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14. ABSTRACT
During the research period stated above, we have expanded our colony of SIRT6 F/F; Villin-Cre; APCmin mice and found that lack of SIRT6 dramatically increases intestinal tumorigenesis in vivo, and this phenotype is driven by an enhanced aerobic glycolysis in the absence of SIRT6. To study in more detail how SIRT6 (and aerobic glycolysis) modulates intestinal tumorigenesis, we have knocked-down SIRT6 in a panel of human CRC cell lines (HCT116, SW620, HT29, SW1116). However, no differences in glycolytic metabolism were found. Alternatively, we have taken advantage of our mouse model and found that lack of SIRT6 leads to an increase in the number of intestinal stem cells (ISC), suggesting that SIRT6 regulates intestinal tumorigenesis by controlling the number of tumor initiating cells. We have confirmed this result by using intestinal organoids derived from our mouse model. In parallel, we generated a SIRT6 F/F, Lgr5-EGFP-Cre; APCmin mouse line to precisely address the role of SIRT6 in the ISCs. Our preliminary data shows that SIRT6 F/F, Lgr5-EGFP-Cre; APCmin mice have increased numbers of ISCs compared to control animals, suggesting that SIRT6 might regulate ISC

15. SUBJECT TERMS
SIRT6, intestinal tumorigenesis, cancer metabolism, intestinal stem cells, tumor initiating cells

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1. INTRODUCTION

Colorectal carcinoma (CRC) is the third leading cause of cancer morbidity and mortality in the United States. Familial Adenomatous Polyposis (FAP) represents one of the most common syndromes associated with high penetrant hereditary CRC. A prominent feature of cancer cells is their increased glucose uptake and reliance on aerobic glycolytic metabolism, a phenomenon described by Otto Warburg decades ago. Though it is a potential candidate for targeting against tumors, little is known about the mechanisms controlling it. Remarkably, we have recently identified the SIRT6 histone deacetylase as a central regulator of glycolytic metabolism: cells lacking SIRT6 undergo a dramatic metabolic switch, increasing glycolysis and inhibiting mitochondrial respiration (Mostoslavsky et al., 2006; Zhong et al., 2010). In this proposal, we will study the role of SIRT6 in colorectal cancer cells. We hypothesize that colon cancer cells might selectively down-modulate SIRT6 to acquire a selective advantage in order to grow under conditions of glycolytic metabolism. Indeed, our preliminary results indicate that loss of SIRT6 provides tumorigenic potential to otherwise normal cells, modulating glycolysis and bypassing classical oncogenic pathways. Furthermore, SIRT6 levels are reduced in human tumors, predominantly in colon cancers. In this proposal, we will determine the precise role for SIRT6 in controlling glucose metabolism and the Warburg effect in the context of APC-dependent colorectal cancers. Overall, our results should provide new insights into the molecular mechanisms regulating colon cancer metabolism. In this context, modulation of SIRT6 activity could provide us in the future with a potential therapeutic approach to tackle cancer development.

2. KEYWORDS

SIRT6, cancer metabolism, aerobic glycolysis, intestinal tumorigenesis, intestinal stem cells, tumor initiating cells.

3. OVERALL PROJECT SUMMARY

Task 1. To Study the role of SIRT6 in controlling the switch to glycolytic metabolism in colorectal cancer cells (months 1-10).

Our recently published work (Sebastian et al., 2012; Zhong et al., 2010) demonstrated that cells lacking SIRT6 exhibit an increase in aerobic glycolysis, which drives tumorigenesis without the activation of a major oncogenic pathway. Moreover, we found that SIRT6 expression is downregulated in human colorectal cancer, leading us to propose that SIRT6 could act as a tumor suppressor in this type of cancer by regulating glucose metabolism. To test this hypothesis, we used a lentiviral system to efficiently knock-down SIRT6 expression in a panel of colorectal cancer cell lines (HCT116, HT29, SW1116 and SW620) and analyzed their glycolytic metabolism by measuring glucose uptake (by using a fluorescent glucose analogue, 2-NBDG) and the expression of key glycolytic genes (Glut1, Pdk1, Pfk1 and Ldha). Interestingly, we have found that SIRT6 downregulation does not increase glucose uptake and metabolism in this

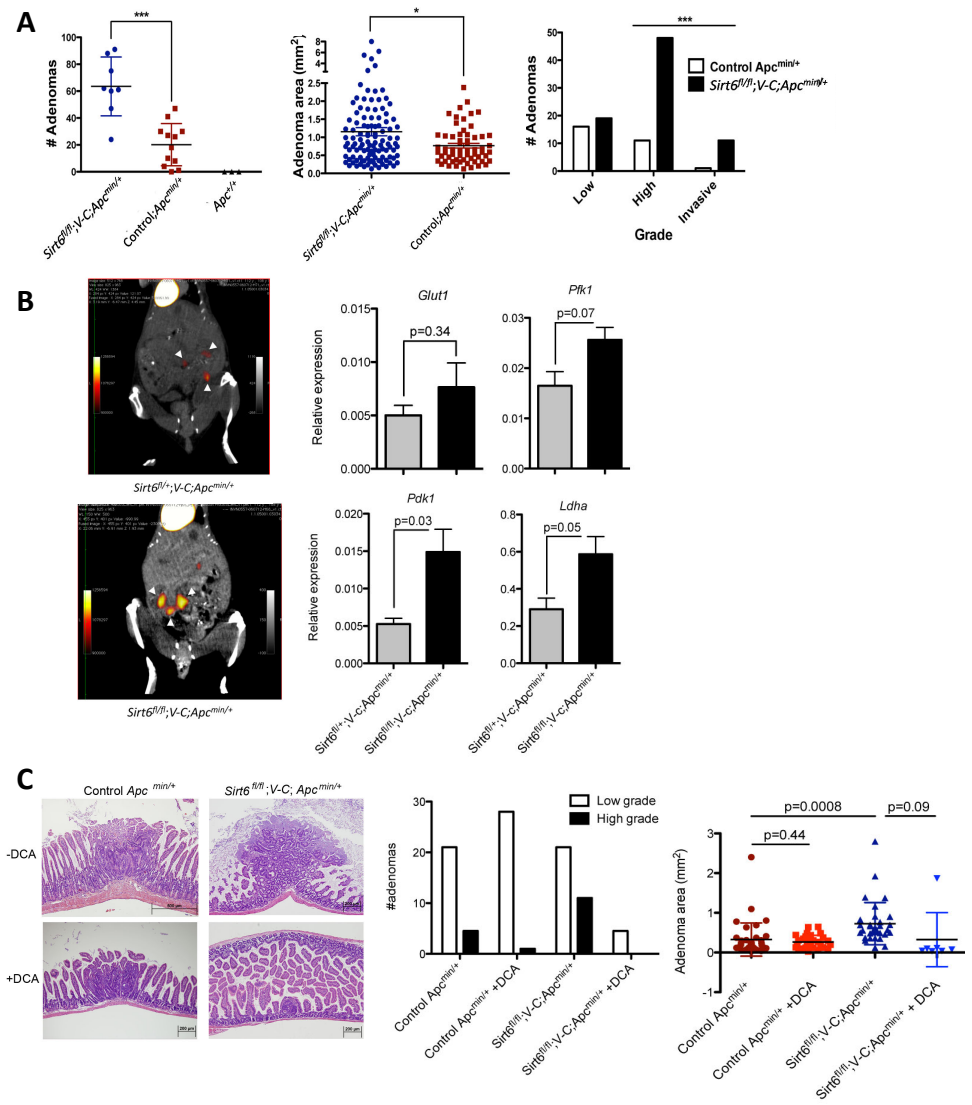

Figure 1. SIRT6 inhibits intestinal tumorigenesis by repressing aerobic glycolysis. A) Number, size and grade of intestinal adenomas in control and Sirt6$^{fl/fl}$; Villin-cre; Apc^{min} mice. B) In vivo glucose uptake (left pannels) and glycolytic gene expression (right pannel) in control and Sirt6$^{fl/fl}$; Villin-cre; Apc^{min} adenomas. C) Representative picture of the size of the adenomas in control and Sirt6$^{fl/fl}$; Villin-cre; Apc^{min} mice treated with DCA (left pannel) and quantification of the number, grade and size of these adenomas (middle and right pannels).

setting (data not shown). In fact, we have observed similar results using cancer cell lines from different tumor types (pancreatic and breast cancer), suggesting that most of established cancer cell lines are already highly glycolytic and, therefore, knocking down SIRT6 does not further increase glucose metabolism. Another possible explanation could be that SIRT6 levels are already very low in these cells (our previous data indicates that SIRT6 expression is downregulated in human colorectal cancer), and thus knocking it down will not have any biological consequence.

Task 2. To evaluate the role of SIRT6 in colon cancer in vivo using a conditional allele of SIRT6 in the context of a murine model of colorectal cancer (months 1-36).

We have crossed our *Sirt6* conditional KO mice with mice expressing Cre recombinase under the *villin* promoter to specifically delete SIRT6 in the intestine of Apc^{min} mice. We have confirmed that deletion of SIRT6 in the intestine of Apc^{min} mice increases the number, size and aggressiveness of intestinal adenomas (Figure 1A). Moreover, glucose uptake (measured by FDG-PET) and expression of several glycolytic genes (Glut1, Pfk1, Pdk1 and Ldha) were upregulated in the adenomas of $Sirt6^{fl/fl}$*; villin-Cre;* Apc^{min} mice (Figure 1B). Finally, treatment with the glycolytic inhibitor DCA (dichloroacetate), specifically inhibited tumor formation in $Sirt6^{fl/fl}$*; villin-Cre;* Apc^{min} mice, as we observed fewer, smaller and lower-grade tumors compared to untreated animals (Figure 1C). These results demonstrated that SIRT6 suppresses intestinal tumorigenesis *in vivo* by inhibiting glucose metabolism reprogramming, and they are part of our manuscript that was published in Cell in December 2012 (see Appendices section) (Sebastian et al., 2012).

Since we didn't observe any change in glycolysis using the human colorectal cancer cell lines proposed in task1 (see above), we decided to use our mouse model as a tool to precisely analyze the role of SIRT6 (and glucose metabolism) on intestinal tumorigenesis. As mentioned before, lack of SIRT6 increases the number of tumors in the intestine of APCmin mice (Figure 1A), suggesting a role for this chromatin factor in tumor initiation. Work done by Hans Clevers laboratory has demonstrated that intestinal stem cells (ISCs) are the cell-of-origin of intestinal cancer in mice (Barker et al., 2009). Therefore, we hypothesized that SIRT6 could be regulating tumor initiation by increasing the number of ISCs, which, upon APC loss, could give rise to intestinal adenomas. To test this possibility, we first performed *in situ* hybridization to detect *Olfm4* mRNA expression (an ISC marker) in the intestine of our mice. Remarkably, we observed a 25% increase in the number of ISCs in the intestine of $Sirt6^{fl/fl}$*; villin-Cre;* Apc^{min} mice compared to control animals (Figure 2). Furthermore, in a preliminary experiment where we treated five controls and one $Sirt6^{fl/fl}$*; villin-Cre;* Apc^{min} mice with DCA, we observed that only in the latter inhibition of glycolysis reduced the number of ISCs (Figure 2), suggesting that increased glycolysis in the absence of SIRT6 might be boosting the number of ISCs.

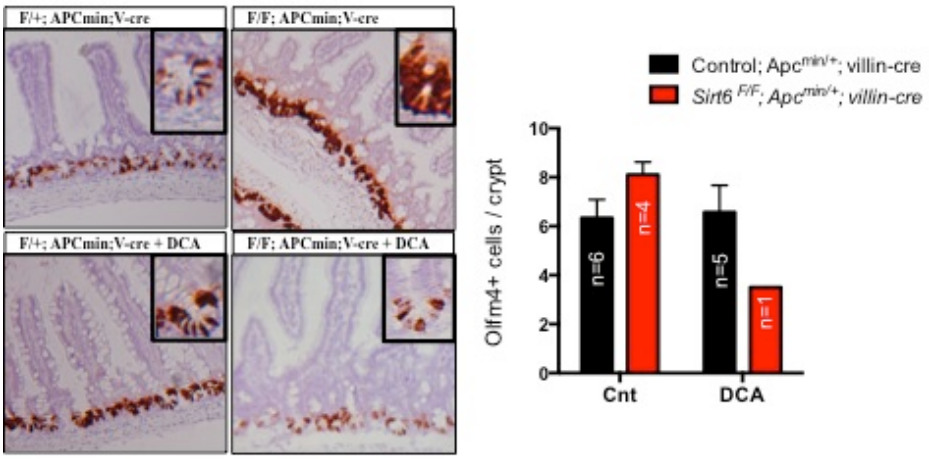

Figure 2. Expansion of ISCs in $Sirt6^{fl/fl}$; Villin-cre; Apc^{min} mice. Representative pictures of Olfm4 *in situ* hybridization showing ISC staining (left pannels) and quantification of Olfm4 postive cells (right pannel) in the intestines of control and $Sirt6^{fl/fl}$; Villin-cre; Apc^{min} mice treated or untreated with DCA.

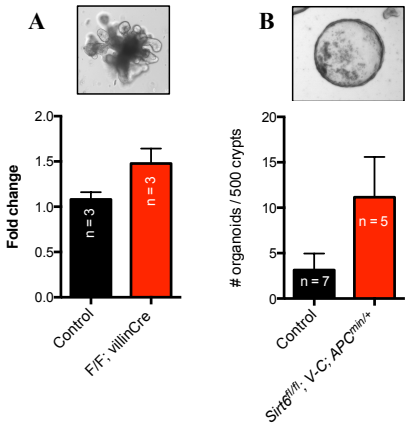

Figure 3. Increased organoid formation in the absence of SIRT6. Representative pictures and quantification of intestinal organoids derived from (A) control and Sirt6$^{fl/fl}$; villin-Cre and (B) control and Sirt6$^{fl/fl}$; villin-Cre; Apc^{min} mice.

Next, we move to an *in vitro* system and derived intestinal organoids from isolated intestinal crypts from control and *Sirt6$^{fl/fl}$; villin-Cre* mice. We observed an increase in the number of organoids in *Sirt6$^{fl/fl}$; villin-Cre* animals, indicating more ISC activity within crypts lacking SIRT6 (Figure 3A). To extend this result to intestinal adenomas, we derived organoids from APCmin mice in the absence of R-spondin, a condition that will only allow crypts from intestinal adenomas to grow and form cyst-like organoids (normal intestinal stem cells are devoid of R-spondin to form organoids). Again, lack of SIRT6 lead to an increase in the number of organoids (Figure 3B), suggesting that SIRT6 regulates the number of adenoma stem cells as well. Together, these preliminary results suggest that SIRT6 might be at the origin of intestinal tumorigenesis by regulating aerobic glycolysis.

To corroborate that the effect of SIRT6 on ISC expansion and intestinal tumorigenesis is cell-autonomous, we planned to specifically delete SIRT6 on ISCs. To do so, we crossed our *Sirt6$^{fl/fl}$* mice with *Lgr5-EGFP-ires-CreERT2* mice (Barker et al., 2007). This system allows us to delete SIRT6 in Lgr5 positive cells (ISCs) as well as to visualize ISCs by GFP expression after tamoxifen injection. In a preliminary experiment, we observed an increase in Lgr5 positive cells in both the intestine and colon of *Sirt6$^{fl/fl}$; Lgr5-EGFP-ires-CreERT2* mice, compared to control animals (Figure 4), suggesting that, indeed, deletion of SIRT6 in ISCs leads to an expansion of the ISC compartment, which represent putative tumor initiating cells.

