FOXO3 Growth Inhibition of Colonic Cells Is Dependent on Intraepithelial Lipid Droplet Density*

Received for publication, March 19, 2013, and in revised form, April 18, 2013 Published, JBC Papers in Press, April 18, 2013, DOI 10.1074/jbc.M113.470617

Wentao Qi†‡1, Philip S. Fitchev§, Mona L. Cornwell§, Jordan Greenberg‡, Maleen Cabe†, Christopher R. Weber§, Hemant K. Roy†, Susan E. Crawford‡, and Suzana D. Savkovic†‡1

From the †Department of Medicine, Division of Gastroenterology, Northshore University HealthSystem Research Institute, Evanston, Illinois 60201, the ‡Department of Pathology, Saint Louis University School of Medicine, St. Louis, Missouri 63104, and the §Department of Pathology, The University of Chicago, Chicago, Illinois 60637

**Background:** The loss of FOXO3 is critical for tumor growth.

**Results:** FOXO3 and lipid droplets (LDs) have feedback regulation, and the loss of FOXO3 leading to increased LDs is key in the growth of colonic cells.

**Conclusion:** FOXO3-dependent LDs provide lipid energy critical for cellular growth.

**Significance:** Identifying regulators of cellular lipid energy could provide new targets for colon cancer treatment.

Forkhead transcription factor FOXO3 plays a critical role in suppressing tumor growth, in part, by increasing the cell cycle inhibitor p27kip1, and Foxo3 deficiency in mice results in marked colonic epithelial proliferation. Here, we show in Foxo3-deficient colonic epithelial cells a striking increase in intracytoplasmic lipid droplets (LDs), a dynamic organelle recently observed in human tumor tissue. Although the regulation and function of LDs in non-adipocytes is unclear, we hypothesize that the anti-proliferative effect of FOXO3 was dependent on lowering LD density, thus decreasing fuel energy in both normal and colon cancer cells. In mouse colonic tumors, we found an increased expression of LD coat protein PLIN2 compared with normal colonic epithelial cells. Stimulation of LD density in human colon cancer cells led to a PI3K-dependent loss of FOXO3 and a decrease in the negative regulator of lipid metabolism in Sirtuin6 (SIRT6). FOXO3 deficiency also led to a decrease in SIRT6, revealing the existence of LD and FOXO3 feedback regulation in colonic cells. In parallel, LD-dependent loss of FOXO3 led to its dissociation from the promoter and decreased expression of the cell cycle inhibitor p27kip1. Stimulation of LD density promoted proliferation in colon cancer cells, whereas silencing PLIN2 or overexpression of FOXO3 inhibited proliferation. Taken together, FOXO3 and LDs might serve as new targets for therapeutic intervention of colon cancer.

Tumor suppressor FOXO3 belongs to the family of Forkhead O transcription factors localized in the nucleus, which regulate the expression of genes involved in the control of cell cycle progression, glucose metabolism, and longevity (1–3). The activity of FOXO3, determined by expression, subcellular localization, or DNA binding, is regulated by a variety of external stimuli such as growth factors, insulin, cytokines, bacteria, and oxidative stress (1, 4–7). During tumorigenesis, the loss of FOXO3 activity promotes tumor growth (8–12). In human colon cancer cells, FOXO3 negatively regulates proliferation, and the inactivation of FOXO3 leads to a decrease of the cell cycle inhibitor p27kip1 and a loss of cell cycle arrest (2, 5, 13). Moreover, several critical regulators in colon cancer progression, such as p53 and members of the Wnt/β-catenin pathway (14, 15), alter FOXO3 activity, further revealing the significance of FOXO3 in colon cancer growth.

Lipid droplets (LDs) are organelles constitutively present in adipocytes, which contain a mixture of triglycerides and cholesterol esters, and serve as lipid stores that provides energy for a number of diverse functions in organisms (16, 17). The lipids within LDs are coated with regulatory proteins known as PERILIPINS, which include a family of PLIN1 and PLIN2–5 (previously called adipose differentiation-related protein, TIP47, S3–12, and OXPAT, respectively; nomenclature according to Kimmel et al. (18) and Refs. 19 and 20). PLINs are key regulators of lipid metabolism, and their expression correlates with LD status (21–23). Moreover, LD surfaces are associated with vimentin filaments forming a vimentin cage, which most likely stabilize LDs, suggesting that changes in LDs status involves cytoskeletal reorganization in the cell (24). Although LDs are infrequently observed in most resting non-adipocytic cells, increased density of LDs has been recently associated with human cancer (21, 22, 25–27). However, the regulation and function of LDs in non-adipocytic cells remains unclear.

