively. PP2A dephosphorylates and activates FOXO1. Activated FOXO1 binds to the promoters of target genes encoding p21, p27 and PPAR γ and initiates subsequent transcriptions. A full colour version of this figure is available at <https://doi.org/10.1530/JME-18-0178>.

change. Therefore, 14-3-3 may control the subcellular localization of FOXO1 and simultaneously affect the function of FOXO1.

Binding of 14-3-3 to p-FOXO1 inhibits the re-entry into nuclei

AKT-mediated FOXO1 phosphorylation induces the binding of 14-3-3, and the resulting complex is easily transported to the cytoplasm. The complex of 14-3-3/FOXO1 could prevent FOXO1 from re-entering nuclei by interfering with the NLS function of FOXO1 (Brownawell *et al.* 2001, Zhao *et al.* 2004, Obsilova *et al.* 2005, Silhan *et al.* 2009). Because the interaction between FOXO1 and 14-3-3 is dependent on the FOXO1 phosphorylation, blocking its phosphorylation in 'gatekeeper' S256 can abolish 14-3-3 binding and destroy the binding site of 14-3-3 (Yaffe 2002, Van Der Heide *et al.* 2004, Tzivion *et al.* 2011). Obsilova *et al.* studied the crystal structure of FOXO4 and verified the effect of 14-3-3 on the conformational change of FOXO NLS using a FOXO4 NLS model (Obsilova *et al.* 2005). Paradoxically, it was found that the binding of 14-3-3 to FOXO4 did not result in any dramatic conformational change. The results also displayed that the FOXO4 forkhead domain was anchored in the central channel of the 14-3-3 dimer, which is consistent with the assumption that 14-3-3 masks DNA

binding. The non-classical bipartite NLS of FOXO1 contains 12 arginine and lysine residues located on both sides of the second AKT/14-3-3 binding motif in the forkhead domain. The two basic clusters are independent and normally both of them are required for nuclear targeting (Obsil & Obsilova 2008). Therefore, 14-3-3 is considered to prevent nuclear reimport of FOXO1 by masking NLS (Brunet *et al.* 2002, Silhan *et al.* 2009).

Binding of 14-3-3 to p-FOXO1 prevents dephosphorylation and degradation

Brunet *et al.* reported that AKT phosphorylation motifs of the N-terminal and forkhead domains in FOXO1 were involved in 14-3-3 binding (Brunet *et al.* 1999). Dobson *et al.* also indicated that the 14-3-3 binding stabilizes FOXO3 by suppressing its dephosphorylation and degradation, as well as the well-defined negative FOXO regulation (Dobson *et al.* 2011). Binding of 14-3-3 involves recognition of the RSXpSXP and RXXXpSXP motifs, where pS represents the phosphorylated serine residue (Hermeking & Benzinger 2006). Therefore, FOXO1 has an ideal 14-3-3 binding site that overlaps the N-terminal AKT phosphorylation motif (Figs 1 and 2). Accordingly, 14-3-3 could prevent FOXO1 from dephosphorylation. Thus, the availability of 14-3-3 determines the fate of degradation or recycling of phosphorylated FOXO1. In general, by associating with NLS and NES of FOXO1, 14-3-3 achieves translocation between nucleus and cytoplasm. In the meantime, because of the overlap of AKT/14-3-3 binding motifs, 14-3-3 could reinforce the masking of NLS after phosphorylation to stabilize the status of FOXO1.

FOXO1 acetylation

FOXO1 can be acetylated by CREB-binding protein (CBP/P300) and P300/CBP-associated factor (P/CAF), while histone deacetylases (HDACs), such as SIRT1 and SIRT2, can deacetylate it (Fig. 2) (Xie *et al.* 2012). The acetylation of K222, K245, K248, K262, K265, K274 and K294 sites of FOXO1 has been reported to regulate its DNA-binding affinity and its sensitivity to AKT phosphorylation (Matsuzaki *et al.* 2005, Qiang *et al.* 2010). Among these sites, K245 and K248 are extremely particular because their acetylation substantially weakens the DNA-binding ability of FOXO1. In addition, the acetylation of K242, K245 and K262 are sufficient to reduce the transcriptional activity of FOXO1 (Wang *et al.* 2014). These three acetylation sites of FOXO1 (K242, K245 and K262) located in the W2 region of the forkhead domain are directly involved in DNA