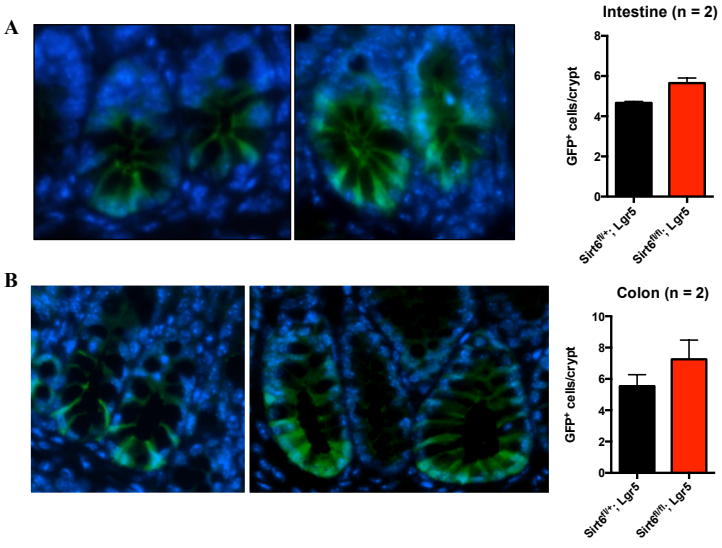

Figure 4. Expansion of Lgr5^{+} ISCs in Sirt6$^{fl/fl}$; Lgr5-EGFP-ires-CreERT2 mice. Representative pictures and quantification of Lgr5^{+} cells in (A) intestine and (B) colon of control and Sirt6$^{fl/fl}$; Lgr5-EGFP-ires-CreERT2 mice. GFP expression was detected on intestine and colon paraffin sections by immunostaining with an anti-GFP atibody.

Task 3. To determine the role of SIRT6 during the early events of APC-mediated cellular transformation using intestinal organoids derived from human FAP-specific iPS cells (months 1-24).

The purpose of this aim was to derive intestinal organoids from FAP-specific iPS cells as an *in vitro* system where to study the role of SIRT6 in human colorectal cancer patients carrying APC mutations. In collaboration with the laboratory of Gustavo Mostoslavsky, we had previously generated iPS cells from these patients, which were used to differentiate intestinal organoids.

However, we needed to mutate the second Apc copy to mimic the normal progression of these patients, who loose the second Apc allele by loss of heterozygosity leading to the development of intestinal adenomas. To do so, we took advantage of the TALEN technology to selectively mutate the Apc locus. We designed TALEN pairs targeting the mutation cluster region of Apc with TALEN Targeter (https://boglab.plp.iastate.edu/node/add/talen) using default parameters. Efficient combinations of TALENs were selected by assessing the frequency of double strand break (DSB)-induced NHEJ events with the SURVEYOR Nuclease Assay (Transgenomic) as described by the manufacturer. TALEN pairs were introduced into iPSCs derived from FAPs patients and surviving iPSCs were allowed to grow and form colonies that were picked up two weeks later and expanded. However, all the clones tested carried mutations in the already mutated allele, suggesting that these cells might require a normal Apc allele to grow. As an alternative, we used iPSCs form healthy donors to sequentially target both Apc alleles. We identified by PCR iPSC clones containing mutations (*indels*) surrounding codon 1320 of APC by PCR, which were confirmed by DNA sequencing of genomic DNA (Figure 5). We selected an iPSC clone carrying a 140-bp deletion in one of the APC alleles that results in a frameshift and the production of a truncated APC protein. Finally, successful targeting of APC was confirmed by western blot analysis using two different antibodies directed to the amino- and carboxy-terminal ends of the protein (Figure 5). We are currently trying to mutate the second allele.

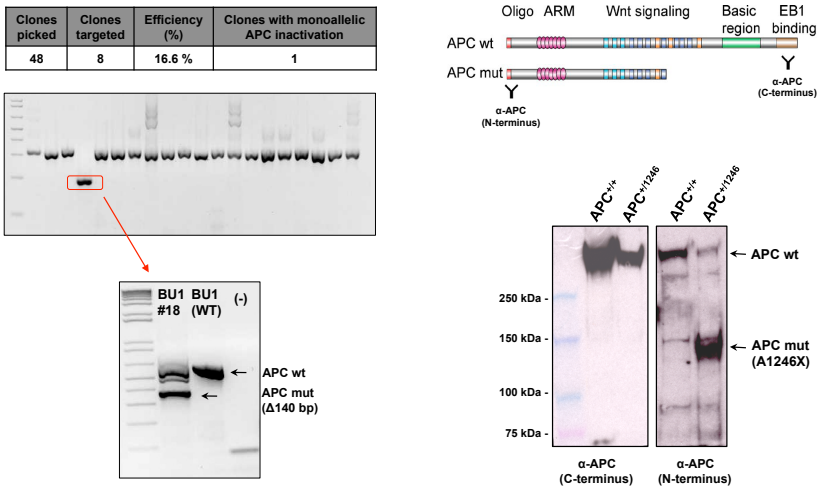

Figure 5. Generation of isogenic disease and control iPSC lines differing exclusively at the APC locus. Following transfection of iPSCs with the TALEN vectors, PCR screening was performed on genomic DNA samples obtained from isolated iPSC clones to discriminate clones with *indels*. A deletion of 140 bp in clone BU1 #18 was confirmed by PCR using a different primer pair and also by direct sequencing. Western Blot analysis of the APC protein in control and mutant iPSCs demonstrates expression of both wild-type and truncated forms of the protein in clone BU1 #18 ($APC^{+/1246}$).

4. KEY RESEARCH ACCOMPLISHMENTS

- Loss of SIRT6 leads to a glucose metabolism reprogramming that drives intestinal tumorigenesis in APCmin mice.
- SIRT6 regulates the number of intestinal and adenoma stem cells, likely by regulating glucose metabolism.

5. CONCLUSION

We have demonstrated that SIRT6 acts as a potent tumor suppressor in colorectal cancer *in vivo* by controlling glucose metabolic reprogramming. Importantly, inhibition of glycolysis in SIRT6-deficient mice dramatically reduces intestinal tumor initiation and growth, suggesting that targeting glycolysis might potentially provide a new approach to modulate cancer growth in those tumors with low SIRT6 levels. Furthermore, preliminary data generated during this year points to SIRT6 as a critical regulator of ISC activity. Using two different mouse models as well as an *in vitro* intestinal organoid system, we have found that lack of SIRT6 leads to an expansion of the ISC compartment. Similarly, SIRT6-deficient intestinal adenomas have increased numbers of adenoma stem cells. These results could imply that SIRT6 regulates intestinal cancer initiation in APCmin mice, since it has been demonstrated that ISCs are the cell-of-origin in this type of tumor. Remarkably, although very few animals have been analyzed so far, this phenotype seems to be dependent of glycolysis, since its inhibition decreases ISCs number only in SIRT6-deficient mice. If confirmed, this result could place, for the first time, cancer metabolism at the origin of cancer. Importantly, an ISC signature identifies colorectal cancer stem cells in human patients and correlates with poorer disease outcome (Merlos-Suarez et al., 2011), indicating that our results could have an enormous impact on those colorectal cancer patients with low levels of SIRT6. In this regard, our work with iPSCs derived from FAP patients represents and invaluable tool to analyze the role of SIRT6 in ISCs and tumor initiation in human samples. Therefore, during the second year we will take advantage of our mouse models and our *in vitro* intestinal organoids (from mouse and from FAP-specific iPSCs) generated during the first year to analyze the role of SIRT6 and glucose metabolism in colorectal cancer in ISCs and colorectal cancer initiation.

6. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

Publications:

1. Lay press: nothing to report
2. Peer-reviewed scientific journals: Sebastián, C., Zwaans, B.M., Silberman, D. M., Gymrek, M. A., Goren, A., Zhong, L., Ram, O., Truelove, J., Guimaraes, A. R., Toiber, D., Cosentino, C., Greenson, J. K., Mac Donald, A., McGlynn, L., Maxwell, F., Edwards, J., Giacosa, S., Guccione, E., Weissleder, R., Bernstein, B. E., Regev, A., Shiels, P. G., Lombard, D. B., and Mostoslavsky, R. The histone deacetylase SIRT6 is a novel tumor suppressor that controls cancer metabolism. *Cell* 2012 Dec 7;151(6):1185-99.

3. Invited articles: Sebastián, C. Tracking Down the Origin of Cancer: Metabolic Reprogramming as a Driver of Stemness and Tumorigenesis. Critical Reviews in Oncogenesis (In Press).
4. Abstracts: nothing to report

Presentations: Massachusetts General Hospital Cancer Center Floor Talk.

7. INVENTIONS, PATENTS AND LICENSES

Nothing to report

8. REPORTABLE OUTCOMES

Nothing to report

9. OTHER ACHIEVEMENTS

Nothing to report

10. REFERENCES

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Zhong, L., D'Urso, A., Toiber, D., Sebastian, C., Henry, R. E., Vadysirisack, D. D., Guimaraes, A., Marinelli, B., Wikstrom, J. D., Nir, T., *et al.* (2010). The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. Cell *140*, 280-293.

Cell

The Histone Deacetylase SIRT6 Is a Tumor Suppressor that Controls Cancer Metabolism

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SUMMARY

Reprogramming of cellular metabolism is a key event during tumorigenesis. Despite being known for decades (Warburg effect), the molecular mechanisms regulating this switch remained unexplored. Here, we identify SIRT6 as a tumor suppressor that regulates aerobic glycolysis in cancer cells. Importantly, loss of SIRT6 leads to tumor formation without activation of known oncogenes, whereas transformed SIRT6-deficient cells display increased glycolysis and tumor growth, suggesting that SIRT6 plays a role in both establishment and maintenance of cancer. By using a conditional SIRT6 allele, we show that SIRT6 deletion in vivo increases the number, size, and aggressiveness of tumors. SIRT6 also functions as a regulator of ribosome metabolism by corepressing MYC transcriptional activity. Lastly, *Sirt6* is selectively downregulated in several human cancers, and expression levels of SIRT6 predict prognosis and tumor-free survival rates, highlighting SIRT6 as a critical modulator of cancer metabolism. Our studies reveal SIRT6 to be a potent tumor suppressor acting to suppress cancer metabolism.

INTRODUCTION

Cancer cells are characterized by the acquisition of several characteristics that enable them to become tumorigenic (Hanahan and Weinberg, 2000). Among them, the ability to sustain uncontrolled proliferation represents the most fundamental trait of cancer cells. This hyperproliferative state involves the deregulation of proliferative signaling pathways as well as loss of cell-cycle regulation. In addition, tumor cells need to readjust their energy metabolism to fuel cell growth and division. This metabolic adaptation is directly regulated by many oncogenes and tumor suppressors and is required to support the energetic and anabolic demands associated with cell growth and proliferation (Lunt and Vander Heiden, 2011).

Alteration in glucose metabolism is the best-known example of metabolic reprogramming in cancer cells. Under aerobic conditions, normal cells convert glucose to pyruvate through glycolysis, which enters the mitochondria to be further catabolized in the tricarboxylic acid cycle (TCA) to generate ATP. Under anaerobic conditions, mitochondrial respiration is abated; glucose metabolism is shifted toward glycolytic conversion of pyruvate into lactate. This metabolic reprogramming is also observed in cancer cells, even in the presence of oxygen, and was first described by Otto Warburg several decades ago (Warburg, 1956; Warburg et al., 1927). By switching their glucose metabolism toward "aerobic glycolysis," cancer cells accumulate glycolytic intermediates that will be used as building blocks

for macromolecular synthesis (Vander Heiden et al., 2009). Most cancer cells exhibit increased glucose uptake, which is due, in part, to the upregulation of glucose transporters, mainly GLUT1 (Yamamoto et al., 1990; Younes et al., 1996). Moreover, cancer cells display a high expression and activity of several glycolytic enzymes, including phosphofructokinase (PFK)-1, pyruvate kinase M2, lactate dehydrogenase (LDH)-A, and pyruvate dehydrogenase kinase (PDK)-1 (Lunt and Vander Heiden, 2011), leading to the high rate of glucose catabolism and lactate production characteristic of these cells. Importantly, downregulation of either LDH-A or PDK1 decreases tumor growth (Bonnet et al., 2007; Fantin et al., 2006; Le et al., 2010), suggesting an important role for these proteins in the metabolic reprogramming of cancer cells.

Traditionally, cancer-associated alterations in metabolism have been considered a secondary response to cell proliferation signals. However, growing evidence has demonstrated that metabolic reprogramming of cancer cells is a primary function of activated oncogenes and inactivated tumor suppressors (Dang, 2012; DeBerardinis et al., 2008; Ward and Thompson, 2012). Despite this evidence, whether the metabolic reprogramming observed in cancer cells is a driving force for tumorigenesis remains as yet poorly understood.

Sirtuins are a family of NAD^+-dependent protein deacetylases involved in stress resistance and metabolic homeostasis (Finkel et al., 2009). In mammals, there are seven members of this family (SIRT1–7). SIRT6 is a chromatin-bound factor that was first described as a suppressor of genomic instability (Mostoslavsky et al., 2006). SIRT6 also localizes to telomeres in human cells and controls cellular senescence and telomere structure by deacetylating histone H3 lysine 9 (H3K9) (Michishita et al., 2008). However, the main phenotype that SIRT6-deficient mice display is an acute and severe metabolic abnormality. At 20 days of age, they develop a degenerative phenotype that includes complete loss of subcutaneous fat, lymphopenia, osteopenia, and acute onset of hypoglycemia, leading to death in less than 10 days (Mostoslavsky et al., 2006). Recently, we have demonstrated that the lethal hypoglycemia exhibited by SIRT6-deficient mice is caused by an increased glucose uptake in muscle and brown adipose tissue (Zhong et al., 2010). Specifically, SIRT6 corepresses HIF-1α by deacetylating H3K9 at the promoters of several glycolytic genes, and consequently, SIRT6-deficient cells exhibit increased glucose uptake and upregulated glycolysis even under normoxic conditions (Zhong et al., 2010). Such a phenotype, reminiscent of the "Warburg effect" in tumor cells, prompted us to investigate whether SIRT6 may protect against tumorigenesis by inhibiting glycolytic metabolism.

Here, we demonstrate that SIRT6 is a tumor suppressor that regulates aerobic glycolysis in cancer cells. Strikingly, SIRT6 acts as a first-hit tumor suppressor, and lack of this chromatin factor leads to tumor formation even in nontransformed cells. Notably, inhibition of glycolysis in SIRT6-deficient cells completely rescues their tumorigenic potential, suggesting that enhanced glycolysis is the driving force for tumorigenesis in these cells. Furthermore, we provide data demonstrating that SIRT6 regulates cell proliferation by acting as a corepressor of c-Myc, inhibiting the expression of ribosomal genes. Finally, SIRT6 expression is downregulated in human cancers, strongly reinforcing the idea that SIRT6 is a tumor suppressor.