This study demonstrates increased LD density in proliferative colonic epithelial cells of Foxo3-deficient mice as well as transformed mouse and human colonic cells. Proliferation of colonic cancer cells depends on the LD-mediated loss of

---

*This work was supported, in whole or in part, by National Institutes of Health Grants NIHRO1-CA160809 (to S. D. S.) and NIHRO1-C644239 (to S. E. C.), Senior Investigator Award CCFA 1953 from the Crohn’s and Colitis Foundation of America (to S. D. S.), and Northshore University HealthSystem and University of Chicago Collaborative Grant (to S. D. S. and C. R. W.), and K08DK088953 (to C. R. W.).

1 Present address: Academy of State Administration of Grain, No. 11 Baizhuanzhuang Ave., Xicheng District, Beijing 100037, Peoples Republic of China.

2 To whom correspondence should be addressed: 1001 University Place, Rm. 304, Evanston, IL 60201. Fax: 847-733-5041; E-mail: SSavkovic@northshore.org.

3 The abbreviations used are: LD, lipid droplet; 4-OHT, 4-hydroxytamoxifen; OA, oleic acid; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
FOXO3, leading to a decrease in the cell cycle inhibitor p27kip1. This is a novel mechanism of proliferation that reveals the existence of a LD and FOXO3 regulatory network, which is critical in providing fuel and energy for colon cancer growth.

EXPERIMENTAL PROCEDURES

Cell Culture—Human colon cancer HT-29 cells (ATCC, Manassas, VA) and non-transformed young adult mouse colon, YAMC, cells (28) were employed. Additional human colon cancer cell lines, DLD1 and DL23 (either stably transfected with vector or FOXO3 and selected with geneticin) (2) were used. FOXO3 overexpression was induced with 4-hydroxytamoxifen (4-OHT, 100 nM) (Sigma).

Treatments—LD accumulation was induced with 1 mg/ml of oleic acid (OA) (Sigma) (29).

Inhibitors—C75, a fatty acid synthase inhibitor (50 μg/ml; Sigma), was used to assess the effect of LDs on proliferation and protein regulation. Blockade of PI3K was accomplished using wortmannin (200 nM; Calbiochem).

Immunofluorescent Staining—Experimental monolayers grown on coverslips were washed, fixed with paraformaldehyde, and immunofluorescently stained for FOXO3 and vimentin as described previously (4, 30).

Oil Red-O Staining—Monolayers were fixed in buffered formalin solution (Sigma) and then stained with Oil Red-O solution (Newcomer Supply, Middleton, WI). After rinsing in distilled water, monolayers were counterstained with Gill’s III and mounted with Prolong Gold antifade reagent (Invitrogen).

BODIPY Staining—Monolayers and frozen sections of mouse colonic tissue were fixed in formalin and incubated with 1 μg/ml of BODIPY (Sigma). Slides were washed, mounted with Prolong Gold antifade reagent (Invitrogen).
Gold antifade reagent (Invitrogen), and observed under fluorescent microscope (Electron Microscopy Sciences, Hatfield, PA).

**Protein Extraction and Immunoblot**—Experimental monolayers and tissue were washed with serum-free media, then total and fractionated protein was extracted as previously described (4, 30). Equal amounts of protein, after the concentration was determined by the Bradford assay (Bio-Rad), were loaded on SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). After blocking, specific antibodies such as FOXO3, pAkt, Akt, SIRT6, and p27kip1 from Cell Signaling Technology, Danvers, MA, PLIN3 from ProSci, Inc., Poway, CA, and PLIN2, Oct-1, and actin from Santa Cruz Biotechnology, were used to perform detection.

**Cell Proliferation**—Proliferation was quantified by MTS assay (Promega, Madison, WI) as previously described (5).

**PLIN2 siRNA**—PLIN2 siRNA (sc-44841, Santa Cruz Biotechnology) (50 nM) or equal amounts of negative control low GC oligonucleotides (Invitrogen) were added to 2 × 10⁵ cells. To avoid interference of lipids from transfection agents such as Lipofectamine, cells were incubated with siRNA overnight in serum-free media alone. The next day, medium-containing OA was added and monolayers were kept for 48 h.

**Chromatin Immunoprecipitation (ChIP) Assay**—The cells, after cross-linkage with 1% formaldehyde, were incubated in lysis buffer, sonicated to shear DNA (200 to 1000 bp), and equal amounts of protein were incubated with anti-FOXO3 antibody (Cell Signaling Technology) as previously described (5).

