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Menin Upregulates FOXO1 Protein Stability by Repressing Skp2-Mediated Degradation in β Cells

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Objectives: Menin, a chromatin binding protein, interacts with various epigenetic regulators to regulate gene transcription, whereas forkhead box protein O1 (FOXO1) is a transcription factor that can be regulated by multiple signaling pathways. Both menin and FOXO1 are crucial regulators of β-cell function and metabolism; however, whether or how they interplay to regulate β cells is not clear.

Methods: To examine whether menin affects expression of FOXO1, we ectopically expressed menin complementary DNA and small hairpin RNA targeting menin via a retroviral vector in INS-1 cells. Western blotting was used to analyze protein levels.

Results: Our current work shows that menin increases the expression of FOXO1. Menin stabilizes FOXO1 protein level in INS-1 cells, as shown by increased half-life of FOXO1 by menin expression. Moreover, menin represses ubiquitination of FOXO1 protein and AKT phosphorylation, We found that menin stabilizes FOXO1 by repressing FOXO1 degradation mediated by S-phase kinase-associated protein 2 (Skp2), an E3 ubiquitin ligase, promoting caspase 3 activation and apoptosis.

Conclusions: Because FOXO1 upregulates the menin gene transcription, our findings unravel a crucial menin and FOXO1 interplay, with menin and FOXO1 upregulating their expression reciprocally, forming a positive feedback loop to sustain menin and FOXO1 expression.

Key Words: menin, FOXO1, ubiquitination, Skp2, β cell

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Menin is mainly a nuclear protein that is encoded by the *MEN1* gene mutated in human multiple neoplasia type 1 syndrome.[1] Based on functional cellular studies and x-ray crystal structure studies, menin functions at least partly as a scaffold protein.[2,3] Menin interacts with various epigenetic regulators including histone methyl transferase MLL1 (mixed lineage leukemia) protein, to regulate transcription of genes such as cyclin-dependent kinase inhibitors (p18ink and p27cip).[4] Moreover, menin can also enhance apoptosis.[5] Furthermore, our previous studies have shown that menin is a key β-cell mass regulator, as ablation of the *Men1* gene reverses preexisting hyperglycemia in diabetes and prevents development of diabetes in streptozotocin-induced diabetes in mice.[6,7]

Forkhead box protein O1 (FOXO1) is a member of forkhead box (FOX)–containing superfamily transcription factors.[8] It has a plethora of activities including regulating cell survival, metabolism such as gluconeogenesis, and β-cell proliferation.[9–11] FOXO1 is regulated by multiple mechanisms including its transcription, methylation, phosphorylation, and ubiquitination.[12–15] Multiple studies have shown that serine/threonine protein kinases such as AKT can phosphorylate FOXO1 at several residues, and phosphorylated FOXO1 increases its binding to 14–3–3 to shuttle to and then sequester in the cytoplasm, repressing the function of FOXO1 as a transcription factor to regulate gene transcription.[16,17] On the other hand, phosphorylated FOXO1 also has increased binding affinity to S-phase kinase-associated protein 2 (Skp2), an E3 ligase, resulting in increased ubiquitnation.[18] The ubiquitinated FOXO1 is targeted for proteasome-mediated degradation, leading to reduced FOXO1 protein half-life and thus stability, lowering the steady FOXO1 protein level and thus function.

Thus, it is clear that both menin and FOXO1 are crucial regulators of β-cell function and metabolism; however, whether or how they interplay to regulate β cells is not clear. This prompted us to investigate how menin regulates FOXO1. Our current study unravels that menin increases FOXO1 protein stability and therefore the steady FOXO1 protein level. Menin represses AKT activity and thus AKT-induced FOXO1 phosphorylation, as well as FOXO1 ubiquitination. Moreover, the menin-induced FOXO1 stability was through suppressing Skp2, an E3 ubiquitin ligase. Furthermore, menin-induced FOXO1 protein level is also crucial for the proapoptotic activity of menin. These findings underscore the importance and underlying mechanism of menin/FOXO1 axis in suppressing β-cell function.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Stable menin- and ShMen1-expressing INS-1 cells were established by transduction with pMX-puro-menin and RetroQ-puro-Shmen1–derived retroviruses. pMX-puro-menin and indicated small hairpin RNA (shRNA) were cotransfected with psi-2 helper plasmid into HEK293T cells for retroviral packaging using the calcium chloride precipitation method. The resulting recombinant virus was collected and transduced into INS-1E cells, followed by selection in 2 μg/mL puromycin

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(Sigma, St Louis, Mo) for 4 days. HEK293T cells were cultured in Dulbecco modified Eagle medium (HyClone, Logan, Utah) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. INS-1 cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 1 mol/L Hepes, 0.2 mol/L L-glutamine, 0.1 mol/L sodium pyruvate, and 55 mmol/L β-mercaptoethanol.

Plasmids and Constructions

pBabe-FOXO1-AAA was generated from pBabe-FOXO1 using the QuikChange Site-Directed Mutagenesis kit (Agilent, Santa Clara, Calif). Retroviral plasmid pMX-puro-menin was constructed by inserting polymerase chain reaction (PCR)–amplified menin complementary DNA (cDNA) into the *Bam*HI/*Not*I site of the retroviral vector pMX-puro. Construction of shRNA for *Men1* was performed, as previously described.[19]

RNAi Transfection

Skp2-siRNA with a sequence of UUU GAG AGC AGU CCA UGU GGG AUG U was purchased from Invitrogen (Carlsbad, Calif). Transfection of Skp2-siRNA and control siRNA was performed according to the standard Lipofectamine 2000 transfection procedure from the manufacturer. Briefly, a mixture of diluted siRNA with Opti-MEM medium into 0.2 μm/L and diluted Lipofectamine 2000 Reagent was incubated for 5 minutes at room temperature, and siRNA-lipid complex was added into cells afterward.