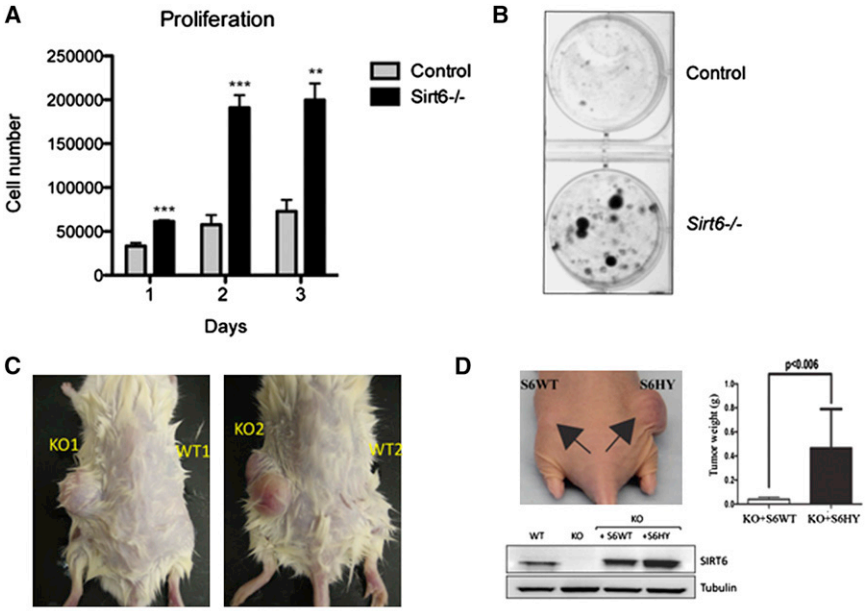
RESULTS

SIRT6-Deficient Cells Are Tumorigenic

We have previously shown that SIRT6 protects from genomic instability (Mostoslavsky et al., 2006) and regulates aerobic glycolysis (Zhong et al., 2010), key features of cancer cells. Therefore, we hypothesized that SIRT6 deficiency could lead to tumorigenesis. To study this possibility, we obtained mouse embryonic fibroblasts (MEFs) from *Sirt6* wild-type (WT) and knockout (KO) embryos and immortalized them by using a standard 3T3 protocol. We found that *Sirt6* KO MEFs showed increased proliferation (Figure 1A) and formed larger colonies when plated at very low density, compared to *Sirt6* WT cells (Figure 1B), indicating that SIRT6 deficiency confers a growth advantage. Next, we injected *Sirt6* WT and KO MEFs into the flanks of SCID mice to assess the ability of these cells to form tumors in vivo. Immortalized MEFs never develop tumors in this setting unless they are transformed with an activated oncogene, such as Ras or Myc ("second hit"). Strikingly, *Sirt6* KO MEFs readily formed tumors (Figure 1C), indicating that SIRT6 deficiency is sufficient to induce transformation of immortalized MEFs. To confirm that this was not due to nonspecific effects of the immortalization process, we immortalized *Sirt6* WT and KO MEFs by knocking down *p53* in passage 3 primary MEFs (Figure S1A available online). Again, *Sirt6* KO MEFs showed increased proliferation (Figure S1B) and were able to form tumors when injected into SCID mice (Figure S1C). Together, these results suggest that SIRT6 acts as a tumor suppressor and, upon loss of cell-cycle checkpoint control, SIRT6 deficiency can lead to tumorigenesis in mice.

Genomic instability can induce transformation by activating oncogenes or inactivating tumor suppressors. Therefore, we first analyzed whether the genomic instability exhibited by SIRT6-deficient cells was responsible for their transformed phenotype. For this purpose, we re-expressed SIRT6 in KO MEFs and injected these cells into nude mice. If genomic instability causes SIRT6-dependent transformation, we would expect tumor formation even in the presence of SIRT6; the effect of mutations occurring on any oncogene or tumor suppressor pathway would not be reverted by simply re-expressing SIRT6 ("mutator phenotype"). However, re-expression of SIRT6 in KO MEFs completely abolished the ability of these cells to form tumors (Figure 1D), suggesting an alternative mechanism for tumor suppression mediated by SIRT6. Furthermore, re-expression of the catalytically inactive SIRT6-H133Y mutant was not able to rescue the tumorigenic phenotype (Figure 1D). Altogether, these results confirm that the tumorigenic capacity of *Sirt6* KO MEFs is specific to the lack of this chromatin regulator and that SIRT6 activity is required for its tumor suppressor function.

Next, we analyzed whether SIRT6 influences tumor growth in the presence of activating oncogenes. For this purpose, we transformed *Sirt6* WT and KO MEFs by expressing an activated form of H-Ras (H-RasV12) and knocking down *p53* expression (shp53). We found that, even in the presence of a strong oncogenic signal such as H-RasV12, *Sirt6* KO cells exhibited



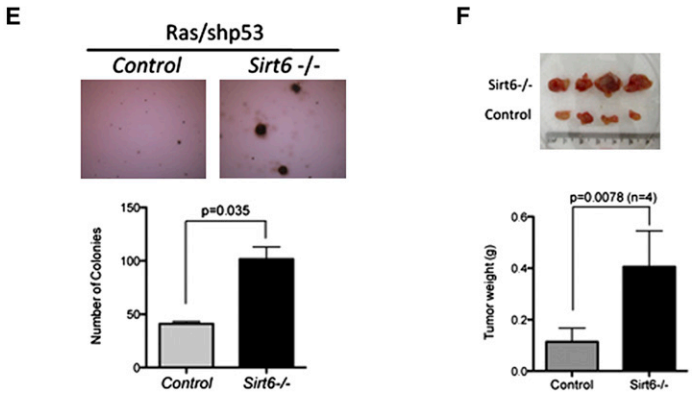


Figure 1. SIRT6-Deficient Cells Are Tumorigenic

(A) *Sirt6* WT and *Sirt6* KO-immortalized MEFs (two independent cell lines for each) were plated, and cells were counted at the indicated times. Errors bars indicate SEM.

(B) *Sirt6* WT and KO cells were plated at very low confluency and assayed for colony formation.

(C) Two independent immortalized cell lines of the indicated genotypes were injected into the flanks of SCID mice to assess their tumorigenic potential.

(D) *Sirt6* KO-immortalized MEFs were transduced with lentiviruses encoding Flag-SIRT6 (WT and HY) and were assayed for in vivo tumor formation as in (C).

(E) Anchorage-independent cell growth of *Sirt6* WT and KO H-RasV12/shp53-transformed MEFs (error bars indicate SD).

(F) The same cells as in (D) were injected into the flanks of SCID mice (n = 4), and the tumors were harvested and weighed (error bars indicate SD). See also Figure S1.

increased anchorage-independent growth in soft agar (Figure 1E) and formed bigger tumors when injected into SCID mice compared to *Sirt6* WT cells (Figure 1F), indicating that SIRT6 deficiency also confers a growth advantage to transformed cells. Taken together, these results strongly suggest that SIRT6 is a tumor suppressor involved in both cancer initiation and tumor growth.

SIRT6-Deficient Cells and Tumors Exhibit Enhanced Aerobic Glycolysis

To identify the mechanism underlying the tumorigenic phenotype associated with SIRT6 deficiency, we focused on SIRT6-dependent regulation of glucose metabolism. Immortalized *Sirt6* KO MEFs showed increased glucose uptake and lactate production (Figure 2A). In addition, re-expression of SIRT6 in these cells reduced glucose consumption (Figure 2B) as well as tumor formation (Figure 1D), suggesting that a switch toward aerobic glycolysis may be involved in the tumorigenic process. Furthermore, acute deletion of SIRT6 by adeno-Cre infection in MEFs derived from *Sirt6* KO conditional mice (Figure 7) increased glucose uptake in these cells (Figure S2A), confirming that this phenotype is specific to the absence of this chromatin factor. To further characterize the glycolytic phenotype of these cells, we measured expression levels of key glycolytic enzymes that are direct targets for SIRT6 (Zhong et al., 2010). When compared to WT MEFs, *Sirt6* KO MEFs expressed higher levels of *Glut1*, *Pfk1*, *Pdk1*, and *Ldha* (Figure 2C). Importantly, the expression of these glycolytic genes was further increased in cells derived from *Sirt6*-deficient tumors (Figure 2C, tumor bar). This may indicate a selective advantage within *Sirt6* KO tumors for cells with increased glycolytic activity. To confirm the reliance of *Sirt6* KO cells on glycolysis, we analyzed their survival after glucose starvation. Whereas nearly all *Sirt6* WT MEFs survived glucose withdrawal, a significant percentage of *Sirt6* KO cells died under these conditions (Figure 2D), indicating that SIRT6 deficiency promotes a state of glucose addiction, a hallmark of cancers undergoing aerobic glycolysis.

Our results indicate that SIRT6 deficiency plays a crucial role in tumor initiation and growth (Figure 1F). To assess whether increased glycolysis is also responsible for the tumor growth phenotype, we analyzed the glycolytic activity of these cells. We found that *Sirt6*KO/H-RasV12/shp53-transformed MEFs uptake more glucose (Figure S2B) and produce more lactate in culture (Figure S2C) when compared to *Sirt6*WT/H-RasV12/shp53 controls. Next, we injected these cells into SCID mice and analyzed glucose uptake in vivo by ^{18}F-fluorodeoxyglucose-positron emission tomography (FDG-PET). Importantly, tumors derived from transformed *Sirt6* KO cells exhibited increased FDG intensity compared to *Sirt6* WT cells (Figure 2E), indicating that SIRT6 deficiency in tumors increases glucose

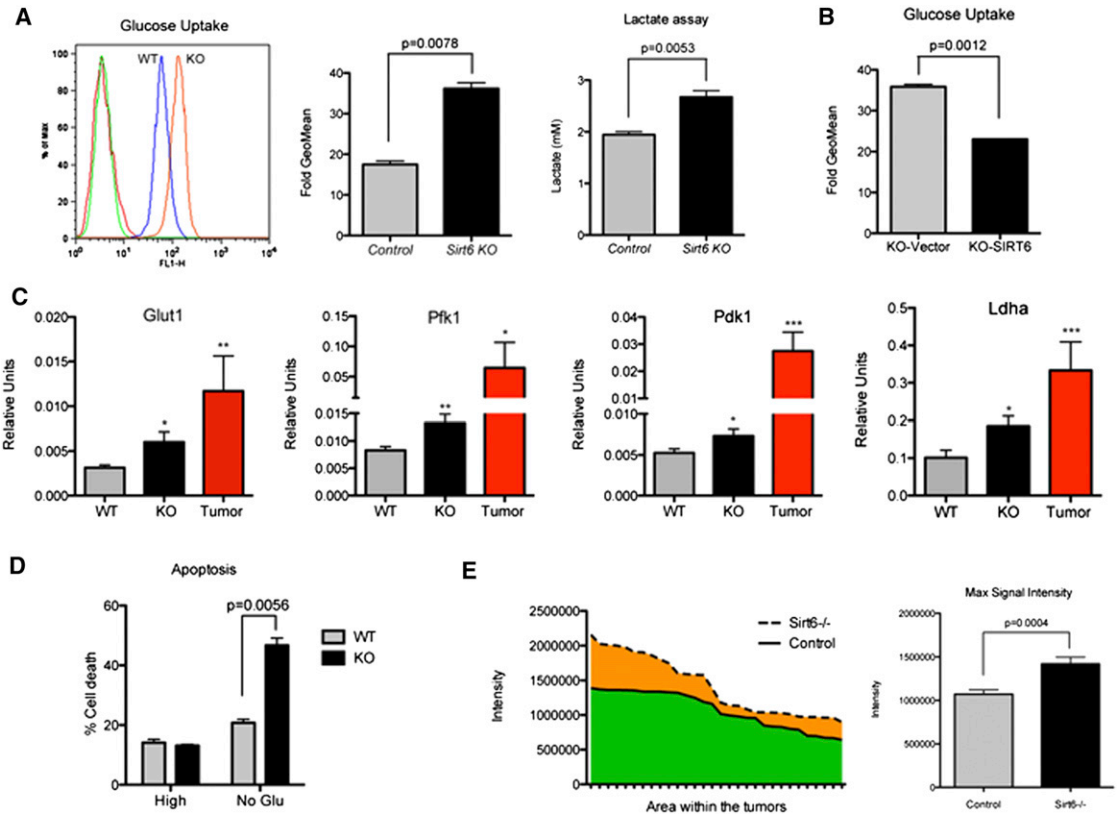


Figure 2. SIRT6-Deficient Cells and Tumors Exhibit Enhanced Aerobic Glycolysis

(A) Glucose uptake (left and middle) and lactate production (right) of *Sirt6* WT and *Sirt6* KO-immortalized MEFs (two independent cell lines; error bars indicate SEM).

(B) Glucose uptake of *Sirt6* KO-immortalized MEFs transduced with either an empty vector or a plasmid encoding Flag-SIRT6 (error bars indicate SD).

(C) Real-time PCR showing the expression of the indicated genes in *Sirt6* WT and *Sirt6* KO (n = 20 experiments from two independent lines) -immortalized MEFs and in the cells derived from *Sirt6* KO tumors (n = 8 experiments from three independent lines) (error bars indicate SEM).

(D) The same cells as in (A) were cultured with or without glucose for 6 days, and cell death was assayed by AnnexinV staining (error bars indicate SEM).

(E) ^{18}FDG-Glucose uptake in *Sirt6* WT and KO H-RasV12/shp53 tumors. Left panel shows FDG-PET intensity of the five sections of each tumor (total of six tumors for each genotype) showing the highest intensity. Right panel shows the average of 30 FDG-PET signals (six tumors, five sections per tumor) for the indicated genotypes (error bars indicate SD).

See also Figure S2.

uptake and glycolysis both in vitro and in vivo. Together, these results strongly suggest that SIRT6 may act as a tumor suppressor by repressing aerobic glycolysis.

Oncogene-Independent Transformation in *Sirt6* KO Cells

The data presented above suggest a role for a SIRT6-dependent glycolytic switch in cancer initiation and progression. Nonetheless, SIRT6 deficiency might promote tumor formation via activation of an oncogenic pathway. To test this possibility, we analyzed the activation of oncogenic signaling pathways in SIRT6-deficient cells. Because deregulation of most oncogenes leads to the activation of the downstream ERK and AKT pathways, we focused on these signaling pathways. Phospho-ERK and phospho-AKT levels were similar in *Sirt6* WT and KO MEFs (Figure 3A, left). In addition, primary MEFs immortalized by knocking down *p53* exhibited the same phenotype (Figure S3A), ruling out nonspecific effect of the immortalization process. Moreover, activation of these pathways in H-RasV12/shp53-transformed MEFs was similar in the presence or absence of SIRT6 (Figure 3A, middle). These results suggest that tumorigenesis in *Sirt6* KO cells is oncogene independent. Importantly, PDK1 and LDHa protein levels were specifically upregulated in both immortalized and transformed *Sirt6* KO cells (Figures 3A and S3A), confirming that these cells are highly glycolytic.