**Animal Studies**—Foxo3-deficient mice, C57BL/6J strain (6), were maintained and genotyped in accordance with approved animal care protocols. Mice were housed in the Biological Resources Laboratory at the Northshore University HealthSystem Research Institute facility and all guidelines and experimental procedures were approved by the animal ethics committee.

**Induction of Mouse Colonic Tumors by Dextran Sulfate Sodium/Azoxymethane**—Colonic tumors were induced in C57BL/6J mice by single intraperitoneal injection of 10 mg/kg of azoxymethane (Sigma) and three cycles of 2.5% dextran sulfate sodium (MP Biomedicals, Solon, OH) (31). Dextran sulfate sodium was added to the drinking water for 5 days, with three-
week intervals. Mice were allowed to develop tumors for 26 weeks following azoxymethane injection.

**Histological Analysis**—Tissue sections were stained with routine hematoxylin and eosin (H&E) or immunohistostained after antigen retrieval with anti-PLIN2 (1:50) for 1 h at room temperature. Labeled polymer rabbit HRP was added, followed by incubation with DAB\(^*\) chromogen, and then counterstained with hematoxylin. Tissues were dehydrated in graded alcohol and xylene, then coverslipped using Permount (Biomeda, Foster City, CA).

**Statistical Analysis**—Data were compared with Student's \(t\) test to determine statistical significance. The results were expressed as mean ± S.D. A \(p\) value of \(<0.05\) was considered statistically significant.

**RESULTS**

**Proliferating Colonic Epithelial Cells in Foxo3-deficient Mice and Colonic Tumors Have Increased LD Density**—We previously showed Foxo3 deficiency in mice led to increased proliferation of colonic epithelia (5). Here, we identified a new function for FOXO3 as a negative regulator of LD density in several organ systems. In Foxo3-deficient mice, increased intracytoplasmic LD density was detected in the liver (Fig. 1A) as well as in colonic epithelia (Fig. 1B). BODIPY staining revealed the LDs localized toward the surface of normal mouse colonic crypts, whereas in the colons of Foxo3-deficient mice, LDs were found along the entire crypts (Fig. 1B). Additionally, elevated expression of the LD coat protein PLIN2, also known to be associated with increased LD density (21–23), was found in Foxo3-deficient colonic cells (Fig. 1C). In normal mouse colonic epithelia, proliferating cells at the base of the crypts, previously known to have attenuated Foxo3 activity (4), exhibited increased PLIN2 staining (Fig. 1D). Moreover, mouse colonic tumors had dramatically increased expression of PLIN2 (Fig. 1D). These data suggest that FOXO3 acts as a potent negative regulator of LD density and colonic tumor cells have a higher capacity for storing LDs, which most likely provide energy to support sustained growth.

**Increased LD Density Leads to Inactivation of FOXO3 in Human Colonic Cancer Cells**—To determine the effects of LD density on FOXO3 activity, human colon cancer cells (mouse

---

**FIGURE 4.** Regulatory network comprised of LD and FOXO3 depended on PI3K and SIRT6. A, protein from HT-29 cells treated with OA were immunoblotted for the substrate Akt (phosphorylated and total) from PI3K. The densitometric analysis graph shows a significant increase in Akt phosphorylation by OA relative to control (*, \(p < 0.05\), \(n = 3\)). B, protein from HT-29 cells treated with OA in the presence of the PI3K inhibitor wortmannin were immunoblotted for FOXO3. Densitometric analysis showed OA-induced FOXO3 degradation was inhibited by wortmannin (*, \(p < 0.05\), \(n = 3\)). C, protein from scraped colonic epithelia of WT and Foxo3 KO mice were immunoblotted for SIRT6. Densitometric analysis revealed a significantly lower SIRT6 in colonic epithelia of Foxo3 KO mice (*, \(p < 0.05\), \(n = 3\)). D, protein from HT-29 cells treated with OA and immunoblotted for SIRT6 revealed degradation in the first 6 h and recovery at 24 h. The densitometric analysis graph shows a significant difference compared with control (*, \(p < 0.05\), \(n = 3\)).
FOXO3 Inhibition of Growth Depends on Lipid Droplet Density

non-transformed colonic cells used as a control) were loaded with LDs using OA. Immunofluorescent staining of neutral lipids (BODIPY) and vimentin showed increases in LD density (Fig. 2A) as well as immunoblotted LD coat protein PLIN2 and PLIN3 in colon cancer cells treated with OA (Fig. 2B). Oil Red-O staining in non-transformed YAMC and cancer HT-29 cells showed a similar finding (Fig. 2C, upper graph). In contrast to YAMC cells, LDs in HT-29 cells assembled in large aggregates and increased 3-fold with OA treatment (Fig. 2C, lower graph).