Immunoprecipitation and Western Blotting

For immunoprecipitation (IP), cells were suspended in lysis buffer (50 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 5% glycerol, 1% NP-40, 1 mmol/L EDTA), supplemented with 1 mM PMSF and 4 μg/mL protease inhibitor cocktail (Sigma). Lysates were centrifuged at 13,000*g* for 10 minutes, and the supernatant was added to 2 μL indicated antibodies and 100 μL protein A agarose beads (Invitrogen) to incubate for 4 hours at 4°C. Afterward, protein A beads were washed by 250 mmol NaCl 4 times. For Western blotting, cells were collected at indicated time points and then were lysed by RIPA lysis buffer (Beyotime, Nantong, China). Cell lysates (90 μL) were mixed with 30 μL sodium dodecyl sulfate loading buffer and boiled for 5 minutes at 100°C for sodium dodecyl sulfate—polyacrylamide gel electrophoresis. Dilutions of antibodies used in the Western blot were performed according to the suppliers' instructions. Horseradish peroxidase–labeled secondary antibody was used at a dilution of 1:3000.

Chemicals and Inhibitors

MG132 was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO). Epidermal growth factor (EGF) was purchased from Merck Millipore (Temecula, Calif) and dissolved in phosphate-buffered saline. FOXO1 inhibitor AS1842856 was purchased from Merck Millipore and dissolved in DMSO.

Antibodies

Antibodies used were antimenin (Bethyl, Montgomery, Tex; A300-105A), anti-FoxO1 (CST, Boston, Mass; no. 2880), anti–p-AKT (CST; no. 4060), anti-AKT (CST; no. 9272), anti–p-FoxO1 (CST; no. 9461), anti-hemagglutinin (HA) (CWbiotech, Beijing, China; CW0092A), antiubiquitin (CST; no. 3936), anti-Skp2 (CST; no. 2652), anti–caspase-3 (CST; no. 9665), and anti–β-actin (Santa Cruz Biotechnology, Dallas, Tex; SC47778).

RESULTS

Menin Increases the Expression of FOXO1

To examine whether menin affects expression of FOXO1, a key transcription factor that regulates gluconeogenesis[9] and suppresses β-cell proliferation,[10] we ectopically expressed menin cDNA via a retroviral vector in INS-1 cells,[20] an insulin-secreting rat β-cell line. Western blotting analysis showed that the menin-overexpressing cells expressed a higher level of menin as compared with the vector-transduced control cell (Fig. 1A, top). Quantification using ImageJ (Version 1.48; National Institutes of Health, Bethesda, Md) scanning showed that the menin-overexpressing cells expressed a higher level of FOXO1 protein (Fig. 1B). We also found that the menin-overexpressing cells had a higher FOXO1 messenger RNA (mRNA) level (Fig. 1C), consistent with our previous finding.[21] To further determine whether menin is required for high expression of FOXO1 at protein and mRNA level, we transduced shRNA targeting menin or control retroviral vector into INS-1 cells, and the resulting cells were subjected to Western blot and quantitative reverse transcription PCR analysis of FOXO1 expression. The results indicate that menin knockdown (KD) using an shRNA showed a reduced FOXO1 protein level (Figs. 1D-E) and the mRNA level (Fig. 1F). These results indicate that menin is crucial for maintaining a high level of FOXO1 expression level in INS-1 cells. To confirm our results, we further performed menin KD or overexpression in another murine β-cell line, TGP61,[22] or a murine endocrine cell line, STC-1.[23] In these 2 additional cell lines, we also found that the FOXO1 expression was upregulated by menin (Supplemental Fig. 1, http://links.lww.com/MPA/A699).

Menin Stabilizes FOXO1 Protein Level in INS-1 Cells

Because menin expression is crucial for maintaining a high-level expression of FOXO1 at the mRNA and protein levels, it is likely that menin increases FOXO1 mRNA transcription, and a higher FOXO1 may lead to more translation of FOXO1 protein, resulting in increased FOXO1 protein level. On the other hand, it is also possible that menin is crucial for increased FOXO1 protein level at a posttranscriptional level such as protein stability. To explore this possibility, we treated control or menin-expressing INS-1 cells with cycloheximide, a compound that blocks cellular protein translation, for various periods to inhibit new protein synthesis. The treated control and menin-expressing cells were subjected to Western blotting analysis, and the results indicate that ectopic menin expression is correlated with a higher FOXO1 protein level (Fig. 2A), reinforced by quantification of the amount of FOXO1 protein at the Western blot (Fig. 2B). To further determine whether menin expression is crucial for the stability of FOXO1 protein, we treated the vector control cells and menin KD cells with cycloheximide for various periods, followed by Western blot to determine the FOXO1 protein level. Consistently, the result indicates that menin KD substantially reduced the stability of the FOXO1 proteins (Fig. 2C, lanes 5–6 vs 2–3; Fig. 2D). Collectively, these results suggest that menin increases FOXO1 protein stability, leading to increased FOXO1 protein level.[24]

Menin Represses Ubiquitination of FOXO1 Protein

It has been reported that FOXO1 protein stability can be regulated by ubiquitin-induced, proteasome-mediated proteolysis. To investigate how menin increases FOXO1 protein stability, we sought to determine whether menin affects FOXO1 ubiquitination. To this end, we transfected cDNA encoding