In order to better understand the transformation process in SIRT6-deficient cells, we directly compared Ras-transformed *Sirt6* WT cells with immortalized *Sirt6* KO cells. To this end, we obtained primary MEFs from WT and KO embryos and infected them in parallel with viruses expressing H-RasV12 plus shp53 or shp53 alone, respectively. As expected, analysis of the ERK and AKT pathways showed strong activation of these proteins in H-RasV12/shp53-transformed *Sirt6* WT cells (Figure 3A, right). However, these oncogenic pathways were not activated in shp53-immortalized KO cells, despite their transformation

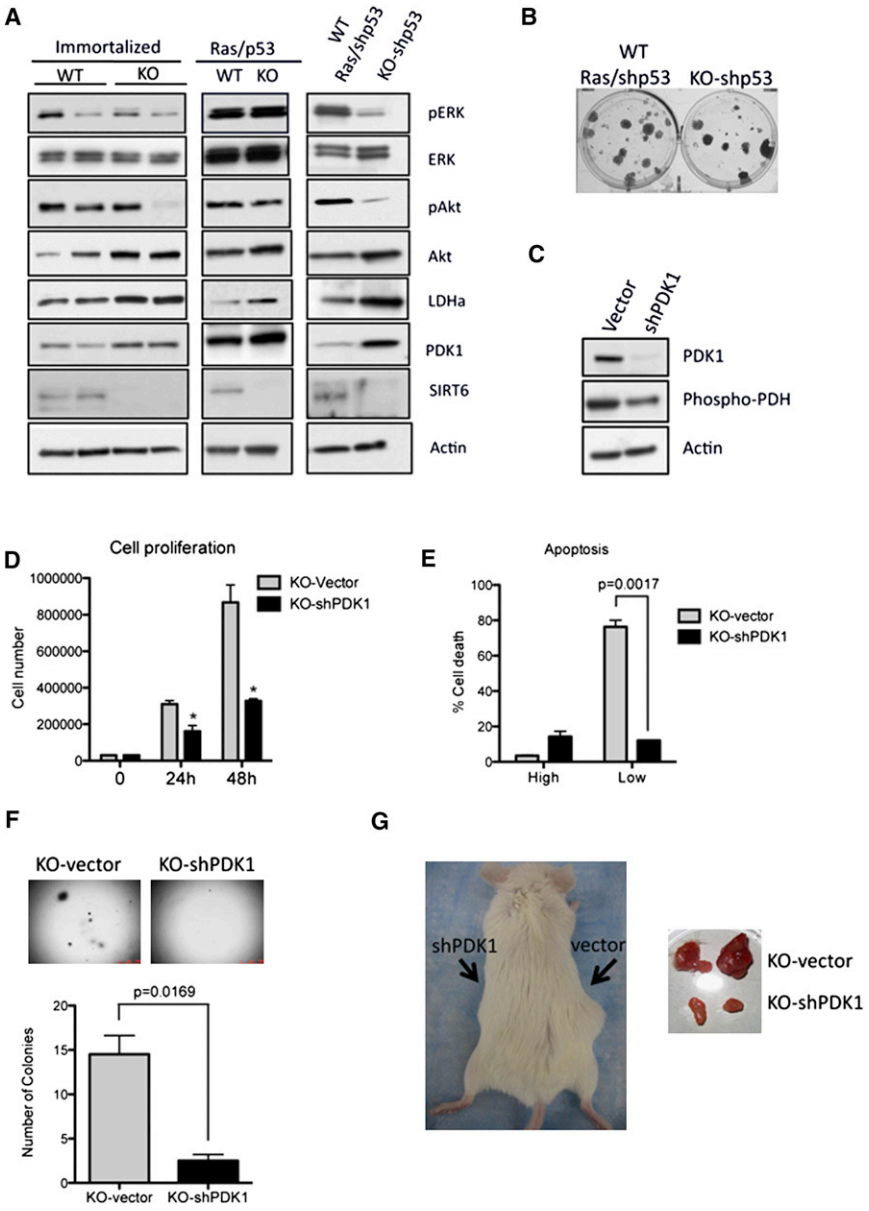


Figure 3. Oncogene-Independent, Glycolysis-Dependent Transformation of SIRT6-Deficient Cells

(A) Western blots showing the activation of ERK and AKT pathways as well as PDK1 and LDHa expression in *Sirt6* WT and KO-immortalized and transformed MEFs.

(B) Colony formation assay with the indicated cell lines.

(C) Western blot of PDK expression and PDH-E1a-Ser293 phosphorylation in *Sirt6* KO-shPDK1 cells.

(D) Cell proliferation of *Sirt6* KO-shVector and *Sirt6* KO-shPDK1 (error bars indicate SD).

(E) Glucose-starvation-induced cell death of *Sirt6* KO-shVector and *Sirt6* KO-shPDK1 cells (error bars indicate SD).

(F) Anchorage-independent cell growth of the same cells as in (E) (error bars indicate SD).

(G) The same cells as in (F) were injected into the flanks of SCID mice (n = 2), and the tumors were harvested and photographed.

See also Figure S3.

capability. Importantly, LDHa and PDK1 expression was higher in *Sirt6* KO cells (Figure 3A), suggesting that enhanced glycolysis, rather than oncogene activation, may be the driving force for tumorigenesis in SIRT6-deficient cells. Further supporting this notion, a colony formation assay indicates similar growth in shp53-immortalized *Sirt6* KO cells and H-RasV12/shp53-transformed WT cells (Figure 3B). Similar to the 3T3-immortalized SIRT6-deficient cells, shp53-immortalized *Sirt6* KO cells gave rise to tumors when injected into SCID mice (Figure S1C).

Inhibition of Glycolysis Suppresses Tumorigenesis in *Sirt6* KO Cells

The above results indicate that SIRT6 acts as a tumor suppressor, potentially by inhibiting a switch toward aerobic glycolysis (Warburg effect). We reasoned that, if this was the case, inhibition of glycolysis would abolish the tumorigenic potential of *Sirt6* KO cells. Because conversion of pyruvate to lactate is rate limiting and represents a downstream step in the glycolytic pathway, we aimed to modulate glycolytic activity in SIRT6-deficient cells by controlling this step. For this purpose, we knocked down the expression of *Pdk1* by using short hairpin RNAs (shPDK1) (Figure 3C). As expected, PDK1 downregulation reduced PDH phosphorylation (Figure 3C). In addition, *Sirt6* KO-shPDK1 cells exhibited reduced proliferative capacity (Figure 3D). Notably, these cells were no longer "glucose addicted," as reflected by the complete rescue of glucose-starvation-induced cell death (Figures 3E and S3B). Moreover, PDK1 downregulation in *Sirt6* KO MEFs inhibited the anchorage-independent cell growth in soft agar (Figure 3F) and severely diminished tumor formation in vivo (Figure 3G). Together, these results demonstrate that SIRT6 may act as a tumor suppressor by blocking a switch toward aerobic glycolysis. In addition, inhibition of glycolysis in SIRT6-deficient cells is sufficient to revert this phenotype, further confirming that these cells have not acquired cancer-driving mutations but rather rely fully on glycolysis for growth.

SIRT6 Controls Cancer Cell Proliferation by Corepressing Myc Transcriptional Activity

In most cancer cells, increased glycolysis per se is not sufficient to provide a growth advantage, suggesting that SIRT6 might be controlling proliferating genes as well. In order to determine

whether this is the case, we used data sets from SIRT6 chromatin immunoprecipitation followed by massively parallel DNA sequencing (ChIP-seq). These data include chromatin maps from two independent cell lines: K562 erythroleukemia cells and human embryonic stem cells (hESCs) (Ram et al., 2011). Gene ontology analysis of SIRT6-bound genomic regions revealed significant enrichment of ribosomal and ribonucleoprotein genes (Figure 4A). Interestingly, the transcription factor MYC has recently been described as a global regulator of ribosome biogenesis (Arabi et al., 2005; Dai and Lu, 2008; Grandori et al., 2005; Grewal et al., 2005), leading us to speculate that SIRT6 and MYC could cooperate in the regulation of ribosomal gene expression. To study this possibility, we first compared the SIRT6 genome-wide binding maps with a published MYC ChIP-seq data set (Ram et al., 2011; Raha et al., 2010) to identify commonly bound genes. Notably, we found that a significantly high percentage of MYC target genes were also enriched for SIRT6 binding (75%; 752/top 1,000 bound genes) (Figure 4B and Table S1). The correlation between SIRT6- and MYC-bound promoters (0.63) was very similar to the one exhibited by the MYC interactors FOS (0.76) and JUN (0.86) (Figure S4A). In contrast, no correlation was observed between SIRT6 and other chromatin modulators, such as EZH2, which is similar to what was observed for Myc (Figure S4B). Moreover, we analyzed the MSigDB gene set collection for their enrichment of overlapping SIRT6-MYC target genes by using the hypergeometric test. We find clear enrichment for genes that fall into ribosome biosynthesis ($p = 9 \times 10^{-8}$), structural constituent of ribosome ($p = 0$), and translation ($p = 1.2 \times 10^{-14}$) GO categories (Figure 4B, yellow dots; Figure 4C), suggesting that SIRT6 might be involved in the regulation of MYC-dependent ribosomal gene expression. Remarkably, ChIP-seq analysis for SIRT6 and MYC in mouse ES cells showed similar cobinding patterns (data not shown), strongly indicating that such a mechanism is evolutionary conserved. We analyzed the cobinding of SIRT6 and MYC on the ribosomal protein genes *Rpl3*, *Rpl6*, *Rpl23*, and *Rps15a* (four of the top hits in overlapping SIRT6/MYC target genes) (Table S1). As expected, MYC binding exhibited a sharp peak on the promoters, colocalizing with the signal of H3K4me3 (a mark of activated as well as poised promoters) (Schneider et al., 2004; Zhou et al., 2011) (Figure 4D). Strikingly, SIRT6 also showed significant enrichment on the promoters of these genes (Figure 4D), suggesting that MYC and SIRT6 are sitting together on the promoter region of ribosomal protein genes. Interestingly, SIRT6 binding extended into the intragenic regions, arguing that SIRT6 may play a role beyond transcriptional initiation, as suggested by our previous work (Zhong et al., 2010).

The co-occupancy of ribosomal gene promoters by MYC and SIRT6 suggests that these two proteins may interact to coordinate expression of target genes. Indeed, Flag-SIRT6 IP in U2OS pulled down MYC, indicating that SIRT6 and MYC can interact (Figure S4C). Although both SIRT2 and SIRT5 exhibited weak interaction with MYC as well, SIRT6 showed the strongest interaction (Figure S4C). Similar results were obtained in 293T cells overexpressing Flag-SIRT6, where MYC was detected in the Flag-IP and vice versa (Figure 4E). To confirm that these proteins interact under physiological conditions, we performed endogenous SIRT6 IP in ES cells. Importantly, MYC was specifically pulled down in the SIRT6 IP (Figure 4F). Altogether, the above results indicate that SIRT6 and MYC interact on the promoter region of ribosomal protein genes. MYC has been described as a transcriptional activator of genes involved in ribosome biogenesis (van Riggelen et al., 2010), whereas we and others have described a role for SIRT6 as a transcriptional repressor (Kawahara et al., 2009; Zhong et al., 2010). Thus, we hypothesized that SIRT6 might act as a corepressor of MYC activity in the context of ribosomal gene expression. To study this possibility, we first tested whether SIRT6 could influence MYC-dependent expression of a luciferase reporter. Indeed, expression of SIRT6 in 293T cells carrying a MYC-luciferase reporter dramatically reduced luciferase expression (Figure 4G), indicating that SIRT6 corepresses MYC activity in this setting. In line with this, we found increased expression of *Rpl3*, *Rpl6*, *Rpl23*, and *Rps15a* in SIRT6-deficient tumors (Figure 4H). Interestingly, the expression of all these genes is not upregulated in immortalized *Sirt6* KO MEFs (Figure S4B), suggesting that the increase in ribosome biogenesis may be a late event during the tumorigenic process in SIRT6-deficient cells. Consistent with this idea, ribosomal gene expression was found to be upregulated in cells derived from *Sirt6* KO tumors (Figure S4D, tumor bar). Similarly, glutamine uptake and glutaminase (*Gls*) expression, which are also regulated by MYC in cancer cells (Dang, 2012), are not upregulated in *Sirt6* KO-immortalized MEFs (Figures S4E and S4F), whereas *Sirt6* KO H-RasV12/shp53-transformed MEFs exhibited increased glutamine uptake (Figure S4E).

We next studied in detail the molecular mechanism by which SIRT6 regulates MYC transcriptional activity. MYC expression and protein stability are not affected by SIRT6 deficiency (Figures S5A and S5B). Similarly, MYC acetylation levels are not changed in *Sirt6* KO cells (Figure S5C). Although we cannot completely rule out by western blot that SIRT6 may deacetylate MYC in a specific residue, this result strongly suggests that SIRT6 is not a main deacetylase for MYC. Moreover, SIRT6 is not regulating the recruitment of MYC to its target promoters because MYC binding to ribosomal gene promoters was not affected in *Sirt6* KO cells (Figure S5D). As mentioned above, SIRT6 has been described as an H3K9 deacetylase. Thus, we analyzed by ChIP the acetylation levels of this histone mark on the promoter region of ribosomal protein genes. Surprisingly, the levels of H3K9 acetylation were not changed on these promoters, which is in contrast with what we observed on glycolytic gene promoters (Figure S5E) (Zhong et al., 2010). However, we found an increase in H3K56 acetylation on the promoter region of ribosomal protein genes in SIRT6-deficient cells (Figure 4I). H3K56Ac is a direct substrate of SIRT6 (Yang et al., 2009; Michishita et al., 2009), and this histone mark has been involved in transcriptional regulation (Xie et al., 2009), indicating that this residue might be a specific substrate of SIRT6 in the context of ribosomal gene expression.

Finally, to fully test whether MYC-dependent gene expression was important for the tumorigenic phenotype in the absence of SIRT6, we knocked down the expression of *c-Myc* in *Sirt6* KO-immortalized MEFs (Figure 5A) and found that, indeed, MYC downregulation in these cells reduced their proliferation

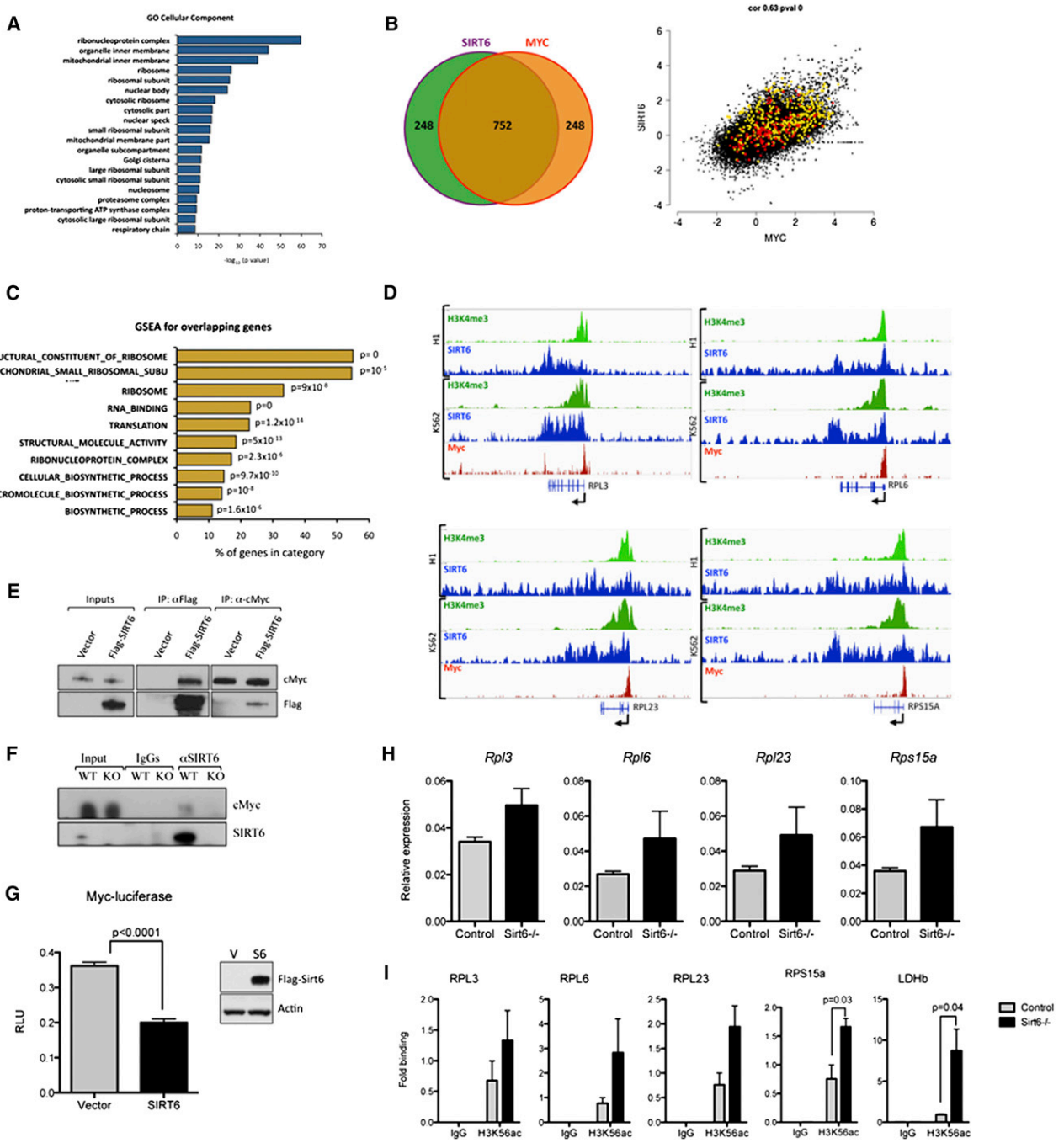


Figure 4. SIRT6 Inhibits Ribosomal Gene Expression by Corepressing MYC Transcriptional Activity

(A) Gene Ontology clustering of SIRT6-bound promoters.