During OA treatment, FOXO3 decreased and remained low for 6 h, whereas the amount appeared to recover after 24 h in cells with dense LDs (Fig. 3A). This was confirmed by immunofluorescent staining in which FOXO3 became cytosolic with dimmer staining in the first 6 h of OA stimulation but recovered with increased localization in the nucleus (Fig. 3B) of cells with dense LDs (at 24 h). Also, fractionation of cellular protein showed that the FOXO3 amount in the nucleus was similar to the control at 48 h (Fig. 3C), suggesting that FOXO3 activity was recovered after the cells were loaded with LDs. Treatment with C75, an inhibitor of fatty acid synthase (32), depleted LDs and inhibited the OA-mediated FOXO3 decrease in HT-29 cells (Fig. 3D). These data support a direct link between LD density and FOXO3 activity by showing the existence of feedback regulation between LDs and FOXO3 in colon cancer cells.

LD and FOXO3 Feedback Regulation Is Dependent on PI3K and SIRT6—As the above data revealed that FOXO3 activity and LD density have feedback regulation, we assessed other potential regulators involved in the network. In colon cancer cells, FOXO3 activity is predominately controlled upstream by the PI3K pathway (2, 4, 5), also known to be critical in colon cancer progression (33). During stimulated LD density, the PI3K immediate target Akt was increasingly phosphorylated in colon cancer cells (Fig. 4A). Additionally, the PI3K inhibitor wortmannin protected the OA-mediated FOXO3 decrease (Fig. 4B), suggesting that the LD-mediated loss of FOXO3 is via the PI3K pathway. These findings suggest a pathway comprised of LD-PI3K-FOXO3 in colon cancer cells.

Because Foxo3 deficiency led to increased LD density, we anticipated that FOXO3 might negatively regulate lipids in colonic cells. The regulation of lipids has been extensively studied in adipocytes and liver; however, this regulation still remains undefined in colonic tissue (non-transformed and transformed). It has been reported that sirtuins (SIRTs), members of the class III family of histone deacetylase, inhibit lipid accumulation and promote lipid mobilization in adipocytes and liver (34, 35), and could be important in tumor growth of different tissues (36–38). Additionally, in liver cells, FOXO3 transcriptionally regulates SIRT6 (39), further supporting the link between FOXO3 and lipid metabolism. Here, our data revealed that SIRT6 expression was significantly decreased in proliferative colonic epithelia of Foxo3-deficient mice (Fig. 4C), suggesting FOXO3-dependent regulation of SIRT6 in colonic cells. Additionally, in colon cancer cells during OA-stimulated LD density, SIRT6 was down-regulated in the first 6 h, whereas expression was recovered at 24 h (Fig. 4D), similar to FOXO3 (Fig. 3, A and B). These data revealed that during stimulated LD density, loss of FOXO3 leads to the down-regulation of SIRT6, a negative regulator of lipid metabolism, suggesting a signaling cascade involving FOXO3-SIRT6-LD in colon cancer cells. Taken together, these data support the existence of a LD-FOXO3 regulatory network in both non-transformed colonic cells and cancer cells, which includes PI3K and SIRT6.

LD-dependent Loss of FOXO3 Leads to Down-regulation of Cell Cycle Inhibitor p27kip1 in Human Colon Cancer Cells—FOXO3 transcriptionally regulates p27kip1, an inhibitor of the cell cycle (2), and its expression is decreased in human colon cancer tissue (40). Although Straub et al. (22) described increased LDs in human colon cancer, their role in the cell cycle was not defined. To understand the role of LD density in colon cancer, cells subjected to OA-stimulated LD density showed decreased p27kip1 expression during the first 24 h and partial recovery at 48 h (Fig. 5A), whereas C75 protected p27kip1 from OA-induced decreased levels (Fig. 5B). Additionally, FOXO3
was not found on the p27kip1 promoter in the first 4 h of OA treatment, whereas it was present on the promoter at 24 h but less than the control (Fig. 5C). These data demonstrate that increased LD density leads to a loss of FOXO3-dependent down-regulation of the cell cycle inhibitor p27kip1 in colon cancer cells, thereby establishing a connection between LD density and the cell cycle. Also, whereas changes in p27kip1 expression correlate, in part, with the FOXO3 decreased expression and recovery (shown in Fig. 3, A–C), p27kip1 is still significantly lower at 24 h and not fully recovered at 48 h, strongly suggesting that in colon cancer cells, when LD density is increased, cell cycle arrest is attenuated.