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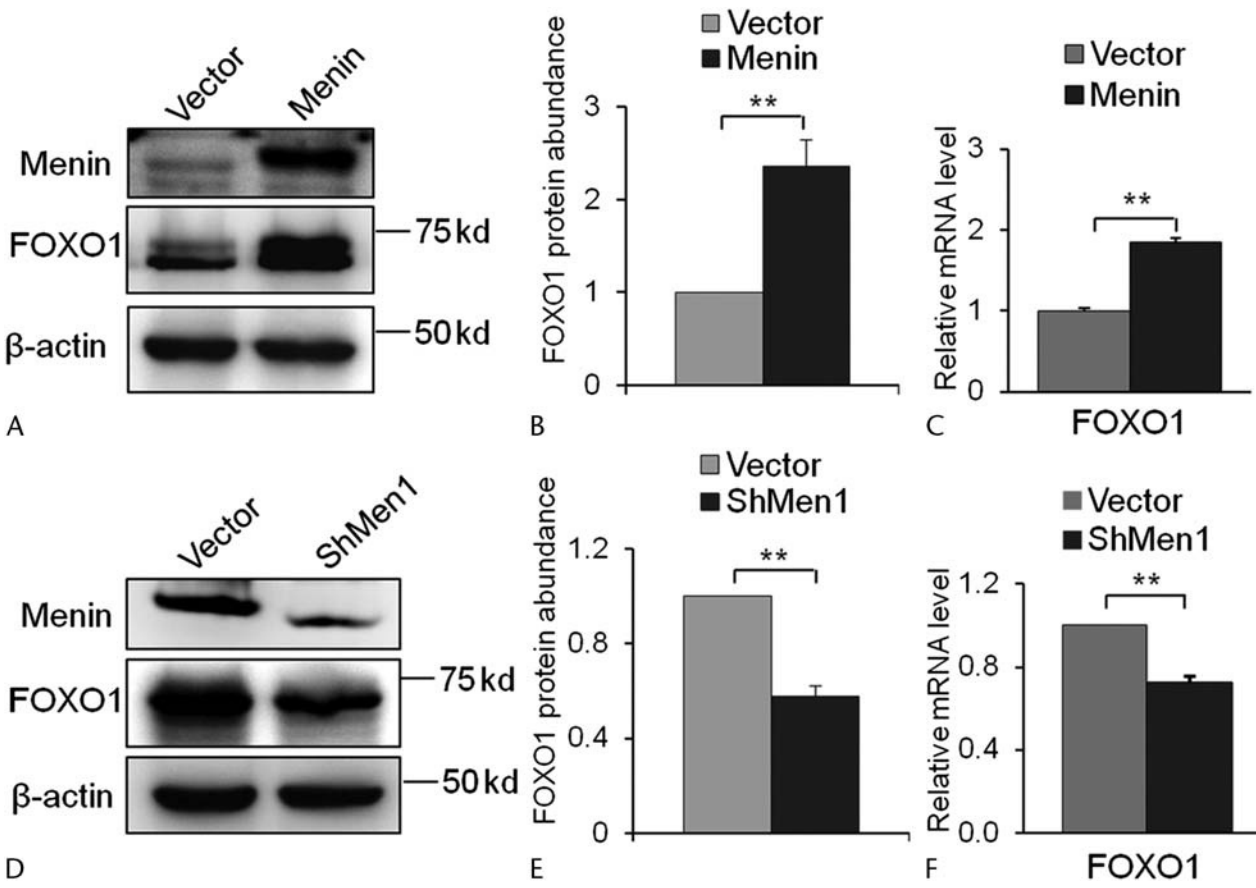

FIGURE 1. Menin increases the stable protein level of FOXO1. A, INS-1 cells were transfected with vector or menin cDNA and selected with puromycin for 4 days. The resulting cells were used for Western blot with indicated antibodies. B, Quantitation of FOXO1 protein level from panel A. Band density analysis was executed with ImageJ, and FOXO1 protein levels were quantified and normalized to β-actin. C, Quantitative reverse transcription PCR analysis of FOXO1 mRNA level in INS-1 cells expressing vector or menin cDNA. Data were normalized to the level of GAPDH and expressed as fold change relative to control. D, INS-1 cells were transfected with vector or ShMen1 DNA and selected with puromycin (2 μg/mL) for 4 days, followed by Western blot with indicated antibodies. E, Quantitation of FOXO1 protein level from panel C. Band density analysis was executed with ImageJ, and FOXO1 protein levels were quantified and normalized to β-actin. F, Quantitative reverse transcription PCR analysis of FOXO1 mRNA level in INS-1 cells expressing vector or ShMen1 cDNA. Data were normalized to the level of GAPDH and expressed as fold change relative to control. Values are means ± SEM of 3 independent experiments per each group. $^{**}P < 0.01$.

HA epitope-tagged ubiquitin alone or in combination with the menin cDNA into HEK293 cells, and then treated them with proteasome inhibitor MG132. The resulting cells were processed for IP with anti-FOXO1 antibody to pull down the endogenous FOXO1, followed by Western blot to determine ubiquitinated FOXO1. We found that transfection of HA-ubiquitin cDNA led to increased ubiquitination of FOXO1 as detected by the anti-HA antibody (Fig. 3A, lane 2), but ectopic expression of menin led to suppression of FOXO1 ubiquitination (Fig. 3A, lane 3). We sought to further investigate whether menin affects FOXO1 ubiquitination in INS-1 β cells. We thus transfected these cells with control vector or menin cDNA and found that ectopic expression of menin in these cells also reduced ubiquitination of the endogenous FOXO1 protein (Fig. 3B). To further confirm whether endogenous menin is crucial for reducing the ubiquitination of endogenous FOXO1 in the cells, we knocked down menin in INS-1 cells, and the vector control and menin KD cells were subjected to IP with the anti-FOXO1 antibody, followed by Western blotting with the antiubiquitin antibody. The results demonstrate that menin KD substantially increased the ubiquitinated FOXO1 (Fig. 3C). Together, these findings demonstrate that menin plays an important role in suppressing FOXO1 ubiquitination, likely leading to reduced proteolysis and increased FOXO1 stability and protein level.

Menin Represses FOXO1 Ubiquitination and AKT Phosphorylation

FOXO1 is phosphorylated at several sites including serine 256 by serine/threonine kinase AKT.[17] The phosphorylated FOXO1 has increased affinity in binding to ubiquitin E3 ligase such as Skp2.[18] As we showed that menin reduced FOXO1 ubiquitination but increased its stability and protein level, coupled with a report that menin can suppress AKT phosphorylation and activation in HEK293 cells,[25] we sought to determine whether ectopic menin expression affects AKT phosphorylation and thus activation in INS-1 β cells. To this end, we examined the level of the phosphorylated (activated) AKT and total amount of AKT protein in INS-1 cells transduced with either control vector or menin-expressing retroviruses, and found that ectopic menin expression reduced the amount of the phosphorylated AKT but did not affect the level of total AKT (Figs. 4A, B). Consistent with reduced AKT phosphorylation, menin expression also reduced FOXO1 phosphorylation at serine 256 (Figs. 4C, D),

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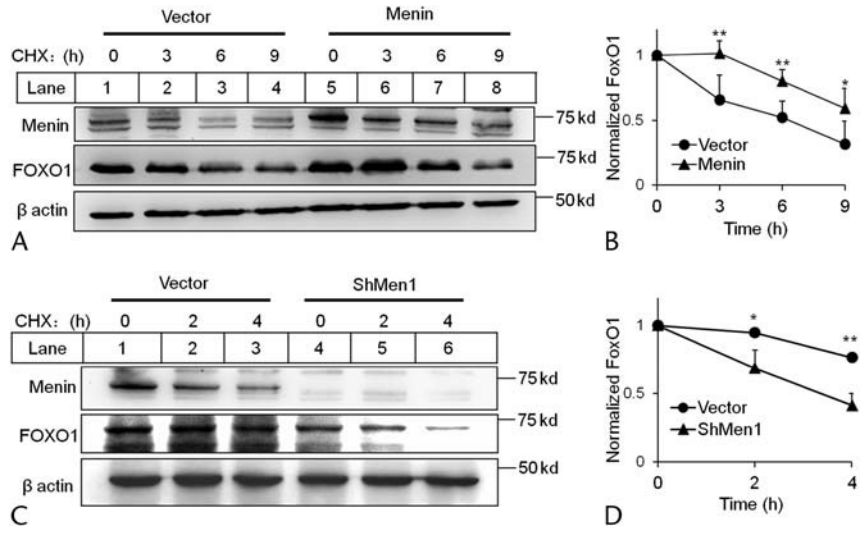