(B) Overlapping of the top 1,000 SIRT6- and MYC-bound promoters.

(C) Gene Set Enrichment Analysis for the overlapping genes described in (B).

(D) H3K4me3, SIRT6, and MYC ChIP signal in the indicated genomic regions in K562 cells and human ES cells (H1).

(E) Flag-SIRT6 and cMYC IPs showing physical interaction between SIRT6 and MYC.

(F) Endogenous SIRT6 was immunoprecipitated, and the interaction with MYC was analyzed by western blot.

(G) A luciferase reporter gene under the regulation of a MYC-responsive element was contrasfected with empty vector or Flag-SIRT6 plasmids in 293T cells, and luciferase expression was analyzed 24 hr later (error bars indicate SEM).

(H) Expression of the indicated genes in *Sirt6* WT and KO H-RasV12/shp53 tumors (n = 4) (error bars indicate SEM).

(I) ChIP analysis of H3K56 acetylation levels in *Sirt6* WT and KO H-RasV12/shp53 MEFs (n = 4, error bars indicate SEM).

See also Figure S4 and Table S1.

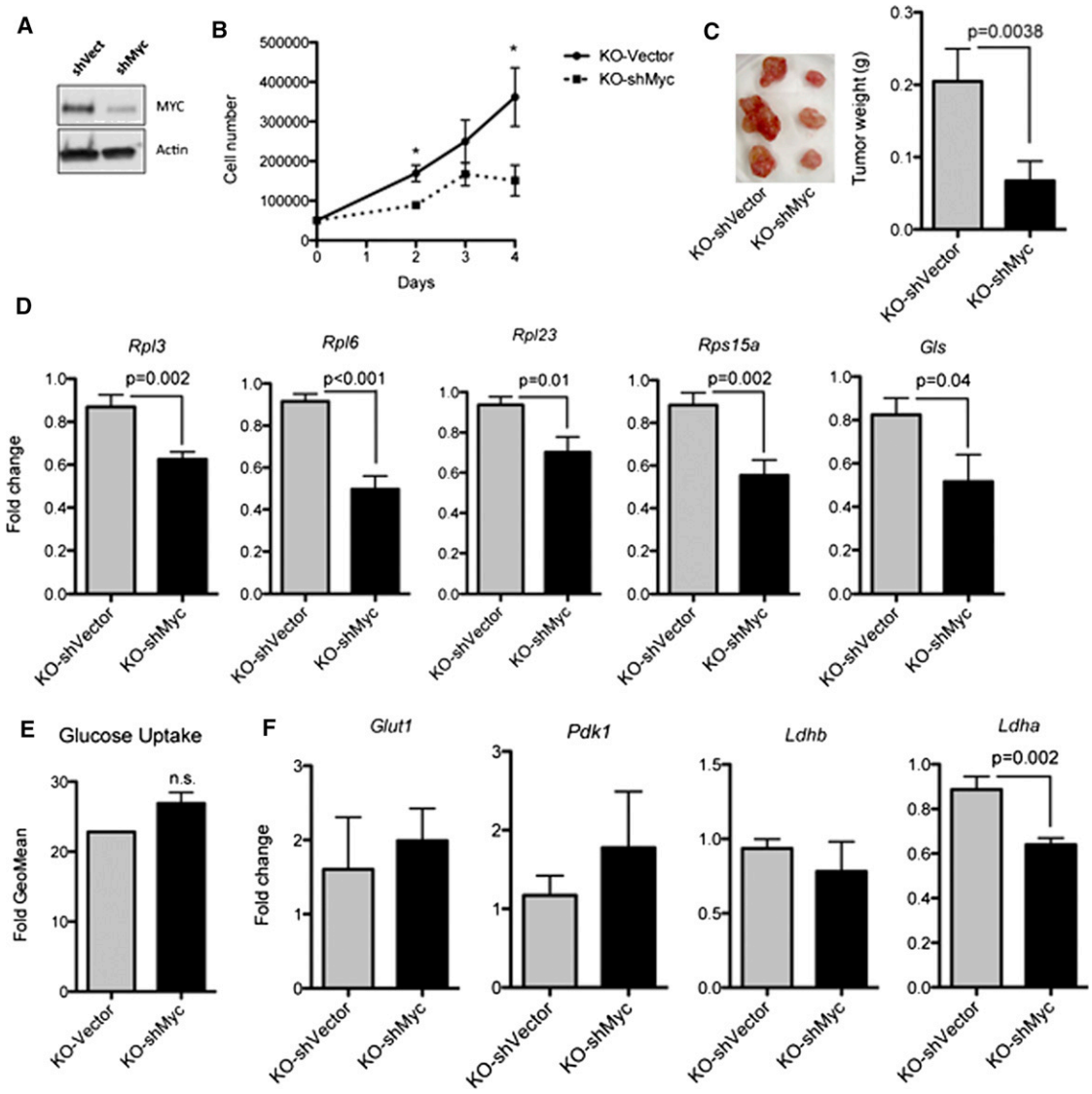


Figure 5. MYC Regulates Tumor Growth of SIRT6-Deficient Cells

(A) Western blot showing MYC levels in *Sirt6* KO-shVector and shMYC cells.

(B) 5×10^5 MEFs were plated in triplicate, and cells were counted at the indicated time points (error bars indicate SD).

(C) 5×10^6 cells of the indicated genotypes were injected into SCID mice, and the tumors were harvested and weighted (error bars indicate SD).

(D) Expression of the indicated genes in *Sirt6* KO-shVector and KO-shMYC cells (n = 9) (error bars indicate SEM).

(E) Glucose uptake was analyzed in the same cells as in (A) (error bars indicate SD).

(F) The same samples as in (D) were used to analyze the expression of the indicated genes (error bars indicate SEM).

See also Figure S5.

(Figure 5B) and, more importantly, dampened tumor growth (Figure 5C). In addition, MYC knockdown decreased ribosomal protein gene expression as well as *Gls* expression (Figure 5D). However, glucose uptake and glycolytic gene expression were unaffected in *Sirt6* KO-shMYC cells. These results indicate that MYC is controlling tumor growth in SIRT6-deficient cells specifically by regulating ribosome and glutamine metabolism, whereas SIRT6's effect on glycolysis likely depends on its function as a HIF-corepressor (Zhong et al., 2010; see Discussion).

Sirt6 Expression Is Downregulated in Human Cancers

The above results indicate a putative role for SIRT6 as a tumor suppressor regulating glycolytic metabolism, suggesting that its expression or activity might be decreased in human cancers. To study this possibility, we analyzed *Sirt6* gene copy number across multiple cancer types by using the Tumorscape database (Beroukhim et al., 2010). Strikingly, *Sirt6* lies within a region in chromosome 19 significantly deleted across the entire data set (Figure 6A) (q value = 0.00011). Additionally, The Cancer Genome Atlas (TCGA) database revealed that *Sirt6* is deleted

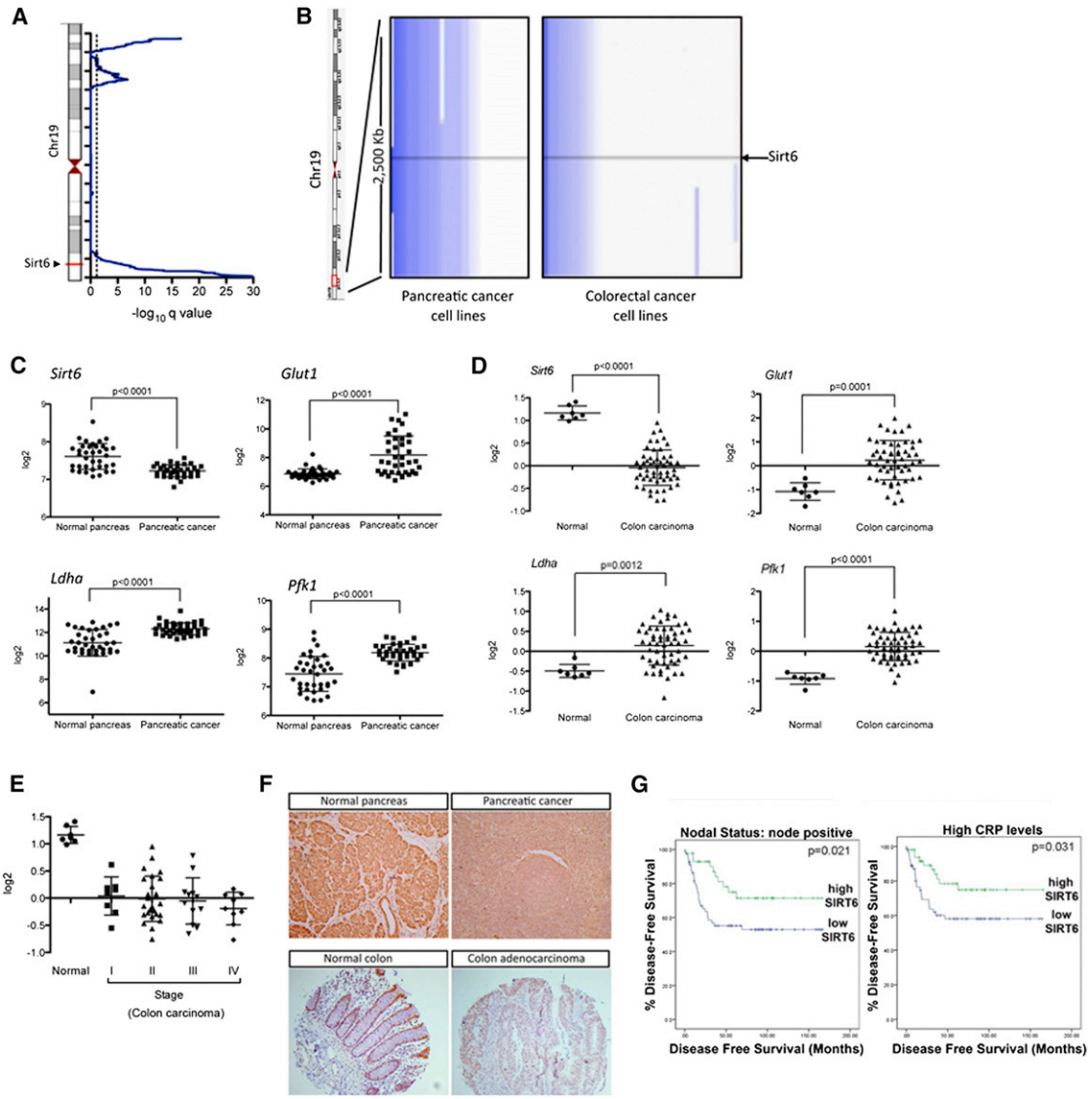


Figure 6. SIRT6 Expression Is Downregulated in Human Cancers

(A) Analysis of gene copy number loss in chromosome 19. Blue line indicates deletion significance ($-\log_{10}$(q value); 0.6 [dotted line] is the significance threshold for deletion). *Sirt6* location within the chromosome is indicated.

(B) *Sirt6* copy number data for pancreatic (left graph, n = 40) and colorectal (right graph, n = 51) cancer cell lines. Color bars indicate degree of copy number loss (blue) or gain (red).

(C) Gene expression of the indicated genes in human pancreatic cancer (GEO data set GSE15471).

(D) Gene expression of the indicated genes in human colon carcinoma (GEO data set GSE31905).

(E) *Sirt6* expression in the same colon carcinoma data set as (D) but classified by stage.

(F) IHC showing SIRT6 expression in pancreatic cancer and colon adenocarcinoma compared to normal tissue.

(G) Kaplan-Meier curves showing disease-free survival rates in patients with node-positive tumors (left) or high CRP serum levels (right) with high and low levels of nuclear SIRT6.

See also Figure S6.

in 20% of all cancers analyzed (q value = 3.87×10^{-110}) and, importantly, that it is located within a peak of deletion in almost 8% of colorectal cancers (Figure S6A) (q value = 0.0119). Next, we used the Cancer Cell Line Encyclopedia (CCLE) (Barretina et al., 2012) to further study gene-copy alterations of *Sirt6* in human cancers. We found that the *Sirt6* locus is deleted in 35% of ~1,000 cancer cell lines collected in this data set and, importantly, in 62.5% and 29% of pancreatic and colorectal cancer cell lines, respectively (Figures 6B and S6B). In accordance with our model, *Sirt6* is not amplified in any of the pancreatic cancer cell lines and only in 4% of colorectal cancer cell lines analyzed (Figures 6B and S6B).