Increased LD Density Promotes the Loss of FOXO3-dependent Proliferation in Colonic Cancer Cells—It had been suggested that targeting lipid synthesis or PLINs could be a new strategy for cancer intervention (22, 41), yet the role of LDs and their coat proteins in proliferation is unclear. As the above data showed, increased LD density, via loss of FOXO3, led to a decrease of the cell cycle inhibitor p27kip1, thus, increased LD density has the potential to stimulate proliferation in colonic cancer cells. In HT-29 cells when LDs were stimulated with OA, proliferation was significantly increased (47 ± 10%), whereas the silencing of PLIN2 by siRNA (by ~35%) decreased proliferation induced by OA (51 ± 4%) (Fig. 6A). These data show, for the first time, that in colon cancer cells, increased LD density stimulates growth, whereas silencing LD coat protein attenuates proliferation. Next, it was determined if proliferation mediated by increased LD density depends mainly on a loss of FOXO3 by employing colon cancer cells overexpressing FOXO3. Thus, OA-stimulated proliferation was assessed in colon DLD1 cancer cells and its clone, DL23, stably transfected with FOXO3 under the 4-OHT inducible promoter (2). Similar to HT-29 cells (Fig. 6A), OA stimulated proliferation in parental DLD1 cells (Fig. 6B). In contrast, OA proliferative stimulus in DL23 cells was overridden when FOXO3 expression was induced with 4-OHT (Fig. 6B). Although 4-OHT alone could induce proliferation in DLD1 cells, in DL23 cells, when FOXO3 is overexpressed, the proliferative effect of 4-OHT was not seen. Additionally, the OA proliferative effect on DL23 cells, when FOXO3 expression was not induced, was insignificant relative to DLD1 cells due to a promoter leak as seen in the immunoblot (Fig. 6B), further supporting that a loss of FOXO3 is critical in the LD-mediated proliferation in colon cancer cells. Taken together, the increased LD density stimulates proliferation mainly by loss of FOXO3 and targeting the LD coat protein could be an efficient strategy in attenuating colon cancer growth.

DISCUSSION

Tumor suppressor FOXO3 is associated with many cellular functions, including the ability to modulate tumor cell growth by blocking cell cycle progression. Two recent studies demonstrated a new function of FOXO members in lipid metabolism in the liver (39, 42); however, a direct or indirect mechanism linking FOXO3 to lipids in other tissues has not been reported. We show a novel LD-FOXO3 regulatory network, which when active, stimulated proliferation in colon non-transformed and cancer cells through a loss of cell cycle arrest, whereas simultaneously providing fuel for growth.

The link between growth and metabolic functions in tumor progression is not well understood. Recently, it was found that FOXO3 has a critical function in lipid storage in adipose tissue.
and skeletal muscles during food intake (43), and the loss of FOXO3 is a potential mechanism for increasing lipid synthesis in the liver (44). Liver-specific FOXO1-, FOXO3-, and FOXO4-deficient mice exhibit elevated LD density (42) and active FOXO3 negatively regulates lipid metabolism through the up-regulation of SIRT6 (39) in liver cells. Recently, it has been found that SIRT6 acts as a tumor suppressor by controlling FOXO3 found that SIRT6 acts as a tumor suppressor by controllingFOXO3 regulation of SIRT6 (39) in liver cells. Recently, it has been demonstrated that SIRT6 regulates lipid metabolism in liver cells. Moreover, because PI3K is important in elevated LD density in colon cancer cells and a similar finding is described in cancer cell metabolism (45) and our data shows that FOXO3 may act as a critical regulator of the cell cycle and lipid-derived energy needed for cancer cell growth. Moreover, because PI3K is important in elevated LD density in colon cancer cells and a similar finding is described in Drosophila-derived cells (46), it is expected that the regulation of FOXO3-dependent LD density is evolutionary-conserved among distant species. Collectively, this study demonstrates that FOXO3 has a dual function in regulating both cellular growth and LD-mediated metabolic needs for sustained growth.