FIGURE 2. Menin stabilizes FOXO1 protein in INS-1 cells. A, INS-1 cells expressing ectopic menin were treated with cycloheximide (20 μg/mL) for the indicated times and lysed for Western blot. B, Quantitation of FOXO1 protein level from panel A. Band density analysis was performed with ImageJ, and FOXO1 protein levels were quantified and normalized to β-actin. The value at the time points 3, 6, and 9 hours were normalized to that of time point 0 hours. C, INS-1 cells stably expressing vector or Men1 shRNA were treated with cycloheximide (20 μg/mL) for the indicated times and lysed for Western blot. D. Quantitation of the FOXO1 protein level from panel C. Band density analysis was executed with ImageJ, and FOXO1 protein levels were quantified and normalized to β-actin. Then the time point 3, 6, and 9 hours were normalized to time point 0 hours. Values are means ± SEM of 3 independent experiments per each group. $*P < 0.05$. $**P < 0.01$.

a known AKT phosphorylation site.[17] We further examined whether menin KD affects AKT activation and FOXO1 phosphorylation, and we found that menin KD indeed increased AKT phosphorylation (Figs. 4E, F), as well as FOXO1 phosphorylation (Figs. 4G, H) but did not affect the total amount of AKT (Figs. 4E, F). We further explored whether stimulation of INS-1 cells with EGF, which can activate PI3K/AKT pathway,[26] can interplay with menin to regulate FOXO1 ubiquitination. To this end, we transfected HEK293 cells with either menin or HA-ubiquitin cDNA alone or their combination, and the resulting cells were stimulated with or without EGF as shown in Figure 4I. The resulting cells were subjected to co-IP with the anti-FOXO1 antibody, followed by Western blot with anti-HA or other indicated antibodies. Our results indicate that ectopic menin expression repressed FOXO1 ubiquitination (Fig. 4I, lane 3 vs 2), as expected. Stimulation of the cells with EGF modestly increased AKT phosphorylation and FOXO1 ubiquitination (Fig. 4I, lane 3). Notably, EGF stimulation in the absence of ectopic menin expression substantially increased FOXO1 ubiquitination and reduced FOXO1 protein level (Fig. 4I, lane 5). Collectively, these results indicate that menin-mediated increase of FOXO1 protein

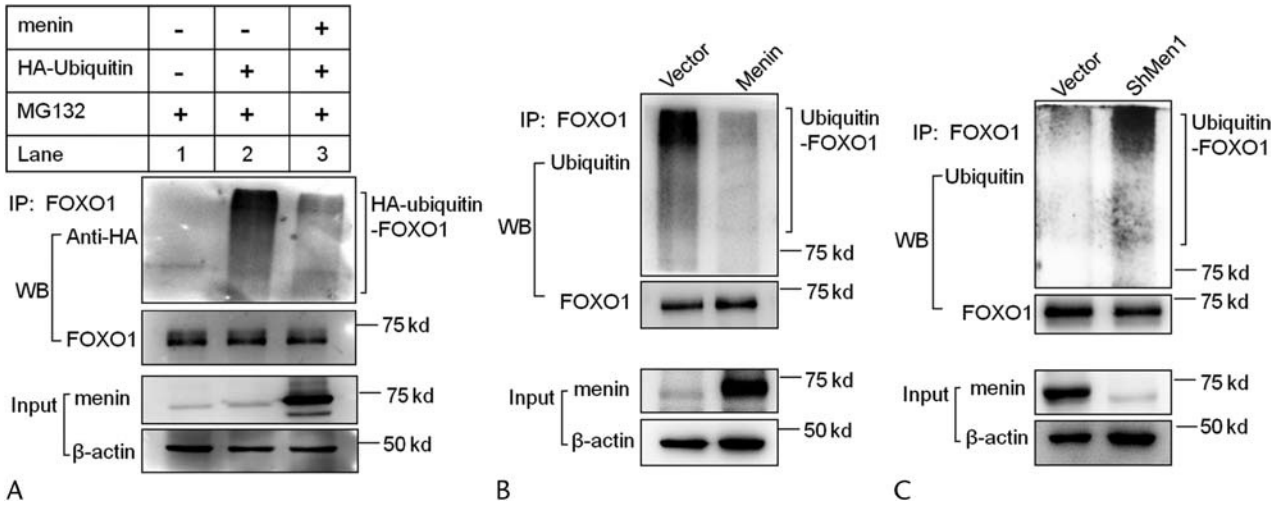

FIGURE 3. Menin represses ubiquitination of FOXO1 protein. A, Equal amounts of menin and/or HA-ubiquitin constructs were transfected into 293T cells, followed by MG132 (20 μmol) treatment for 4 hours and lysed for IP and Western blot with the indicated antibodies. B, INS-1 cells expressing vector or ectopic menin were treated with MG132 (20 μmol) for 4 hours, followed by IP for FOXO1 and Western blot for ubiquitin. C, INS-1 cells expressing vector or Men1 shRNA were treated with MG132 (20 μmol) for 4 hours, followed by IP for FOXO1 and Western blot for ubiquitin.