recognition and/or FOXO-DNA complex stabilization (Lai *et al.* 1993). It has been reported that acetylation of positively charged lysine residues in W2 region may inhibit the binding of FOXO to DNA (Matsuzaki *et al.* 2005). Accordingly, Brent *et al.* showed that the W2 region in the C-terminal of the forkhead domain was necessary for DNA binding, and FOXO1 acetylation mediated by CBP/P300 reduced the DNA-binding affinity (Brent *et al.* 2008). By using acetylation-defective (3K-to-3R) and acetylation-mimicking (3K-to-3A and 3K-to-3Q) mutants, Matsuzaki *et al.* confirmed that the acetylation of FOXO1 did reduce the affinity of DNA binding (Matsuzaki *et al.* 2005). To date, it has been identified that the acetylation of FOXO1 regulates its activity through three different mechanisms: (1) acetylation of lysine residues in the forkhead domain of FOXO1 reduces its binding ability to DNA; (2) FOXO1 acetylation promotes the phosphorylation of S256 of human FOXO1 mediated by AKT, which leads to nuclear exclusion; (3) FOXO1 acetylation can lead to nuclear exclusion independent of its phosphorylation state. On the contrary, FOXO1 deacetylation promotes nuclear retention (Matsuzaki *et al.* 2005, Qiang *et al.* 2010). Besides, Sewastianik *et al.* confirmed FOXO1 acetylation facilitated its proapoptotic activity and tumour-suppressive function in DLBCL cells (Sewastianik *et al.* 2016). Taken together, the acetylation and deacetylation of FOXO1 harmoniously alter its function and transcriptional activity during adipocyte differentiation.

Interaction between acetylation and phosphorylation of FOXO1 in adipocyte differentiation

The interaction of acetylation and phosphorylation in the regulation of FOXO1 were also found in adipocyte differentiation. Matsuzaki *et al.* have shown that FOXO1 acetylation leads to an increase in AKT-mediated phosphorylation of FOXO1 at S253, thereby further diminishing FOXO1 binding to DNA (Matsuzaki *et al.* 2005). In addition, Qiang *et al.* also confirmed that acetylated FOXO is more likely to be translocated to the cytoplasm (Qiang *et al.* 2010). Moreover, the N-terminal AKT phosphorylation site also regulates the binding of FOXO proteins to CBP/P300. Mahmud *et al.* reported that the addition of growth factor and the phosphorylation of N-terminal AKT phosphorylation motifs interfered with the interaction between CBP/P300 and FOXO3 (Mahmud *et al.* 2002), which may block the FOXO3 acetylation and directly affect the transcriptional activation mediated by FOXO3 (Van Der Heide *et al.* 2004). In the nucleus, FOXO1 recognizes and binds to the promoter of target

genes. When CBP/P300 is recruited into the FOXO1-DNA complex, it can result in two consequences. On the one hand, CBP/P300-mediated histone acetylation facilitates the transcription of FOXO1-targeted genes (Yang *et al.* 2009); on the other hand, the CBP/P300-mediated FOXO1 acetylation will then attenuate its DNA-binding affinity (Matsuzaki *et al.* 2005). Therefore, AKT can effectively phosphorylate the acetylated FOXO1 (Fig. 2). Subsequently, p-FOXO1 interacts with 14-3-3 in the nucleus, which deactivates NLS. Meanwhile, p-FOXO1 interferes with the CBP/P300 recruitment to initiate a series of protein-protein interactions, such as 14-3-3 and exportin/CRM1, which eventually leads to the cytoplasmic retention of FOXO1 (Vogt *et al.* 2005).