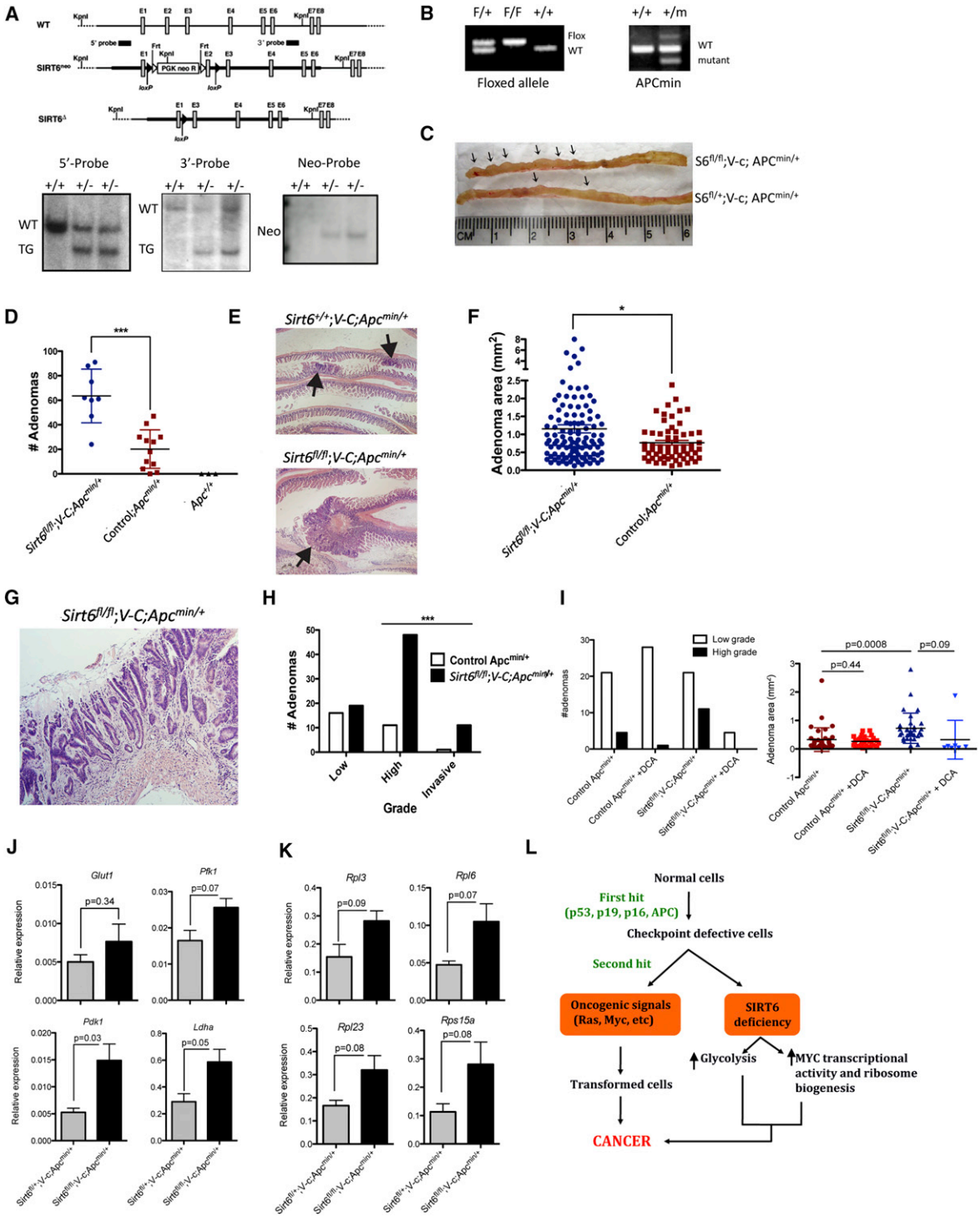


Figure 7. SIRT6 Functions as a Tumor Suppressor In Vivo

(A) Strategy to target the *Sirt6* locus (top). Southern blot (5′, 3′, and Neo probes) of KpnI-digested genomic DNA showing the targeted allele in the heterozygous cells ($^{+/-}$) (bottom).

(B) PCR showing the presence of the *Sirt6* floxed allele (left) and the mutant *Apc* allele (right).

(C) Representative image of a intestine section from *Sirt6*$^{fl/fl}$*;V-c;Apc*$^{min/+}$ and *Sirt6*$^{fl/+}$*;V-c;Apc*$^{min/+}$ mice. Arrows indicate the presence of polyps.

(D) Adenoma number in the intestines of mice of the indicated genotype.

(E) Hematoxylin and eosin staining (H&E) showing the adenoma size in the indicated mice.

(F) Adenoma area in *Sirt6*$^{fl/fl}$*;V-c;Apc*$^{min/+}$ and Control*;Apc*$^{min/+}$ mice.

The significant loss of the *Sirt6* locus in pancreatic and colorectal cancer suggests that SIRT6 expression might be downregulated in these types of cancer. Indeed, we found that *Sirt6* expression is downregulated in a pancreatic ductal adenocarcinoma data set of 36 individual cases (Badea et al., 2008) compared to their matched normal tissue (Figure 6C, $p < 0.0001$). Moreover, the analysis of a data set containing 55 colorectal carcinomas (Anders et al., 2011) also showed decreased *Sirt6* expression when compared to normal colon samples (Figure 6D, $p < 0.0001$). Remarkably, the expression of the SIRT6-target genes *Glut1*, *Ldha*, and *Pfk1* is significantly upregulated in these samples.

Although we cannot rule out that activation or inhibition of other pathways could also be responsible for the increased glycolytic gene expression, our results indicate that the pancreatic and colorectal tumors analyzed are highly glycolytic, and such increase in glycolysis strongly correlates with selective downregulation of *Sirt*6 expression in these tumors. Furthermore, the analysis of additional data sets (Oncomine and GEO) also reveals decreased expression of *Sirt6* in pancreatic and colon cancer as well as in rectal adenocarcinoma (Figure S6C) ($p < 0.0001$). These observations suggest a general role for SIRT6 as a tumor suppressor in these carcinoma types. Interestingly, *Sirt6* expression is also downregulated in pancreatic intraepithelial neoplasia and colon adenomas ($p < 0.0001$), a phenotype that correlates with high expression of glycolytic genes in these samples (Figures S6C and S6D). This further suggests that SIRT6 downmodulation is an early event during tumorigenesis, thereby indicating that this glycolytic switch may play a role in initiation of tumor development. In line with this evidence, classification of the 55 colorectal carcinomas described above showed that *Sirt6* expression is downregulated in early stages and, importantly, its low expression is maintained during cancer progression, indicating that SIRT6 downregulation might be required for both tumor initiation and maintenance (Figure 6E).

To further validate these observations, we used immunohistochemistry to analyze SIRT6 expression in a set of human pancreatic and colorectal cancers. Whereas normal pancreatic ducts and colon crypts exhibited strong SIRT6 staining, pancreatic ductal adenocarcinoma and colorectal carcinoma tissues had a clear decrease in SIRT6 protein levels (Figure 6F). Taken together, the results derived from human data sets strongly indicate that SIRT6 may act as a tumor suppressor in human pancreatic and colorectal cancer. Furthermore, selective downregulation of SIRT6 in tumors may provide an important selective advantage through modulation of glycolytic metabolism.

In order to determine whether SIRT6 expression levels could be correlated with cancer progression and/or survival, we performed IHC for SIRT6 expression in samples from 253 colorectal carcinomas (CRCs), collected over a period of 11 years at the Department of Surgery, Western Infirmary, Glasgow. Two independent observers scored tumors by using the histoscore method (see Experimental Procedures). When patients were categorized according to nodal status, there was no significant difference in patient outcome due to SIRT6 expression levels in node-negative patients. However, in node-positive patients, low levels of SIRT6 were associated with shorter time to relapse ($p = 0.021$, 96 months versus 128 months; Figure 6G, left). Furthermore, these patients were 2.3 times more likely to relapse than those patients whose tumors expressed high levels of SIRT6 ($p = 0.024$). Patients were also categorized by C-reactive protein (CRP) serum levels, a known marker of colon cancer progression. In the subgroup with high levels of CRP, patients with low levels of nuclear SIRT6 had shorter time to relapse than those patients with high levels of nuclear SIRT6 ($p = 0.031$, 101 versus 131 months; Figure 6G, right). These patients were 2.2 times more likely to relapse than patients whose tumors expressed high levels of SIRT6 ($p = 0.036$). These results suggest that decreased disease-free survival time is associated with low tumor levels of nuclear SIRT6 in patients with more aggressive tumors (node-positive tumors and high CRP serum levels).

SIRT6 Acts as a Tumor Suppressor In Vivo

The above results strongly indicate that SIRT6 functions as a tumor suppressor, suggesting that its absence would lead to tumorigenesis in vivo. However, *Sirt6* germline KO mice die early in life (Mostoslavsky et al., 2006), thus preventing the use of this mouse model to experimentally confirm this hypothesis. To overcome this issue, we took advantage of conditional gene-targeting technology to inactivate SIRT6 in a tissue-specific manner and generated mice with one or both floxed alleles for *Sirt6* (Figure 7B, left; Figures 7A–7C and 7I–7J). In parallel, we analyzed a previously described mouse strain with a floxed *Sirt6* allele with similar results (Figures 7D–7H) (Kim et al., 2010a).

To determine the role of SIRT6 in tumorigenesis in vivo, we focused on a model of colorectal adenomatosis, utilizing the well-established Apc^{min} mouse (see Experimental Procedures) (Moser et al., 1990; Su et al., 1992). We have generated mouse lines carrying the APC mutation in the presence or specific intestinal deletion of SIRT6, hereafter referred to as control ($Sirt6^{+/+}$ or $Sirt6^{fl/+}$);*V-C;*$Apc^{min/+}$ and $Sirt6^{fl/fl}$;*V-C;*$Apc^{min/+}$, respectively (Figure 7B, right). Strikingly, we found that $Sirt6^{fl/fl}$;*V-C;*$Apc^{min/+}$ mice developed a 3-fold increase in the number of adenomas when compared to $Apc^{min/+}$ control animals (Figures 7C and 7D) ($p < 0.0001$) and that these adenomas were on average 2-fold larger than those observed in control mice ($p = 0.017$) (Figures 7E and 7F). Furthermore, pathologic analysis of the polyps showed that the lesions were of higher grade in the absence of SIRT6, resulting in many invasive tumors, a phenotype rarely observed in $Apc^{min/+}$ animals (Figures 7G and 7H). Importantly, glucose uptake (measured by FDG-PET scanning) and expression of glycolytic genes were upregulated in the adenomas from $Sirt6^{fl/fl}$;*V-C;*$Apc^{min/+}$ mice (Figures 7J and S7A), suggesting that SIRT6 suppresses intestinal tumorigenesis by inhibiting glycolysis. Remarkably, treatment with the PDK1 small-molecule inhibitor dichloroacetate (DCA) (Bonnet et al., 2007) specifically inhibited tumor formation in $Sirt6^{fl/fl}$;*V-C;*

(G and H) (G) Representative image and (H) quantification of the grade of the tumors in the indicated mice.
(I) Grade (right) and area (left) of the adenomas in $Sirt6^{fl/fl}$;*V-c;*$Apc^{min/+}$ and Control;$Apc^{min/+}$ mice untreated or treated with DCA (5 g/l).
(J and K) Expression of several glycolytic and ribosomal genes in adenomas (n = 3) of $Sirt6^{fl/fl}$;*V-c;*$Apc^{min/+}$ and $Sirt6^{fl/+}$;*V-c;*$Apc^{min/+}$ mice (error bars indicate SEM).
See also Figure S7.

Apc$^{min/+}$ mice, as we observed fewer, smaller, and lower-grade tumors compared to untreated animals (Figures 7I and S7B). In contrast, DCA treatment had little to no effect on control *V-C;Apc*$^{min/+}$ mice, strongly indicating that glycolysis plays a dominant and driving role in SIRT6-deficient tumors. Finally, ribosomal gene expression and *Gls* expression were also upregulated in the adenomas from *Sirt6*$^{fl/fl}$*;V-C;Apc*$^{min/+}$ mice (Figures 7K and S4G). Together, our results demonstrate that SIRT6 inhibits the initiation and progression of colorectal cancer in vivo by repressing aerobic glycolysis and ribosomal gene expression (Figure 7L).

DISCUSSION

The data presented here reveal a role for SIRT6 as a tumor suppressor. By using a combination of in vitro and in vivo studies, as well as data from several human cancer databases, we have demonstrated that loss of SIRT6 leads to tumorigenesis, and its expression is selectively downregulated in several human cancers. Mechanistically, SIRT6 represses aerobic glycolysis (Warburg effect), dampening cancer initiation and growth. Moreover, we describe a key role of this sirtuin in controlling cancer cell proliferation by corepressing MYC transcriptional activity and the expression of ribosomal genes.

Given their absolute dependency on NAD^+, sirtuins have evolved as critical modulators of stress responses, DNA repair, and metabolism, sensing changes in metabolic cues in order to exert adaptive responses (Finkel et al., 2009). In this context, these proteins represent good candidates to control tumorigenesis and cancer growth. Indeed, SIRT1, SIRT2, and SIRT3 have been described to have tumor-suppressive activity by controlling genomic stability and cellular metabolism (Kim et al., 2011; Martinez-Pastor and Mostoslavsky, 2012). Here, we show that SIRT6 functions as a first-hit tumor suppressor, and lack of this chromatin factor leads to tumor initiation and growth. Several lines of evidence support this model. First, SIRT6 deficiency, even in nontransformed cells, causes tumorigenesis (Figure 1C). Importantly, this appears to be specific to SIRT6 because the tumor-suppressive effect of other sirtuins has been observed only in transformed cells (Bell et al., 2011; Fang and Nicholl, 2011; Finley et al., 2011; Kim et al., 2011). Although immortalized SIRT2-deficient cells also exhibit tumorigenic potential (Kim et al., 2011), this phenotype might be related to the accumulation of genomic instability in these cells, leading to the activation of oncogenic signals, a phenotype not observed in *Sirt6* KO cells (as discussed below). Second, SIRT6 deficiency promotes tumor growth in transformed cells (H-RasV12/shp53) (Figure 1F), indicating that SIRT6 is also controlling the proliferation of cancer cells. Third, *Sirt6* gene copy number, as well as mRNA expression, is downregulated in several human cancer databases (Figure 6), arguing for a positive selection within the tumor for cells that exhibit low levels of SIRT6. Strikingly, analysis of colon carcinomas from patients followed during a span of 11 years showed that low levels of SIRT6 correlated with shorter relapse, even in more aggressive tumors. Finally, deletion of SIRT6 in an in vivo model of colon carcinoma increases adenoma number and size and, strikingly, promotes aggressiveness (Figure 7), fully confirming the role of SIRT6 as a tumor suppressor. Interestingly, it has been shown that male mice overexpressing SIRT6 have increased life span compared to control animals (Kanfi et al., 2012). Our results, in combination with those of Kanfi et al. (2012), suggest that SIRT6 overexpression may extend life span at least in part by acting as a tumor suppressor. In contrast, a decline in NAD^+ levels during aging could potentially decrease SIRT6 activity, thus leading to increased susceptibility to tumor formation.

SIRT6 is a chromatin-bound factor that was first described as a suppressor of genomic instability by promoting base excision DNA repair (BER) (Mostoslavsky et al., 2006). Recent studies have demonstrated that SIRT6 is involved in DNA double-strand break (DSB) repair by regulating the activity of C-terminal-binding protein (CtBP) interacting protein (CtIP) (Kaidi et al., 2010) and poly(ADP-ribose) polymerase 1 (PARP1) (Mao et al., 2011), further supporting a role for SIRT6 as a key DNA repair factor. Genomic instability is a known characteristic of cancer cells. Surprisingly, our data indicate that chromosome instability likely does not account for the increased tumorigenic potential in SIRT6-deficient cells because reintroduction of SIRT6 in *Sirt6* KO cells completely abolishes tumor formation (Figure 1D). Furthermore, we have not observed activation of known oncogenic pathways in SIRT6-deficient cells (Figure 3A). Although we cannot rule out activation of other signaling pathways, our data strongly suggest that the genomic instability observed in SIRT6-deficient cells is not the major driving force for tumorigenesis in this setting.