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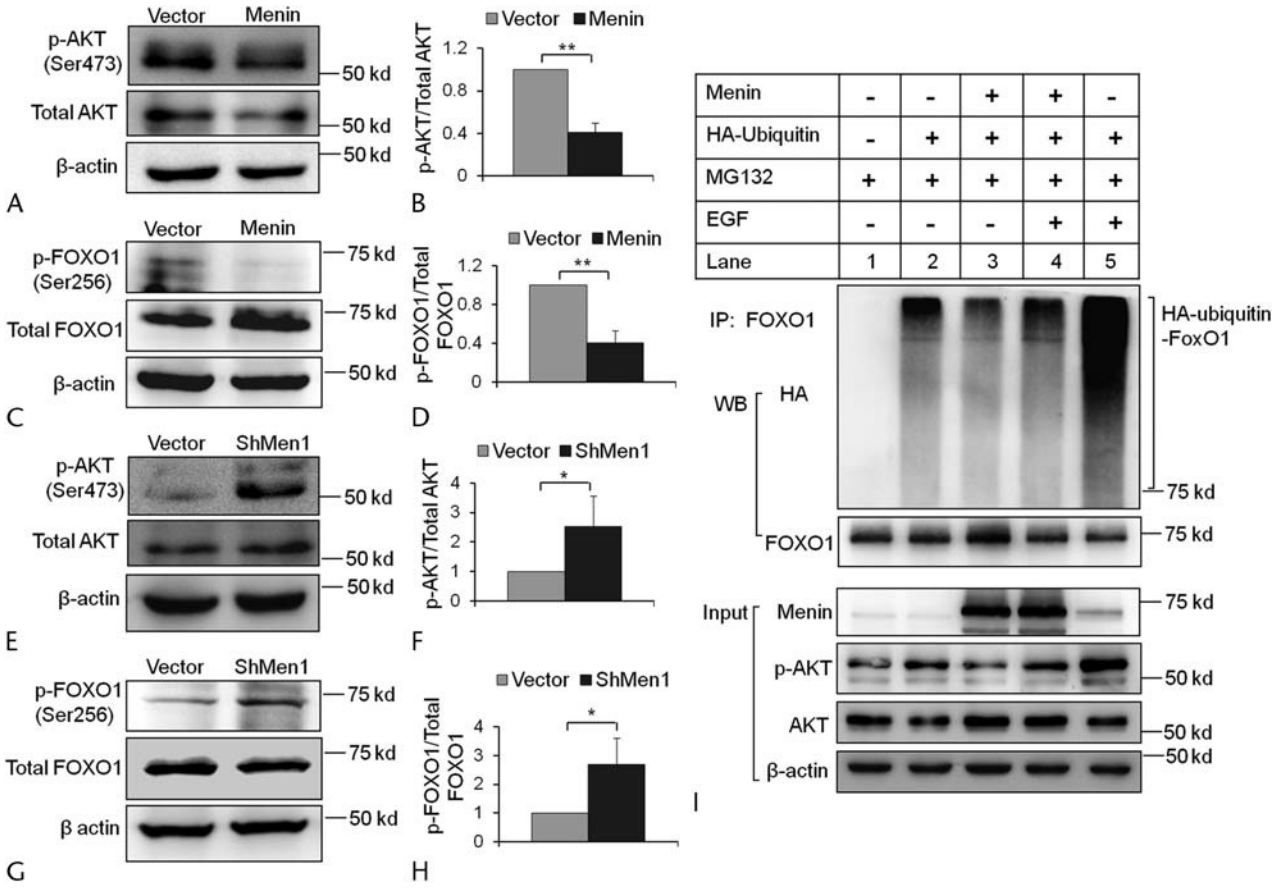

FIGURE 4. Menin represses FOXO1 ubiquitination and AKT phosphorylation. A, C, INS-1 cells expressing vector or ectopic menin were lysed for Western blot with indicated antibodies. B and D, Quantitation of p-AKT or p-FOXO1 protein level from panel A or C. Band density analysis was executed with ImageJ, and p-AKT or p-FOXO1 protein levels were quantified and normalized to total AKT or total FOXO1. E and G, INS-1 cells expressing vector or sShMen1 were lysed for Western blot with indicated antibodies. F and H, Quantitation of p-AKT or p-FOXO1 protein level from panel E or G. Band density analysis was executed with ImageJ, and p-AKT or p-FOXO1 protein levels were quantified and normalized to total AKT or total FOXO1. I, Equal amounts of menin and/or HA-ubiquitin constructs were transfected into 293T cells in the presence or absence of EGF (100 ng/mL) for 24 hours, followed by MG132 (20 μmol) treatment for 4 hours and lysed for IP and Western blot with the indicated antibodies. $*P < 0.05$. $**P < 0.01$.

stability may at least partly result from menin-mediated suppression of AKT and AKT-induced phosphorylation, leading to reduced FOXO1 ubiquitination and increased stability.

Menin Stabilizes FOXO1 by Repressing Skp2-Mediated FOXO1 Degradation

It has been reported that Skp2 plays a role in mediating FOXO1 degradation by acting as an E3 ligase.[18] We sought to determine whether menin affects FOXO1 stability via influencing Skp2. To this end, we examined the association between FOXO1 and Skp2, which binds phosphorylated FOXO1 to induce its ubiquitination and degradation using co-IP with INS-1 cells transduced with either control vector DNA or menin cDNA, followed by Western blot using anti-Skp2 or FOXO1 antibodies. The results showed that ectopic menin expression substantially reduced Skp2 binding to FOXO1 (Fig. 5A, lane 4). Consistently, KD of menin increased Skp2 binding to FOXO1 (Fig. 5B, lane 4 vs 3). Furthermore, to determine whether Skp2 mediates menin KD-induced degradation of FOXO1, we knocked down Skp2 expression using siRNA targeting Skp2 in either control or menin KD cells. The resulting cells were subjected to Western blotting. Our results indicate that Skp2 expression was suppressed by the siRNA treatment (Fig. 5C, lanes 3–4). Notably, although in control cells, menin KD reduced the amount of total FOXO1 (Fig. 5C, lane 2), as expected, Skp2 KD abolished menin KD-induced reduction of the FOXO1 protein level (Fig. 5C, lane 4; Fig. 5D). Together, these results demonstrate that menin increases FOXO1 protein stability at least partly by inhibiting Skp2-mediated ubiquitination and proteasome-dependent proteolysis, likely via repressing AKT activity.