Histone deacetylase: SIRT6

Sirtuins (SIRT6) participate in the regulation of metabolic pathway by deacetylating proteins (Lavu *et al.* 2008, Kelly 2010). Till date, seven SIRT6s were reported to be identified. Among these SIRT6s, SIRT1 plays the most critical role in the regulation of metabolic pathways by deacetylating FOXO1 in the nucleus (Fig. 2) (Schug & Li 2011, Carafa *et al.* 2012). SIRT2 and SIRT 3 deacetylate FOXO1 in the cytoplasm and mitochondria, respectively (Daitoku *et al.* 2011). Oliveira *et al.* reported that the expression of AMPK and FOXO1 in the murine adipose tissue was increased after treatment with resveratrol, a natural activator of the SIRT6s family extracted from traditional Chinese medicine *Polygonum cuspidatum* (Oliveira Andrade *et al.* 2014). SIRT6s also regulate the expression of adiponectin (Qiang *et al.* 2007) and TNF- α (Gillum *et al.* 2011, Lin *et al.* 2012), as well as take part in the energy balance at the level of hypothalamus (Çakir *et al.* 2009, Sasaki & Kitamura 2010). In addition, SIRT6s play significant roles in lipogenesis, lipid hydrolysis and fatty acid mobilization during fasting (Picard *et al.* 2004, Chakrabarti *et al.* 2011).

SIRT1, a mammalian ortholog of yeast SIR2, is a NAD-dependent deacetylase that regulates several transcription factors, co-repressors and co-activators. In particular, SIRT1 deacetylates FOXO1 to increase nuclear retention and FOXO1 activity (Qiang *et al.* 2010). FOXO1 has a conserved LXXLL motif in their C-terminal region for its binding to SIRT1. The disruption of the LXXLL motif of FOXO1 enhances acetylation of FOXO1 and inhibits the transcriptional activity of FOXO1 in liver (Nakae *et al.* 2006). In addition, SIRT1 interacts with nuclear receptor's co-repressors to inhibit the transcriptional activities of nuclear receptors, such as PPAR γ (Bai *et al.* 2008, Lee *et al.* 2017). Under the stimulation of growth factor,

SIRT1 is mainly distributed in the nuclei and directly binds to FOXO1. Subsequently, it deacetylates FOXO1 and ultimately increases the transcriptional activity of FOXO1. However, with elongation of deacetylation time, degradation caused by ubiquitin proteasome system also occurs and thus reducing its transcriptional activity (Nakae *et al.* 2006).

SIRT2 inhibits adipocyte differentiation by deacetylating FOXO1 in the cytoplasm. Jing *et al.* showed that SIRT2 knockdown during clonal expansion of 3T3-L1 adipocyte differentiation enhanced the acetylation of FOXO1 and promoted AKT-mediated phosphorylation, which caused subsequent FOXO1 nuclear exclusion. Whereas the overexpression of SIRT2 antagonized the above-mentioned processes (Jing *et al.* 2007). Moreover, the results of the Oil Red O staining also showed that SIRT2 knockdown cells exhibited a rapid lipid accumulation after 4 days of differentiation (Jing *et al.* 2007). The mechanisms underlying the decreasing level of SIRT2 in clonal expansion includes increased acetylation of FOXO1 and direct interaction between SIRT2 and FOXO1 in the cytoplasm, which is consistent with the sigmoid expression pattern of FOXO1 during adipocyte differentiation. This interaction enhances the insulin-stimulated phosphorylation of FOXO1 and regulates the translocation of FOXO1 (Jing *et al.* 2007). Meanwhile, SIRT2 also promotes the binding of FOXO1 to PPAR γ , thereby it inhibits the transcriptional activity of PPAR γ (Fei & Qiang 2009).

FOXO1 methylation and O-GlcNAcylation

Methylation is known as a type of arginine modification that positively or negatively influences protein or RNA activity (Poornima *et al.* 2016). Protein arginine methyltransferase-1 (PRMT1) mediates FOXO1 methylation at conserved R248 and R250 in the phosphorylation motif of AKT, suggesting that this methylation interferes with the ability of AKT to phosphorylate the S253 FOXO1 (S256 in human FOXO1) during adipocyte differentiation (Yamagata *et al.* 2008). The knockdown of PRMT1 promoted the decrease of FOXO1 function through its increased nucleus exclusion and protein degradation. PRMT1 knockdown also inhibited the hepatic gluconeogenesis by facilitating the phosphorylation of FOXO1 (Choi *et al.* 2012). Lv *et al.* also reported the knockdown of PRMT1-induced glucose-stimulated insulin secretion, promoting the methylation and nuclear localization of FOXO1 in INS-1 cells (Lv *et al.* 2015). However, the effect of PRMT1 on FOXO1

methylation in 3T3-L1 or other precursor adipocytes has not been reported yet, which needs to be further explored according to the known mechanism.