Several studies showed increased LD density within cancer cells, yet the relationship between LDs and the cell cycle is unclear. Góczé and Freeman (47) found that LD size changed throughout the cell cycle, but the mechanism was undefined. As in yeast cell cycle kinases, Cdk1/Cdc28 and Cdk1 regulate the flow of lipid precursors into LDs (48), and as our study reveals that LDs control the cell cycle in human cells, LDs and the cell cycle most likely have feedback regulation among different species. However, the role of the LD coat protein in modulation of the cell cycle is not understood, as PLIN2 was found to control the levels of lipids involved in maintaining LDs (23, 49). The increased number of LDs, together with the elevated expression of the primary enzyme catalyzing the synthesis of fatty acids in some tumors (22, 50), and the effective treatment of some tumors with inhibitors of fatty acid synthesis (51) underscore the importance of understanding the interplay between LDs and the cell cycle. Using a Foxo3-deficient mouse model of colon tumorigenesis would be helpful in elucidating the potential role of LDs as a therapeutic target to control the growth of colon cancers. This study also provides evidence that inhibition of fatty acid synthesis may not be the only mechanism for suppressing the LD-dependent proliferation as the silencing of PLIN2 effectively attenuated the growth of colon cancer cells and showed LD coat proteins as molecules with therapeutic potential against tumor progression.

LDs were previously viewed as storage depots for lipids in adipose tissue, but emerging studies in Drosophila demonstrated the vital importance of LDs as energy stores in non-adipocytes in which lipogenic and lipolytic enzymes were found to be critical in the turnover of lipids (52). Also, abnormal LD dynamics, not currently understood, are associated with the pathophysiology of many metabolic diseases, such as obesity, diabetes, atherosclerosis, fatty liver, and cancer (53). Through the last decade, obesity has become a global health issue, clearly associated with an increased risk of colon cancer (54). The current study highlights (a) the importance of LDs not only in adipocytes but also in colon epithelial cells, (b) the critical role of LD in colon tumor growth, and (c) the significance of LD density regulation by tumor suppressors. In conclusion, this study revealed the importance of a novel LD-FOXO3 regulatory network in the growth of colon cells, both non-transformed and cancer cells, and this LD accumulation is a mechanism by which colon cancer cells support their high fuel and energy demands during tumor progression. This growth regulatory pathway could serve as a new target for therapeutic intervention in colon cancer.

**Acknowledgments**—We thank Dr. Burgering (University Medical Center Utrecht, The Netherlands) for providing DLD1 and DL23 cells and Dr. Stanford Peng (Roche Palo Alto LLC, Palo Alto, CA) for providing Foxo3-deficient mice that established the breeding colony.

**REFERENCES**

1. Lee, R. Y., Hench, J., and Ruvkun, G. (2001) Regulation of *C. elegans* DAF-16 and its human ortholog FKHL1 by the daf-2 insulin-like signaling pathway. *Curr. Biol.* **11**, 1950–1957.
2. Medema, R. H., Kops, G. J., Bos, J. L., and Burgering, B. M. (2000) AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404**, 782–787.
3. Brunet, A., Sweeney, L. B., Sturgill, J. F., Chua, K. F., Greer, P. L., Lin, Y., Tran, H., Ross, S. E., Mostoslavsky, R., Cohen, H. Y., Hu, L. S., Cheng, H. L., Jedrzychowski, M. P., Gygi, S. P., Sinclair, D. A., Alt, F. W., and Greenberg, M. E. (2004) Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* **303**, 2011–2015.
4. Snoeks, L., Weber, C. R., Turner, J. R., Bhattacharyya, M., Wasland, K., and Savkovic, S. D. (2008) Tumor suppressor Foxo3a is involved in the regulation of lipopolysaccharide-induced interleukin-8 in intestinal HT-29 cells. * Infect Immun.* **76**, 4677–4685.
5. Qi, W., Weber, C. R., Wasland, K., Roy, H., Walli, R., Joshi, S., and Savkovic, S. D. (2011) Tumor suppressor FOXO3A mediates signals from the EGFr receptor to regulate proliferation of colon cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **300**, C264–C272.
6. Lin, L., Hron, J. D., and Peng, S. J. (2004) Regulation of NF-κB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. *Immunity* **21**, 203–213.
7. Kops, G. J., Dansen, T. B., Polderman, P. E., Saarloos, I., Wirtz, K. W., Coffer, P. J., Huang, T. T., Bos, J. L., Medema, R. H., and Burgering, B. M. (2002) Forkhead transcription factor FOXO3A protects quiescent cells from oxidative stress. *Nature* **419**, 316–321.
8. Arden, K. C. (2006) Multiple roles of FOXO transcription factors in mammalian cells point to multiple roles in cancer. *Exp. Gerontol.* **41**, 709–717.
9. Greer, E. L., and Brunet, A. (2005) FOXO transcription factors at the interface between longevity and tumor oncogene** **24**, 7410–7425.
10. Hu, M. C., Lee, D. F., Xia, W., Golfman, L. S., Ou-Yang, F., Yang, J. Y., Zou, Y., Bao, S., Hanada, N., Saso, H., Kobayashi, R., and Hung, M. C. (2004) IκB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell* **117**, 225–237.
11. Potente, M., Urbich, C., Sasaki, K., Hofmann, W. K., Heeschen, C., Aicher, A., Kollipara, R., DePinho, R. A., Zeiher, A. M., and Dimmeler, S. (2005) Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. *J. Clin. Invest.* **115**, 2382–2392.
12. Yang, J. Y., and Hung, M. C. (2009) A new fork for clinical application. Targeting forkhead transcription factors in cancer. *Clin. Cancer Res.* **15**, 752–757.
13. Delpuech, O., Griffiths, B., East, P., Essafi, A., Lam, E. W., Burgering, B., Downward, J., and Schulze, A. (2007) Induction of Mxi1-SRα by FOXO3a contributes to repression of Myc-dependent gene expression. *Mol. Cell. Biol.* **27**, 4917–4930.
14. You, H., Yamamoto, K., and Mak, T. W. (2006) Regulation of transactivation-independent proapoptotic activity of p53 by FOXO3a. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 9051–9056.
15. Hoogerboom, D., Essers, M. A., Polderman, P. E., Voets, E., Smits, L. M., and Burgering, B. M. (2008) Interaction of FOXO with β-catenin inhibits β-catenin/T cell factor activity. *J. Biol. Chem.* **283**, 9224–9230.
16. Martin, S., and Parton, R. G. (2006) Lipid droplets. A unified view of a...
FOXO3 Inhibition of Growth Depends on Lipid Droplet Density