Menin Promotes Caspase 3 Activation and Apoptosis

FOXO1 is a proapoptotic factor because it can induce apoptosis by increasing expression of some proapoptotic factors such as Bim[27] and lead to activation of caspases such as caspase 3. To explore whether menin and FOXO1 affect apoptosis in INS-1 cells, we measured the activation of caspase 3, a marker of apoptosis, in various settings. We found that ectopic menin expression in INS-1 cells led to detection of increased cleavage of caspase 3 based on Western blotting (Figs. 6A, B). Consistently, ectopic expression of constitutively active FOXO1-AAA mutant in INS-1 cells increased the level of caspase 3 cleavage (Figs. 6C, D). We

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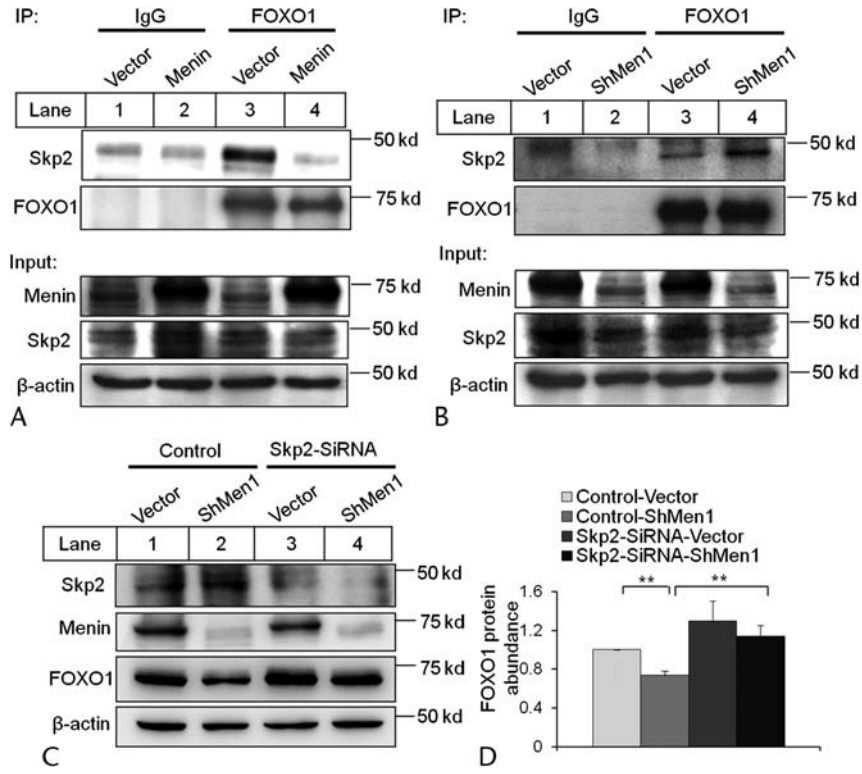

FIGURE 5. Menin stabilizes FOXO1 by repressing Skp2-mediated FOXO1 degradation. A, Lysates from INS-1 cells expressing vector or ectopic menin were immunoprecipitated with anti-FOXO1 antibody, followed by Western blot using indicated antibodies. B, Lysates from INS-1 cells expressing vector or Men1 shRNA were immunoprecipitated with anti-FOXO1 antibody, followed by Western blot using indicated antibodies. C, INS-1 cells expressing vector or Men1 shRNA were respectively transfected with Control or Skp2-SiRNA and lysed for immunoblotting with indicated antibodies after 48 hours. D, Quantitation of FOXO1 protein level from panel C. Band density analysis was executed with ImageJ, and FOXO1 protein levels were quantified and normalized to β actin.**$P < 0.01$.

further tested whether an inhibitor of FOXO1, AS184256,[28] affects menin-induced apoptosis of cells. To this end, we treated the control and menin-overexpressing INS-1 cells with control DMSO or the FOXO1 inhibitor, and the resulting cells were subjected to Western blot for detecting cleavage of caspase 3. The results indicate that, although menin expression increased caspase 3 cleavage (Fig. 6E, lane 2), treatment of the cells with the FOXO1 inhibitor substantially reduced cleavage of caspase 3 (Fig. 6E, lane 4). In aggregate, these findings suggest that menin-induced protein stability of FOXO1 leads to increased activation of caspase 3, likely explaining the proapoptotic effect of menin in β cells.

DISCUSSION

There are 3 major novel points in our current findings. First, we found that, for the first time, menin upregulates FOXO1 protein stability and thus increases FOXO1 protein level. Second, our studies unravel a mechanism whereby menin increases FOXO1 protein stability at least partly through suppressing Skp2-mediated ubiquitination and thus proteolysis. Third, complimentary with the previous reports, menin can upregulate FOXO1 mRNA and protein level,[21,29] our new findings show that menin can also increase FOXO1 protein level by reducing FOXO1 ubiquitination and proteasome-mediated degradation.

The New Role of Menin in Controlling FOXO1 Protein Stability

In the current study, we have unraveled a novel means whereby menin regulates FOXO1 protein level posttranscriptionally via increasing Skp2-mediated ubiquitination (Fig. 6F). These findings are strongly supported by several lines of evidence. First, ectopic menin expression increased FOXO1 protein level (Fig. 1A). Notably, menin KD decreased FOXO1 protein level (Fig. 1D) and led to reduction in FOXO1 protein stability as shown by decrease in FOXO1 protein half-life (Fig. 2C). However, the current studies do not rule out that menin also regulates FOXO1 transcription, and it is conceivable that menin may regulate transcription of FOXO1, an important transcription factor that regulates human β cells and metabolism, but the mechanism remains elucidated. Nevertheless, the current study is the first to unravel the key role of menin in regulating FOXO1 protein stability. These findings reveal a new layer of regulation of FOXO1 by menin, a key regulator of β-cell function and pancreatic islets.

Mechanism Whereby Menin Upregulates FOXO1 Protein Stability

It has been reported that menin binds AKT and represses AKT phosphorylation (activation).[25] Consistently, our findings indicate that menin repressed AKT-dependent phosphorylation of FOXO1. It is known that AKT-mediated phosphorylation increases E3 ligase Skp2 binding to FOXO1, and thus triggers FOXO1 ubiquitination and proteasome-mediated proteolysis.[18] Our findings indicate that menin increases FOXO1 stability and protein level at least partly through repressing Skp2-mediated degradation, as menin KD-induced reduction of FOXO1 was rescued by Skp2 KD (Fig. 5C). However, we cannot rule out the possibility that besides the menin-inhibited AKT reducing