O-GlcNAcylation means that an O-GlcNAc group is added to the serine/threonine residues of protein and it is a reversible post-translation modification of proteins (Xie *et al.* 2012). Fardini *et al.* found that the O-GlcNAcylation of FOXO1 enhanced the transcriptional activity of FOXO1 by promoting nuclear retention whereas inhibited AKT activity through the expression of IGFBP1 (Fardini *et al.* 2014). O-GlcNAcylation involves O-linked b-N-acetyl glucosamine transferase (OGT) and O-linked b-N acetyl-glucosaminidase (OGA), which are responsible for the addition and removal of O-GlcNAc, respectively (Xie *et al.* 2012). Housley *et al.* identified FOXO1 residues S550, T648, S654 and T317 as GlcNAcylated products by electron transfer dissociation tandem mass spectrometry (ETD-MS/MS). Only the mutation of T317 to alanine could reduce the transcriptional activity of FOXO1 by high glucose, while the mutation of other residues had no such effect (Housley *et al.* 2008). Later, Fardini *et al.* identified a new O-GlcNAcylation site T646 on FOXO1, whereas its mutation fails to impact the FOXO1 O-GlcNAcylation and transcriptional activity, suggesting that O-GlcNAcylated residues remain to be explored (Fardini *et al.* 2015). Housley *et al.* showed that high glucose upregulated mRNA expression of *Pepck* and *G6pc* by increasing GlcNAcylation of FOXO1 in the absence of insulin (Housley *et al.* 2008). Moreover, Whelan *et al.* reported that increasing O-GlcNAc in 3T3-L1 adipocytes could reduce the interaction of PI3K with IRS1 and IRS2, as well as the phosphorylation of AKT, suggesting O-GlcNAcylation of FOXO1 is crucial in adipogenesis regulation (Whelan *et al.* 2010). Similar to methylation, most of the studies on FOXO1 O-GlcNAcylation were limited to the mechanism of glucogenesis on hepatocytes and islet β cells under simulated hyperglycemia, but little is known about FOXO1 O-GlcNAcylation in adipocyte differentiation, which should be further researched.

FOXO1 and the cell cycle

FOXO1 is thought to inhibit adipocyte differentiation via cell cycle control. FOXO1, together with PI3K and AKT proteins, was reported to be fundamental signal transduction elements of insulin or IGFs signalling in 3T3-L1 adipocytes (Nakae *et al.* 2003, 2008b, Obsil & Obsilova 2008). FOXOs can upregulate negative regulators of the G1/S transition of the cell cycle, such as p21, p27 and p130 (Huang & Tindall 2007a). Scientists have shown

that FOXO1 can arrest hepatic stellate cells in G0/G1 phase via p21 and p27 (Adachi *et al.* 2007). Nakae *et al.* reported that the downstream proteins of FOXO1 include cell cycle inhibitors, such as p21/p27/pRB, and 3T3-L1 cells were arrested in G0/G1 phase by overexpression of FOXO1 (Nakae *et al.* 2003). While AKT phosphorylates FOXO1, it also elicits the phosphorylation of p27 at T157 (Tsujimura & Obata 2000, Liang *et al.* 2002, Shin *et al.* 2002). Notably, when p27 was located in the cytoplasm, it did not prevent the progress of the cell cycle but can promote cell migration and movement (Wu *et al.* 2006). The re-localization of p27 in rodent cells was observed after PI3K/AKT activation (Fujita *et al.* 2003). Meanwhile, AKT-phosphorylated FOXO1 induces phosphorylation of p21 at T145. Like p27, the phosphorylation of p21 leads to the cytoplasmic localization of p21 (Zhou *et al.* 2001). In general, FOXO1 regulates adipocyte differentiation through eliciting cell cycle arrest and upregulating the expression of p21 and p27 in a sigmoid activation pattern, which plays an inhibitory role in the early period of adipocyte proliferation and the clonal expansion.