dynamic organelle. *Nat. Rev. Mol. Cell Biol.*, 7, 373–378

17. Dugall, I., and Hajdúch, E. (2007) A new look at adipocyte lipid droplets. Towards a role in the sensing of triacylglycerol stores? *Cell Mol. Life Sci.*, 64, 2452–2458

18. Kimmel, A. R., Braasemel, D. L., McAndrews-Hill, M., Szaltry, C., and Londos, C. (2010) Adoption of PERILIPIN as a unifying nomenclature for the mammalian PAT-family of intracellular lipid storage droplet proteins. *J. Lipid Res.*, 51, 468–471

19. Londos, C., Szaltry, C., Tansey, J. T., and Kimmel, A. R. (2005) Role of PAT proteins in lipid metabolism. *Biochimie*, 87, 45–49

20. Braasemel, D. L. (2007) Thematic review series. Adipocyte biology. The perilipin family of structural lipid droplet proteins. Stabilization of lipid droplets and control of lipolysis. *J. Lipid Res.*, 48, 2547–2559

21. Heid, H. W., Moll, R., Schwetlick, I., Rackwitz, H. R., and Keenan, T. W. (2008) Expression of p53 activity by interacting with SirT1 (SIRT1). *Proc. Natl. Acad. Sci. U.S.A.*, 105, 1430–1436

22. Straub, B. K., Herpel, E., Singer, S., Zimbelmann, R., Breuhahn, K., and Machado De Oliveira, R., Leid, M., McBurney, M. W., and Guarente, L. C. A. (1998) Increased levels of phosphatidylinositol 3-kinase activity in colorectal tumors. *Cancer Res.*, 58, 7330–7337

23. McIntosh, A. L., Senthivinayagam, S., Moon, K. C., Gupta, S., Lwande, J. S., Kalluri, R., Cherry-Goeppinger, S., Warth, A., Lehmann-Koch, J., Longerich, T., Heid, H., and Schirmacher, P. (2010) Sirt1 histone deacetylase expression is associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Mod. Pathol.*, 22, 922–932

24. Yusof, J., Wei, W., Xiao, X., Guo, J., Xie, X., Li, L., Kong, Y., Lv, N., Jia, W., Zhang, Y., and Xie. X. (2012) Expression of SIRT1 is associated with lymph node metastasis and poor prognosis in both operable triple-negative and non-triple-negative breast cancer. *Med. Oncol.*, 29, 3240–3249

25. Liu, X., Wang, D., Zhao, Y., Bu, Z., Zheng, Z., Wang, L., Wang, H., Hu, W., Roeder, R. G., and Zhu, W. G. (2011) Methyltransferase Set7/9 regulates p53 activity by interacting with SirT1 (SIRT1). *Proc. Natl. Acad. Sci. U.S.A.*, 108, 1925–1930