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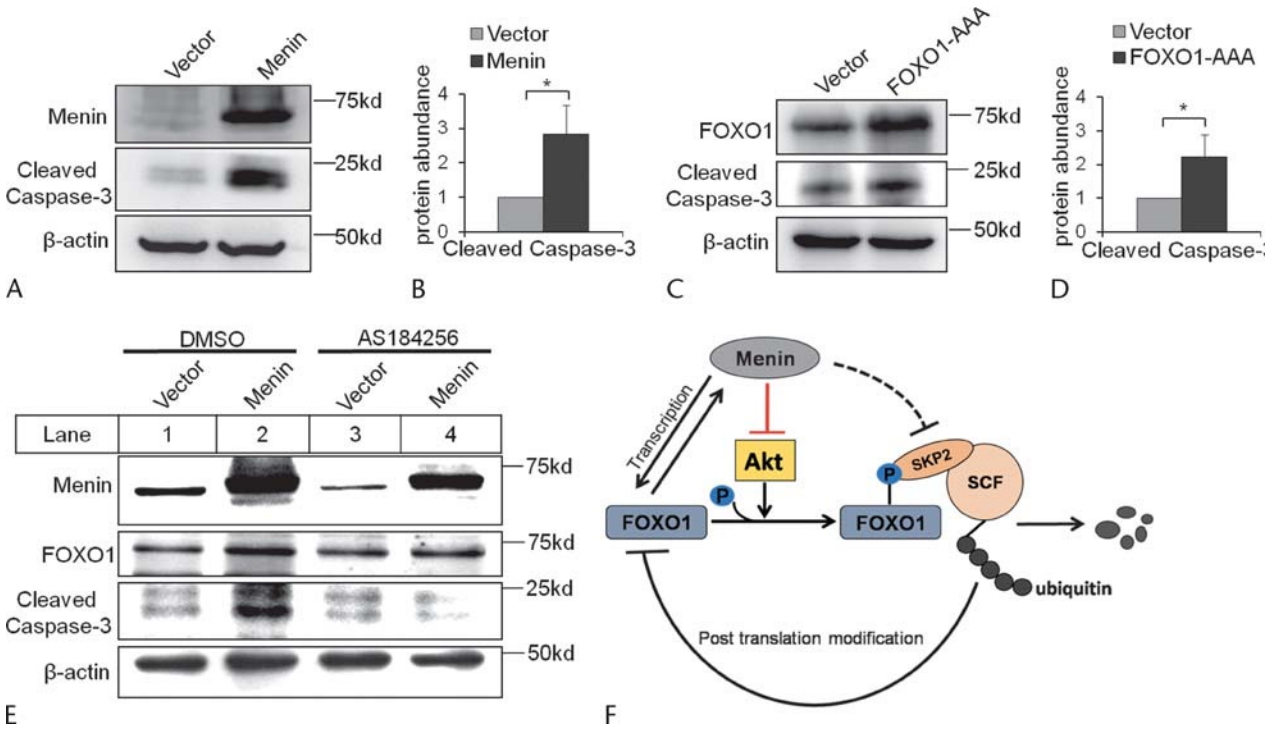

FIGURE 6. Menin promotes caspase 3 activation. A, INS-1 cells expressing vector or ectopic menin were lysed for Western blot with indicated antibodies. B, Quantitation of cleaved caspase-3 protein level from panel A. Band density analysis was executed with ImageJ, and cleaved caspase-3 protein levels were quantified and normalized to β actin. C, INS-1 cells were transfected with vector or FOXO1-AAA cDNA, followed by Western blot with indicated antibodies. D, Quantitation of cleaved caspase-3 protein level from panel C. Band density analysis was executed with ImageJ, and cleaved caspase-3 protein levels were quantified and normalized to β-actin. E, Control or ectopic menin-expressing INS-1 cells were treated with either DMSO or FOXO1 inhibitor (AS184256, 100 nM) for 3 days before Western blot with the indicated antibodies. F, A working model for regulation of FOXO1 degradation by menin in INS-1 cells. $*P < 0.05$.

binding of Skp2 to FOXO1 to increase the stability, menin itself may also binds FOXO1 to attenuate Skp2 association with FOXO1 to reduce FOXO1 degradation. On the other hand, it remains unknown that whether the protein kinase A (PKA) pathway regulates menin-upregulated FOXO1 stability, as we recently reported, PKA can also directly phosphorylate FOXO1,[17] and this remains to be investigated. It also remains to be investigated what is the relative weight of contributions to the increased FOXO1 protein level by either menin-regulated FOXO1 gene transcription or FOXO1 protein stability. By the same token, what is the relative weight of contributions to FOXO1-regulated cell proliferation and survival by each mode of the regulation warrants further investigation. Moreover, we cannot rule out that the expression levels of Menin, FOXO1, Skp2 and some related genes are different between INS-1 or other related cell lines and the primary islets owing to their adaptation to cell culture. Nevertheless, the current studies unravel a critical role of menin in regulating β cells and likely metabolism via posttranslational regulation of FOXO1.

The Intricate Interplay Between Menin and FOXO1, Forming a Positive Feedback Loop

Both menin and FOXO1 are critical proteins that regulate β-cell proliferation and well-being, controlling the islet mass.[10] Although it was reported that FOXO1 binds the menin gene promoter to upregulate menin transcription,[29] and we previously reported that menin increases FOXO1 function by blocking PKA-mediated FOXO1 phosphorylation and thus inhibition,[17] little was previously known whether menin regulates FOXO1 protein level. Our current findings indicate that menin increases FOXO1 protein level and thus FOXO1 activity. Because we failed to observe that menin binds to the FOXO1 promoter (Supplemental Fig. 2, http://links.lww.com/MPA/A699), we cannot rule out the possibility that menin can also upregulate the transcription of FOXO1. However, our findings are consistent with the notion that menin increases FOXO1 protein level by increasing FOXO1 protein stability. These new findings, coupled with the report that FOXO1 can directly upregulate menin expression,[29] are consistent with the concept that menin and FOXO1 interplay, in a positive feedback loop, as shown in Figure 6F, to sustain a brake to reduce β-cell proliferation and islet mass. These findings are also consistent with our earlier findings that menin is a prodiabetic factor, as ablation of the Men1 gene reverses preexisting hyperglycemia in diabetic mice[6] and even prevents the development of streptozotocin-induced diabetes in mice.[7] Although it is unclear whether menin-regulated FOXO1 level affects cell survival and there are many other downstream proteins such as p18, p27, and caspase 8,[4,30] it is plausible that menin promotes apoptosis at least partly through upregulating FOXO1 protein stability. FOXO1 is also important for promoting gluconeogenesis via inducing expression of several key enzymes.[9] Thus, menin-mediated regulation of FOXO1 stability may explain the proapoptotic effect of menin in regulating β cells.