Cyclin-dependent kinase 1 (CDK1) phosphorylates FOXO1 at S249 (Liu *et al.* 2008, Yuan *et al.* 2008). Yuan *et al.* demonstrated that CDK1 did not affect the phosphorylation of S256, but pS249 inhibited the interaction between pS256 and 14-3-3, which led to nuclear retention and activation of FOXO1-dependent transcription (Yuan *et al.* 2008). Intriguingly, CDK2 phosphorylates FOXO1 at S249 as well, resulting in the cytoplasmic localization and inhibition of FOXO1 (Huang *et al.* 2006, Huang & Tindall 2007a). Recently, Kim *et al.* reported CDK2 could phosphorylate FOXO1 in MIN6 cells and human β -cells at the S256 residue, a key to FOXO1 shuttling, which may explain why CDK2-mediated phosphorylation of FOXO1 leads to cytoplasmic retention (Kim *et al.* 2017). More experiments are required to further illustrate the relationship between CDKs and FOXO1.

FOXO1 activates the transcription of p21 and p27 and simultaneously inhibits the transcriptional activity of cyclins D1 and D2 (Greer & Brunet 2008). It was reported that FOXO1 mutant (H215R) failed to bind to the FOXO-recognition element (FRE) sequence and was therefore recruited to the promoters of cyclins D1 and D2 to inhibit their transcription activity (Ramaswamy *et al.* 2002). A conservative FRE, (G/C)(T/A)AA(C/T)AA, was identified by high-affinity DNA-binding studies (Gilley *et al.* 2003). In fact, functional FRE sites have been recognized in the promoters encoding fatty acid synthetase (FAS) ligands, insulin-like growth factor-binding protein 1 (IGFBP1) and some other proteins (Accili & Arden 2004, Greer &

Brunet 2005). FOXO1 may inhibit its own expression by a crosstalk with other transcriptional regulators or by genes containing FRE-independent FOXO-binding elements in their promoters (Ramaswamy *et al.* 2002, Schmidt *et al.* 2002, Huang & Tindall 2007b). In addition to being involved in cell cycle regulation, cyclin D1 has been demonstrated to inhibit PPAR γ -mediated lipogenesis through HDACs recruitment (Fu *et al.* 2005). Altogether, these data suggest that FOXO1 plays a dominant role in cell cycle control of adipocyte differentiation.

FOXO1 and lipogenesis

FOXO1 is also deemed to inhibit adipocyte differentiation through suppressing lipogenesis. The anti-lipogenic effect of FOXO1 seems to be controlled by insulin signals. When the insulin receptor IRS or the insulin target AKT were absent, the activity of FOXO1 was reinforced, resulting in the blocking of adipocyte differentiation (Accili & Taylor 1991, Miki *et al.* 2001, Tseng *et al.* 2004). FOXO1 regulates the expression and transcriptional activity of PPAR γ and CCAAT-enhancer-binding proteins (C/EBPs), two pivotal transcription factors in lipogenesis of terminal differentiation (Cristancho & Lazar 2011). Rapid activation of C/EBP β and C/EBP δ upregulates the expression of PPAR γ at the early stage of differentiation (Farmer 2006). Meanwhile, the mRNA level of PPAR γ increases during adipocyte differentiation (Liu *et al.* 2014). PPAR γ modulates the expression of genes encoding enzymes which are involved in glucolipid metabolism and effectors in lipogenesis, such as GLUT-4 (MacDougald & Lane 1995). Translocation of GLUT-4 from cytoplasm to cell membrane regulates glucose uptake in adipose tissue and skeletal muscle (Jackson *et al.* 2000, Singh *et al.* 2011). Activated FOXO1 binds to the PPAR γ promoter and inhibits the transcriptional activity of PPAR γ by competitively suppressing the formation of functional PPAR γ /retinoid X receptor (RXR) complex, thus inhibiting the adipose differentiation (Armoni *et al.* 2006, Kousteni 2012). Meanwhile, the inhibition of FOXO1 activity in WAT enhances glucose tolerance, insulin sensitivity and energy consumption in both normal and high-fat diet transgenic mice with the deletion of TAD (Nakae *et al.* 2008a). All of these indicate that FOXO1 plays an important role in lipogenesis.