We have recently shown that SIRT6 is a master regulator of glucose homeostasis (Zhong et al., 2010). Here, we further extend these observations and demonstrate that SIRT6 represses tumorigenesis by inhibiting a glycolytic switch (Warburg effect), recently proposed as a "new hallmark" of cancer cells (Hanahan and Weinberg, 2011; Ward and Thompson, 2012). In support of this model, we have found that, similar to what we observe in normal cells (Zhong et al., 2010), SIRT6 deficiency in transformed cells also increases aerobic glycolytic metabolism, and such effect is specifically selected by cancer cells in order to proliferate (Figures 2E and S2). This phenotype is likely HIF-1α dependent, as previously described (Zhong et al., 2010). However, pinpointing its precise contribution to the glycolytic phenotype observed in *Sirt6* KO-transformed cells may be difficult. HIF-1α is involved in multiple processes—besides controlling glycolysis—that may impact in tumorigenesis. Moreover, HIF-1α and HIF-2α have overlapping functions, and how these two factors influence tumorigenesis still remains highly controversial (Keith et al., 2012). Interestingly, SIRT3 has been also described as a tumor suppressor regulating mitochondrial reactive oxygen species (ROS) production and, indirectly, HIF-1α stability and aerobic glycolysis (Finley et al., 2011; Bell et al., 2011; Kim et al., 2010b). However, *Sirt6* expression is not downregulated in human breast cancers with low levels of SIRT3 (Finley et al., 2011) (Figure S6E), suggesting that loss of expression of these two sirtuins might be mutually exclusive in the context of human cancer. Importantly, the role of SIRT3 as a tumor suppressor regulating aerobic glycolysis is only observed in already transformed cells (Finley et al., 2011), whereas activation of this glycolytic switch in nontransformed SIRT6-deficient cells also leads to tumor formation in vivo. Furthermore, we demonstrated that inhibition of glycolysis—by

means of knocking down *Pdk1* and inhibiting PDK1 activity—completely inhibited tumor formation in the context of SIRT6 deficiency (Figures 3G and 7I), confirming that increased aerobic glycolysis is the driving force for tumorigenesis in SIRT6-deficient cells (and further arguing against a mutator phenotype behind this phenotype). Thus, it is reasonable to hypothesize that SIRT6 deficiency promotes both tumor establishment and progression by modulating glucose metabolism. Further supporting this idea, SIRT6 levels are downregulated in pancreatic and colon premalignant lesions (Figures S6C and S6D), and low *Sirt6* expression is selectively maintained in late stages of colon cancer (Figure 6E). Interestingly, SIRT1 has also been involved in colorectal cancer by modulating the activity of β-catenin (Firestein et al., 2008), suggesting that sirtuins might have evolved to regulate different aspects of the tumorigenic process.

In addition to controlling glucose metabolism in cancer cells, our current work unravels SIRT6 as a regulator of ribosomal gene expression. One of the main features of cancer cells is their high proliferative potential. In order to proliferate, cancer cells readjust their metabolism to generate biosynthetic precursors for macromolecular synthesis (DeBerardinis et al., 2008). However, protein synthesis also requires the activation of a transcriptional program leading to ribosome biogenesis and mRNA translation (van Riggelen et al., 2010). As a master regulator of cell proliferation, MYC regulates ribosome biogenesis and protein synthesis by controlling the transcription and assembly of ribosome components as well as translation initiation (Dang, 2012; van Riggelen et al., 2010). Our results show that SIRT6 specifically regulates the expression of ribosomal genes. In keeping with this, SIRT6-deficient tumor cells exhibit high levels of ribosomal protein gene expression. Beyond ribosome biosynthesis, MYC regulates glucose and glutamine metabolism (Dang, 2012). Our results show that glutamine—but not glucose—metabolism is rescued in SIRT6-deficient/MYC knockdown cells, suggesting that SIRT6 and MYC might have redundant roles in regulating glucose metabolism.

Overall, our results indicate that SIRT6 represses tumorigenesis by inhibiting a glycolytic switch required for cancer cell proliferation. Inhibition of glycolysis in SIRT6-deficient cells abrogates tumor formation, providing proof of concept that inhibition of glycolytic metabolism in tumors with low SIRT6 levels could provide putative alternative approaches to modulate cancer growth. Furthermore, we uncover a role for SIRT6 as a regulator of ribosome biosynthesis by corepressing MYC transcriptional activity. Our results indicate that SIRT6 sits at a critical metabolic node, modulating both glycolytic metabolism and ribosome biosynthesis (Figure 7L). SIRT6 deficiency deregulates both pathways, leading to robust metabolic reprogramming that is sufficient to promote tumorigenesis bypassing major oncogenic signaling pathway activation.

EXPERIMENTAL PROCEDURES

All experimental procedures are described in detail in the Extended Experimental Procedures.

Immortalized and Transformed MEFs

Primary MEFs were generated from 13.5-day-old embryos as described (Mostoslavsky et al., 2006). These cells were immortalized by using the standard 3T3 protocol or, alternatively, by knocking down *p53* expression. Primary MEFs were transformed by expressing H-RasV12 and knocking down *p53* expression.

Xenograft Studies

5×10^6 cells in 200 μl of 50% matrigel were injected subcutaneously into the flanks of SCID mice (Taconic Farms, Inc., Hudson, NY) or athymic nude (*Foxn1*^{nu}/*Foxn1*^{nu}) mice (Jackson Laboratories). Mice were checked for the appearance of tumors twice a week, and the tumors were harvested when they reached 10 mm in size.

Genome-wide Overlap of SIRT6 and MYC Binding

Briefly, ChIP-seq data sets (aligned to hg19) for SIRT6 and MYC were obtained from Ram et al. (2011) and Raha et al. (2010), respectively. The 1,000 top bound genes in the SIRT6 and MYC data sets were selected, and the overlapping genes were subjected to hypergeometric test analysis by using the MSigDB gene set collection C5 (GO gene sets, Broad Institute). H3K4me3, SIRT6, and MYC ChIP graphs were done by using the CRome Software (Broad Institute).

Human Data Sets

Sirt6 gene copy number data were obtained from the Tumorscape (Beroukhim et al., 2010) and Cancer Cell Line Encyclopedia (Barretina et al., 2012) (Broad Institute) by using the Integrated Genomics Viewer (IGV). Expression levels of *Sirt6* and glycolytic genes in human cancer were obtained from data sets collected in GEO-NCBI and Oncomine portals.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.10.047.

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Short title: Metabolic Reprogramming at the Origin of Cancer.

Tracking Down the Origin of Cancer: Metabolic Reprogramming as a Driver of Stemness and Tumorigenesis

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ABSTRACT

Metabolic reprogramming has recently emerged as a fundamental trait of cancer cells. Initially thought to be a consequence of rapid cell proliferation, recent data has reset this idea by demonstrating that metabolic reprogramming can actually drive tumorigenesis. The cancer stem cell (CSC) theory predicts that only a small subpopulation of cancer cells with stem cell properties, which derive from the cancer cell of origin, possesses tumor initiating potential. However, whether metabolic reprogramming drives tumor formation by regulating the genesis of CSCs is not known. Importantly, the metabolic properties of stem cells and cancer cells are strikingly similar and metabolic reprogramming is a key factor controlling stemness in these cells. Here I review our current understanding of cancer metabolism and how it mirrors the metabolic requirements of stem cells. I integrate these two concepts and discuss some data demonstrating that metabolic reprogramming regulates CSCs function, suggesting that metabolic regulation of stemness could be at the origin of cancer.

Key Words: Cancer Cell of Origin, Cancer Metabolism, Cancer Stem Cells, Metabolic Reprogramming, Stem Cells.

Abbreviations:
AMPK: AMP-activated protein kinase; **ATP5D**: ATP synthase H+ transporting mitochondrial F1 complex; **ATP5f1**: ATP synthase H+ transporting mitochondrial Fo complex; **EGFR**: epidermal growth factor receptor; **FBP1**: fructose-1,6-bisphosphatase; **FH**: fumarate

dehydrogenase; **GCL**: glutathione cysteine ligase; **GLS1**: glutaminase1; **GLUT1**: glucose transporter 1; **GOT1**: glutamic-oxaloacetic transaminase; **HK2**: hexokinase2; **IDH**: isocitrate dehydrogenase; **LDH-A**: lactate dehydrogenase-A; **LSCs**: leukemia stem cells; **ME1**: malic enzyme1; **MEK**: MAPK/ERK kinase; **MSCs**: mesenchymal stem cells; **NCI**: National Cancer Institute; **NDUFA3**: NADH dehydrogenase (ubiquinone) alpha subcomplex 3; **NF-κB**: nuclar factor-kappaB; **NSCs**: neural stem cells; **OAA**: oxaloacetate; **OCT4**: octamer-binding transcription factor 4; **PDAC**: pancreatic ductal adenocarcinoma; **PDH**: pyruvate dehydrogenase; **PDK1**: pyruvate dehydrogenase kinase1; **PFKFB4**: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; **PI3K**: phosphoinositide-3-kinase; **PKCζ**: protein kinase Cζ; **PFK1**: phosphofructokinase1; **SDH**: succinate dehydrogenase; **2-HG**: 2-hydroxyglutarate; **SLC5A1**:solute carrier family 5A1; **SLC7A1**: solute carrier family 7A1; **TIGAR**: TP53-inducible glycolysis and apoptosis regulator; **UCP2**: uncoupling protein 2; **UDP**: uridine diphospahte; **VHL**: Von Hipple-Lindau

I. INTRODUCTION

In their path to become cancerous, normal cells must overcome several roadblocks before they get to their final destiny, the fully transformed and malignant state. Along this journey, normal cells will increase their proliferation in an uncontrolled manner, avoid cell death and tumor suppression mechanisms, create a new and more permissive microenvironment to support their growth and acquire invasive and metastatic properties.[1] Research done over the last 25 years has shed light on the nature of this journey, where activation of oncogenes and loss of tumor suppressors are key molecular events controlling the acquisition of these capabilities. Furthermore, the recent development of next-generation sequencing techniques has revealed many genes that are mutated in different types of cancer, and has identified some of the genes driving normal cells in their way to become cancer cells.[2] Mutations in these genes will lead to the activation of one or more oncogenic pathways responsible for tumorigenesis. Importantly, recent data has demonstrated that most of these pathways intersect to regulate the metabolic requirements of cancer cells.[3] During the tumorigenic process, cells need to fuel cell growth and division to sustain their increased proliferation. To do so, cancer cells readjust their metabolism to obtain the energy and metabolites required to fulfill both energetic and anabolic demands.

Despite being previously thought to be a mere consequence of a faster proliferation, recent work has shown that this metabolic reprogramming is a fundamental trait of all cancer cells and it is directly regulated by oncogenes and tumor suppressors.[3,4] In line with this, specific metabolic adaptations of tumor cells are required for tumorigenesis and, thus, metabolic reprogramming has been upgraded to be a hallmark of cancer.[1] However, whether this metabolic switch can on its own drive a normal cell along a tumorigenic journey remains as yet poorly understood.

Most human tumors arise in tissues with a very defined cellular organization, where stem cells give rise to several committed progenitors, which in turn will generate terminally differentiated cells carrying on different tissue functions.[5] Moreover, these stem cells have the ability to self-renew, thus maintaining their pool within a tissue, which is crucial for tissue homeostasis. In the same way, and based on a growing amount of data, it has been proposed that tumors originating from these tissues follow a similar hierarchical structure, where only a few cells with self-renewal and differentiation potential give rise to and maintain the whole tumor. This implies that only a small percentage of cells within the tumor (cancer stem cells or CSCs) are able to generate and propagate the tumor as well as to differentiate into multiple cell lineages contributing to the heterogeneity observed in most human cancers. The CSC theory also has profound clinical implications. Due to their unique capability of generating a new tumor and their resistance to most current antiproliferative therapies, CSCs have been proposed to be responsible of metastatic dissemination and tumor relapse. Therefore, it is of special relevance to know the precise mechanisms of the genesis of these cells in order to design more effective therapeutic approaches.

Given the fact that CSCs have stem cell properties (self-renewal and differentiation potential), it is conceivable to think that they derive from normal stem cells which, when transformed, will

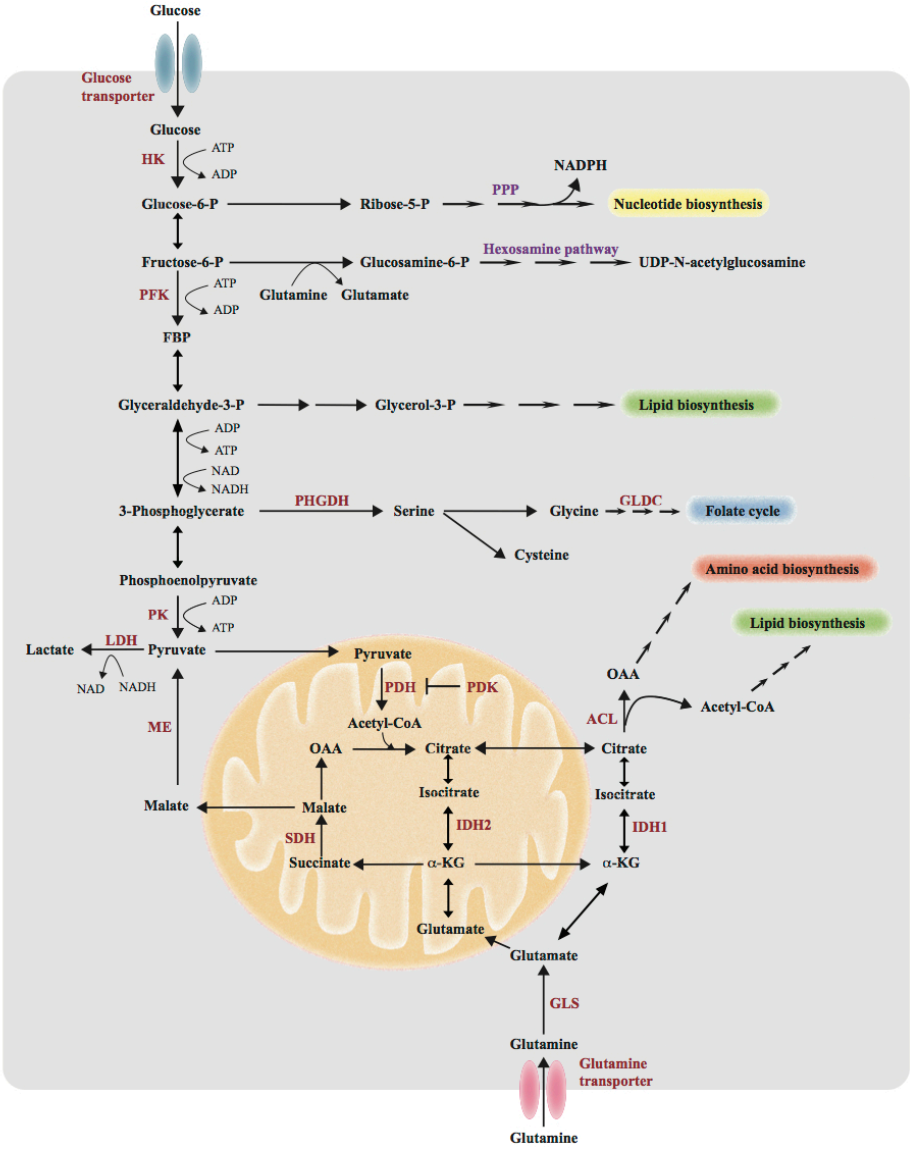
give rise to the tumor. However, another scenario is possible: in the same way that a differentiated cell can be reprogrammed into an induced pluripotent stem cell (iPS cell), it can also be the original target of a tumorigenic event and acquire stem cell properties to become a CSC. These two models predict that CSCs directly derive from the cancer cell of origin, the initial cell that will give rise to a tumor cell with stem cell properties. Intensive research done in the stem cell field has contributed to our understanding of stem cell biology as well as the underlying mechanisms governing self-renewal, differentiation and reprogramming.[6,7] Importantly, stem cell metabolism has recently come to the stage as a critical regulator of stemness and cellular reprogramming.[8,9] Indeed, the metabolic demands of stem cells strikingly mirror the ones observed in cancer cells, suggesting that metabolic reprogramming might be involved in the genesis of CSCs and tumor initiation.