Together with the previous study that FOXO1 upregulates the menin gene transcription, our findings unravel an important interplay between menin and FOXO1. By upregulating their expression reciprocally, menin and FOXO1 form a positive feedback loop to sustain menin and FOXO1 expression and suppress β-cell function.

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REFERENCES

1. Lemmens I, Van de Ven WJ, Kas K, et al. Identification of the multiple endocrine neoplasia type 1 (MEN1) gene. The European Consortium on MEN1. *Hum Mol Genet*. 1997;6:1177–1183.
2. La P, Desmond A, Hou Z, et al. Tumor suppressor menin: the essential role of nuclear localization signal domains in coordinating gene expression. *Oncogene*. 2006;25:3537–3546.
3. Huang J, Gurung B, Wan B, et al. The same pocket in menin binds both MLL and JUND but has opposite effects on transcription. *Nature*. 2012; 482:542–546.
4. Karnik SK, Hughes CM, Rozenblatt-Rosen O, et al. Menin regulates pancreatic islet growth by promoting histone methylation and expression of genes encoding p27Kip1 and p18INK4c. *Proc Natl Acad Sci U S A*. 2005; 102:14659–14664.
5. Schnepp RW, Mao H, Sykes SM, et al. Menin induces apoptosis in murine embryonic fibroblasts. *J Biol Chem*. 2004;279:10685–10691.
6. Yang Y, Gurung B, Wu T, et al. Reversal of preexisting hyperglycemia in diabetic mice by acute deletion of the Men1 gene. *Proc Natl Acad Sci U S A*. 2010;107:20358–20363.
7. Yang Y, Wang H, Hua X. Deletion of the Men1 gene prevents streptozotocin-induced hyperglycemia in mice. *Exp Diabetes Res*. 2010; 2010:876701.
8. Martins R, Lithgow GJ, Link W. Long live FOXO: unraveling the role of FOXO proteins in aging and longevity. *Aging Cell*. 2016;15:196–207.
9. Nakae J, Kitamura T, Silver DL, et al. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest*. 2001;108:1359–1367.
10. Talchai SC, Accili D. Legacy effect of foxo1 in pancreatic endocrine progenitors on adult β-cell mass and function. *Diabetes*. 2015;64: 2868–2879.
11. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*. 1999; 96:857–868.
12. Yamagata K, Daitoku H, Takahashi Y. Arginine methylation of FOXO transcription factors inhibits their phosphorylation by Akt. *Mol Cell*. 2008; 32:221–231.
13. Zhang X, Gan L, Pan H, et al. Phosphorylation of serine 256 suppresses transactivation by FKHR (FOXO1) by multiple mechanisms. Direct and indirect effects on nuclear/cytoplasmic shuttling and DNA binding. *J Biol Chem*. 2002;277:45276–45284.
14. Li F, Xie P, Fan Y, et al. C terminus of Hsc70-interacting protein promotes smooth muscle cell proliferation and survival through ubiquitin-mediated degradation of FoxO1. *J Biol Chem*. 2009;284:20090–20098.
15. Kato S, Ding J, Pisck E, et al. COP1 functions as a FoxO1 ubiquitin E3 ligase to regulate FoxO1-mediated gene expression. *J Biol Chem*. 2008; 283:35464–35473.
16. Biggs WH 3rd, Meisenhelder J, Hunter T, et al. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A*. 1999;96: 7421–7426.
17. Tang ED, Nuñez G, Barr FG, et al. Negative regulation of the forkhead transcription factor FKHR by Akt. *J Biol Chem*. 1999;274:16741–16746.
18. Huang H, Regan KM, Wang F, et al. Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. *Proc Natl Acad Sci U S A*. 2005;102:1649–1654.
19. Gao SB, Feng ZJ, Xu B, et al. Suppression of lung adenocarcinoma through menin and polycomb gene-mediated repression of growth factor pleiotrophin. *Oncogene*. 2009;28:4095–4104.
20. Kong X, Yan D, Sun J, et al. Glucagon-like peptide 1 stimulates insulin secretion via inhibiting RhoA/ROCK signaling and disassembling glucotoxicity-induced stress fibers. *Endocrinology*. 2014;155:4676–4685.
21. Muhammad AB, Xing B, Liu C, et al. Menin and PRMT5 suppress GLP1 receptor transcript and PKA-mediated phosphorylation of FOXO1 and CREB. *Am J Physiol Endocrinol Metab*. 2017;313:E148–E166.
22. Pettengill OS, Memoli VA, Brinck-Johnsen T, et al. Cell lines derived from pancreatic tumors of Tg(Ela-1-SV40E)Bri18 transgenic mice express somatostatin and T antigen. *Carcinogenesis*. 1994;15:61–65.
23. Rindi G, Grant SG, Yiangou Y, et al. Development of neuroendocrine tumors in the gastrointestinal tract of transgenic mice. Heterogeneity OF HORMONE EXPRESSION. *Am J Pathol*. 1990;136:1349–1363.
24. Xie Q, Chen J, Yuan Z, et al. Post-translational regulation of FOXO. *Acta Biochim Biophys Sin (Shanghai)*. 2012;44:897–901.
25. Wang Y, Ozawa A, Zaman S, et al. The tumor suppressor protein menin inhibits AKT activation by regulating its cellular localization. *Cancer Res*. 2011;71:371–382.
26. Carpenter G, Cohen S. Epidermal growth factor. *J Biol Chem*. 1990;265: 7709–7712.
27. Yang Y, Zhao Y, Liao W, et al. Acetylation of FoxO1 activates Bim expression to induce apoptosis in response to histone deacetylase inhibitor depsipeptide treatment. *Neoplasia*. 2009;11:313–324.
28. Zou P, Liu L, Zheng L, et al. Targeting FoxO1 with AS1842856 suppresses adipogenesis. *Cell Cycle*. 2014;13:3759–3767.
29. Zhang H, Li W, Wang Q, et al. Glucose-mediated repression of menin promotes pancreatic β-cell proliferation. *Endocrinology*. 2012;153:602–611.
30. La P, Yang Y, Karnik SK, et al. Menin-mediated caspase 8 expression in suppressing multiple endocrine neoplasia type 1. *J Biol Chem*. 2007;282: 31332–31340.

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