FOXO1 and microRNA

MicroRNA (miRNA) is a small non-coding RNA with a length of approximately 18–24 nt (Maute *et al.* 2013). It

binds to the 3'-untranslated region (UTR) of target mRNA to perform mRNA cleavage and translational repression of target genes (Bartel 2004). miRNA is involved in various important cell processes, including early development (Reinhart *et al.* 2000), fat metabolism (Xu *et al.* 2003), cell proliferation and cell differentiation (Kawasaki & Taira 2003). It has been reported that miR-143, miR-375, miR-103 and miR-107 enhanced adipocyte differentiation (Esau *et al.* 2004, Ling *et al.* 2011, Trajkovski *et al.* 2011), whereas miR-27b, let-7 and miR-138 repressed adipogenesis (Karbiener *et al.* 2009, Sun *et al.* 2009, Yang *et al.* 2011). Furthermore, Ahn *et al.* demonstrated that miR-146b facilitated adipocyte differentiation through downregulation of SIRT1 mRNA level and promotion of FOXO1 acetylation (Ahn *et al.* 2013, Li *et al.* 2017). Dong *et al.* reported that both transcriptional and translational levels of several novel lipogenic genes were upregulated by overexpression of miR-15a/b in the porcine preadipocytes, which promoted adipocyte differentiation and lipogenesis through suppression of FOXO1 (Dong *et al.* 2014). Dong *et al.* also reported that the expression level of miR-15a/b was relatively high during the early stage of adipocyte differentiation and decreased after culture for 4 days, which was consistent with the dynamic change in expression level of FOXO1 as mentioned above.

In recent years, a large number of studies have found and characterized different modification types of RNA base in coding and non-coding RNA, which have been termed as 'epitranscriptomics' (Frye *et al.* 2016). With the innovative chemical and biochemical techniques, the dynamic RNA modifications have been recognized in the transcriptome, including N1-methyladenosine (m1A), N6-methyladenosine (m6A), inosine (I), pseudouridine (Ψ), 5-methylcytosine (m5C) and 5-hydroxymethylcytosine (hm5C) (Song & Yi 2017). It has been reported that the RNA modifications on miRNA are mainly m5C and m6A (Li & Mason 2014). Meanwhile, Xhemalce *et al.* thought that RNA modifications on miRNA mainly affect the specificity and efficacy of miRNA through methylation and epitranscriptomic changes (Xhemalce *et al.* 2012). Consequently, we believe that with further studies of RNA modification on certain related miRNA of FOXO1, the corresponding FOXO1 regulation mechanisms will be clarified, and the understanding of the regulation mechanism of FOXO1 in adipocyte differentiation will be further deepened.

Conclusion

In general, FOXO1 regulates adipocyte differentiation through the control of the cell cycle and lipogenesis in

Figure 3

FOXO1 regulates adipocyte differentiation via cell cycle control and adipogenesis. FOXO1 participates in cell cycle regulation through p21 and p27, and takes part in lipogenesis by inhibiting the transcriptional activity of PPAR γ to regulate adipocyte differentiation. A full colour version of this figure is available at <https://doi.org/10.1530/JME-18-0178>.

a periodic dynamic fluctuation manner (Fig. 3). These two events are influenced by the translocation of FOXO1, which determines its activity and has been shown to be associated with its post-translational modifications and interactions with 14-3-3 and SIRT1s and so on. Given the poor links between the regulatory mechanisms in which FOXO1 is involved, current studies are almost devoted to distinct parts of FOXO1 regulation during adipocyte differentiation. We summarized the molecular mechanism of FOXO1 transcriptional activity regulation during adipocyte differentiation. FOXO1 acetylation, methylation and O-GlcNAcylation could interact with its phosphorylation and alter the DNA-binding affinity of FOXO1, which further influences the transcription activation by FOXO1. Moreover, acetylation and phosphorylation of FOXO1 synergistically facilitate its nuclear shuttling, whereas methylation and O-GlcNAcylation of FOXO1 interfere with FOXO1 phosphorylation. In view of the mechanism as described above, FOXO1 could elicit cell cycle arrest and upregulates cell cycle inhibitors p21 and p27 in a sigmoid activation pattern in the early period of adipocyte differentiation

and the clonal expansion. While during the terminal differentiation, activated FOXO1 binds to the PPAR γ promoter and inhibits the transcriptional activity of PPAR γ by competitively suppressing the formation of functional PPAR γ /RXR/DNA complex, thereby inhibiting lipogenesis and adipocyte differentiation. By providing a more comprehensive overview of the regulatory network and linking separate events of FOXO1 regulation in adipocyte differentiation, our work is expected to provide more FOXO1-related targets, such as miRNA modifications, for further researches.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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