26. Vazquez-Ortiz, G., Leong, W. I., Park, O., Ki, S. H., Gao, B., and Deng, C. X. (2010) Hepatic-specific disruption of SIRT6 in mice results in fatty liver formation due to enhanced glycolysis and triglyceride synthesis. *Cell Metab.*, 12, 224–236

27. Picard, F., Kurtev, M., Chung, N., Topark-Ngarm, A., Senawong, T., Machado De Oliveira, R., Leid, M., McBurney, M. W., and Guarente, L. (2004) Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-γ. *Nature*, 429, 771–776

28. Rodgers, J., T. and Puigserver, P. (2007) Fasting-dependent glucose and lipid metabolic response through hepatic sirtuin 1. *Proc. Natl. Acad. Sci. U.S.A.*, 104, 12861–12866

29. Nosho, K., Shima, K., Irahara, N., Kure, S., Firestein, R., Baba, Y., Toyoda, S., Chen, L., Hazra, A., Giovannucci, E. L., Fuchs, C. S., and Ogino, S. (2009) SIRT1 histone deacetylase expression is associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Mod. Pathol.*, 22, 922–932

30. Kim, H. S., Xiao, C., Wang, R. H., Lahusen, T., Xu, X., Vassilopoulos, A., Vazquez-Ortiz, G., Leong, W. I., Park, O., Ki, S. H., Gao, B., and Deng, C. X. (2010) Hepatic-specific disruption of SIRT6 in mice results in fatty liver formation due to enhanced glycolysis and triglyceride synthesis. *Cell Metab.*, 12, 224–236

31. Gunter, M. J., and Leitzmann, M. F. (2006) Obesity and colorectal cancer. *Cancer Res.*, 66, 7330–7337

32. Rashid, A., Pizer, E. S., Moga, M., Milgraum, L. Z., Zahurak, M., Pasternack, G. R., Kuhajda, F. P., and Hamilton, S. R. (1997) Elevated expression of fatty acid synthase during S phase in human breast cancer cells. *Cancer Res.*, 57, 7458–7463

33. Vazquez-Ortiz, G., Leong, W. I., Park, O., Ki, S. H., Gao, B., and Deng, C. X. (2010) Hepatic FOXOs regulate lipid metabolism via modulation of expression of the nicotinamide phosphoribosyltransferase gene. *J. Biol. Chem.*, 286, 14681–14690

34. Huang, Y. N., Qi, J. H., Xiang, L., and Wang, Y. Z. (2012) Construction of adiponectin-encoding plasmid DNA and overexpression in mice in vivo. *Gene*, 502, 87–93

35. Zhang, K., Li, L., Qi, Y., Zhu, X., Gan, B., DePinho, R. A., Avrett, T., and Guo, S. (2012) Hepatic suppression of Foxo1 and Foxo3 causes hypoglycemia and hyperlipidemia in mice. *Endocrinology*, 153, 631–646

36. Sebastián, C., Zwaans, B. M., Silberman, D. M., Gynre, M., Goren, A., Zhong, L., Ram, O., Truelove, J., Guimaraes, A. R., Toiber, D., Cosentino, C., Greenson, J. K., MacDonald, A. I., McGlynn, L., Maxwell, F., Edwards, J., Giacosa, S., Guccione, E., Weissleder, R., Bernstein, B. E., Regov, A., Shiel, P. G., Lombard, B. D., and Mostoslavsky, R. (2012) The histone deacetylase SirT6 is a tumor suppressor that controls cancer metabolism. *Cell*, 151, 1185–1199

37. Vereshchagina, N., and Wilson, C. (2006) Cytoplasmic activated protein kinase Akt regulates lipid-droplet accumulation in *Drosophila* nurse cells. *Development*, 133, 4731–4736

38. Gocze, P. M., and Freeman, D. A. (1994) Factors underlyng the variability of lipid droplet fluorescence in MA-10 Leydig cells. *Cytometry*, 17, 151–158

39. Santos-Rosa, H., Grimsey, N., Peak-Chew, S., and Siniossoglou, S. (2004) Fatty acid synthase inhibition triggers apoptosis during S phase in human breast cancer cells. *J. Biol. Chem.*, 279, 4731–4735

40. Ciaparrone, M., Yamamoto, H., Yao, S., Chen, L., Hazra, A., Giovannucci, E. L., Fuchs, C. S., and Ogino, S. (2009) SIRT1 histone deacetylase expression is associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Mod. Pathol.*, 22, 922–932