Here, I first provide an overview of our current knowledge in the cancer metabolism field, including new data suggesting that metabolic reprogramming can actually be a driver of tumorigenesis. Next, I outline the most important features of stem cell metabolism and how it parallels the metabolic adaptations exhibited by cancer cells. Then, I put together these two concepts and present recent data indicating that metabolic rewiring is also a hallmark of CSCs. Finally, and based on these data, I discuss the possibility of metabolic reprogramming being at the origin of cancer by controlling the genesis of tumor initiating cells.

II. CANCER CELL METABOLISM

The observation that cancer cells exhibit a different cellular metabolism than normal cells is not new. More than fifty years ago, Otto Warburg described that cancer cells shift their glucose metabolism towards lactate production, instead of fully oxidizing glucose in the tricarboxylic

acid (TCA) cycle, to sustain the rapid proliferation of these cells.[10] However, this important finding was for long forgotten and kept aside of mainstream cancer research, which was mostly focused in this period on the discovery of oncogenes and tumor suppressors. Recently, tumor metabolism has emerged as a key feature of cancer cells, and it has become clear that most of the core metabolic pathways are rewired during tumorigenesis (Figure 1). Importantly, oncogenes and tumor suppressors directly control this metabolic reprogramming, which is absolutely required for tumorigenesis. As described below, these metabolic adaptations allow cancer cells to meet three basic needs to sustain cell growth and proliferation: adenosine triphosphate (ATP) production, biosynthesis of precursors to build up macromolecules and maintenance of cellular redox status.



A. Glucose metabolism and the Warburg effect

Under aerobic conditions, normal cells metabolize glucose to pyruvate through glycolysis, which will enter into the mitochondria to feed the TCA cycle to generate ATP. When oxygen is scarce, mitochondrial respiration is inhibited and pyruvate is converted to lactate in the cytosol. Warburg's work demonstrated that this metabolic shift remained in cancer cells despite the presence of oxygen (Warburg effect or aerobic glycolysis),[10] and represents the best-known example of metabolic reprogramming in cancer cells. Glucose oxidation via mitochondrial respiration yields 36 molecules of ATP, while only 2 molecules of ATP are generated through aerobic glycolysis. This raises two obvious questions: how do cancer cells compensate for the lack of energy? And, why would cancer cells utilize this energetically inefficient pathway? Most cancer cells exhibit an increase in their glycolytic flux, which will allow them to uptake more glucose and catabolize it faster, compensating the lower efficiency of ATP generation by aerobic glycolysis. This increased flux through aerobic glycolysis also leads to the accumulation of glycolytic intermediates that will be used in biosynthetic pathways (Figure 1). In this way, glycolysis contributes to the hexosamine pathway, enhances uridine diphosphate (UDP)-glucose synthesis, provides glycerol and acetyl-CoA for lipid biosynthesis, diverts 3-phosphoglycerate into the serine/glycine biosynthetic pathway and feeds the pentose phosphate pathway (PPP), which will support nucleotide biosynthesis and generate NADPH (nicotinamide adenine dinucleotide phosphate) for reductive biosynthetic reactions and redox balance.[3,4] Thus, by shifting glucose metabolism towards aerobic glycolysis, cancer cells obtain the energy and building blocks essential to sustain their proliferation.

The enhanced aerobic glycolysis displayed by cancer cells is driven by an increase in the expression and activity of many of the enzymes regulating glycolysis. Most cancer cells exhibit

an increase in glucose uptake due to the upregulation of glucose transporters, mainly GLUT1.[11,12] The expression of key glycolytic enzymes, including phosphofructokinase 1 (PFK1), pyruvate kinase M2 (PKM2) and lactate dehydrogenase-A (LDH-A) is also increased, leading to the high glycolytic flux and lactate production observed in cancer cells. Importantly, the flux of pyruvate into the TCA cycle is decreased by an upregulation of pyruvate dehydrogenase kinase-1 (PDK-1) expression and the concomitant decrease in pyruvate dehydrogenase (PDH) activity, allowing pyruvate to be diverted towards lactate production.[4]

As mentioned before, many oncogenic pathways converge to regulate this metabolic switch[3]. Activation of the PI3K pathway, and especially its downstream effector AKT1,[13] stabilization of HIF1 (hypoxia inducible factor 1), mainly through mutations in its negative regulator VHL,[14] increased expression of MYC,[15] suppression of the metabolic sensor AMPK,[16,17] and loss or mutation of the tumor suppressor p53,[18] are among the most important events that promote aerobic glycolysis in cancer cells by directly regulating the expression and activity of several glycolytic enzymes.

B. Glutamine Metabolism

Glutamine is utilized by the cell as a bioenergetic substrate and nitrogen donor, and it is essential to support cell proliferation.[19] Importantly, it has recently been shown that transformation induces glutaminolysis and that many cancer cells rely on this amino acid for survival.[20, 21] Once in the cell, glutamine is converted to glutamate by glutaminase (GLS1), which will be converted to a-ketoglutarate (a-KG), a key TCA cycle intermediate. In this way, glutamine feeds the TCA cycle acting as a carbon source to support the biosynthesis of other amino acids and fatty acids (Figure 1). In tumor cells with defective mitochondria or under hypoxia, in which the TCA cycle is inhibited, glutamine can still contribute to lipid biosynthesis by the reductive carboxylation of

a-KG into citrate by a reverse enzymatic reaction of isocitrate dehydrogenase (IDH).[22, 23] Lastly, glutamine, together with glucose, is required for hexosamine biosynthesis.[24]

Similar to glucose, glutamine is utilized to control the redox status of the cell besides supporting anaplerotic reactions. The enzyme glutathione cysteine ligase (GCL) converts the glutamate generated by glutaminase into reduced glutathione (GSH), which is an essential antioxidant controlling the redox state of all cellular compartments.[25] Furthermore, glutamine-derived carbons can exit the TCA cycle as malate, which will be used by malic enzyme 1 (ME1) to generate NADPH (Figure 1).[26]

Little is known about the precise mechanisms governing the fate of glutamine in cancer cells. The oncogene MYC has recently emerged as a crucial regulator of glutaminolysis by controlling both the uptake and catabolism of glutamine. MYC directly regulates the expression of the glutamine transporters SLC5A1 and SLC7A1 and indirectly controls the levels of GLS1 by repressing the expression of *microRNA-23A* and *microRNA-23B*, which inhibit GLS1.[20,21] Furthermore, KRAS has been involved in glutamine metabolism reprogramming in pancreatic ductal adenocarcinomas (PDACs).[27,28] In this type of cancer, glutamine is converted to aspartate instead of a-KG, which will be used to generate oxaloacetate to feed the TCA cycle. Moreover, OAA will be converted into malate and then pyruvate, thus increasing the NAPDPH/$NADP^+$ ratio and contributing to the maintenance of the cellular redox status. KRAS drives this switch by directly regulating the expression of aspartate transaminase (GOT1), the enzyme catalyzing the conversion of aspartate into OAA.[27]

C. Serine and glycine metabolism

The serine-glycine biosynthetic pathway represents an essential metabolic adaptation in cancer cells.[29] A glycolytic intermediate, 3-phosphoglycerate, fuels this pathway in a reaction catalyzed

by the enzyme phosphoglycerate dehydrogenase (PHGDH),[30-32] thus shunting part of the carbon derived from glucose to serine biosynthesis and the folate cycle (Figure 1). Activation of this pathway supports protein, nucleotide and GSH biosynthesis, and contributes to the cellular anaplerotic flux of glutamine into the TCA cycle by accepting the amino group from glutamate required for the generation of a-KG.[29,31]

Despite being known for decades that cancer cells exhibit an increased *de novo* serine-glycine biosynthesis,[33,34] the molecular mechanisms underlying this metabolic adaptation have just started to be elucidated. Increased expression of PHGDH has been described in triple-negative breast cancer, and a focal amplification of the genomic locus encoding this enzyme has been described in breast cancer and melanoma.[30-32] The glycolytic enzyme PKM2 also supports the serine biosynthetic pathway,[35, 36] and phosphorylation and inhibition of PHGDH by PKCz has been reported to suppress the flux through this pathway limiting tumor growth.[37] Increased expression of key enzymes regulating the serine-glycine biosynthetic pathway (including PHGDH) has also been observed in tumors overexpressing the histone 3 methyltransferase G9a, which drives H3K9 monomethylation (a mark for active transcription) on the promoter region of serine metabolism genes.[38] Finally, the transcription factor p73 has recently been described as a regulator of this biosynthetic pathway. However, in this case, p73 does not directly regulate the expression of any of the enzymes of this pathway, but instead it controls glutaminase 2 (GLS2) expression, thus favoring the conversion of glutamine into glutamate feeding serine biosynthesis.[39]

Glycine derived from the serine-glycine biosynthetic pathway can be further catabolized in the glycine cleavage pathway to fuel the folate cycle (Figure 1). Recent work has demonstrated that the glycine cleavage system is essential for tumorigenesis and tumor growth. Glycine

metabolism correlates with cell proliferation in the NCI-60 cancer cell lines, and it is associated with poor prognosis in breast cancer patients.[40] Moreover, glycine decarboxylase (GLDC), a key enzyme of glycine catabolism, has been found to be overexpressed in non-small cell lung cancer tumor initiating cells, thus driving tumorigenesis of this particular cancer type.[41]

D. Metabolic reprogramming in the driver's seat

It was for long assumed that all the metabolic changes exhibited by cancer cells were merely an adaptation to their increased proliferation and, as such, they were not regarded as essential features required for tumorigenesis. This idea started to be challenged more than a decade ago with the discovery of the first oncogenic mutations affecting metabolic enzymes. Early in 2000, Baysal et al. described mutations on the TCA cycle enzyme succinate dehydrogenase (SDH) in hereditary parangliomas.[42] Two years later, mutations in another mitochondrial enzyme, fumarate hydratase (FH), were reported to be associated with leiomyomatosis and kidney cancers.[43] Mutations in these enzymes lead to the accumulation of succinate and fumarate, respectively, both of which increase the stability of HIF proteins by inhibiting dioxygenases and prolyl hydrolases.[44] It was proposed that this increase in HIF protein levels could be driving the transformation of the cells carrying these mutations. However, these TCA cycle intermediates can also inhibit dioxygenases involved in histone and DNA demethylation, likely altering the epigenetic landscape of these cells, which could enforce the activation of an oncogenic transcriptional program leading to tumorigenesis. In line with this, it has recently been found that some mutations in another metabolic enzyme, isocitrate dehydrogenase (IDH), alter its enzymatic activity resulting in the generation of the oncometabolite 2-hydroxyglutarate (2-HG),[45,46] which can also inhibit dioxygenases including histone and DNA demethylases leading to changes in gene expression and tumorigenesis in gliomas, acute myeloid leukaemia,

sarcomas,[47-49] and a growing list of tumors. Whether these mutations act mainly by reshaping the epigenetic landscape rather than affecting metabolic pathways is currently under intense investigation.

As described before, activation of oncogenic pathways directly impinges on core cellular metabolism, in addition to their effects on cell proliferation. However, given the connection between these two processes, it has been difficult to discern cause from effect. Recently, our laboratory has demonstrated that the histone deacetylase SIRT6 acts as a tumor suppressor by controlling cancer metabolism.[50] SIRT6 co-represses HIF1a activity and directly regulates the expression of key glycolytic genes by acting as a H3K9 deacetylase.[50,51] Similarly, SIRT6 inhibits ribosome biogenesis by deacetylating H3K56 at the promoters of MYC-dependent ribosomal genes.[50] Importantly, loss of this tumor suppressor promotes a robust metabolic reprogramming that is sufficient to drive tumorigenesis bypassing major oncogenic signaling pathway activation, putting cancer metabolism in the "driver's seat".[52] In line with this, upregulation of GLDC leads to cellular transformation by increasing glycine, glucose and pyrimidine metabolism, and this enzyme is overexpressed in non-small cell lung cancer tumor initiating cells, arguing for a driver role of glycine metabolism in tumorigenesis.[41] Finally, recent data indicates that overexpression of the glucose transporter GLUT3 in nonmalignant breast cells enables the activation of EGFR, MEK, AKT and β1-integring signaling pathways and promote transformation, raising the possibility of increased glucose metabolism being upstream of the activation of oncogenic pathways.[53]

The work discussed above has provided sufficient data to confirm that metabolic reprogramming can drive tumorigenesis in otherwise non-transformed cells. However, the cells used in these experiments are immortalized cells, indicating that another hit is required for transformation.

Cellular immortalization is achieved by bypassing cellular senescence, a strong tumor suppressive mechanism.[54] Importantly, activation of glycolytic enzymes and glucose metabolism favors escape from oncogene-induced senescence,[55-57] suggesting that, indeed, metabolic reprogramming *per se* could, in principle, be sufficient for a normal cell to achieve fully transformation.

III. STEM CELL METABOLISM: FOLLOWING THE CANCER PATH

Stem cells have unique energetic and biosynthetic demands, and the ability to regulate their metabolism is essential to control their fate. Recent data has demonstrated that metabolic reprogramming plays a pivotal role in stem cell self-renewal, differentiation and quiescence.[9,58] Notably, stem cell and cancer cell metabolism are strikingly similar, primarily oriented to sustain proliferation, build biomass and control their redox state.[59] Moreover, in the same way metabolic reprogramming can drive tumorigenesis, metabolic adaptation in stem cells has emerged as a driver of stemness and pluripotency.[9, 58]

A. Metabolism in pluripotent stem cells

One of the first observations made regarding stem cell metabolism was the increased aerobic glycolysis exhibited by mouse and human embryonic stem cells (ESCs) compared to differentiated cells.[60-62] Importantly, stimulation of glycolysis in pluripotent stem cells (PSCs) promotes stemness,[63-65] while inhibition of this metabolic pathway halts proliferation and induces cell death.[60] As in cancer cells, this high glycolytic flux provides precursors for anaplerotic reactions. Carbon tracing studies have shown that mouse and human ESCs have a very active PPP, which utilizes carbon from glucose to build up nucleotides and lipids.[61,62] As mentioned before for cancer cells, NADPH generated by this active PPP is a critical antioxidant mechanism

required for ESC survival upon oxidative stress and control of cell fate.[66,67] Notably, consistent with this increased glycolytic flux, mitochondrial respiration and energy production is less coupled in human PSCs compared to differentiated cells.[62,68] This reduced coupling is associated with a decrease in ROS (reactive oxygen species) production, which reduces DNA damage and suppresses PSCs differentiation.[62,69,70] In line with this, reprogramming of differentiated cells into induced pluripotent stem cells (iPSCs) is accompanied with a switch from mitochondrial respiration to glycolysis, and the reprogramming efficiency is higher in those somatic cells with closer metabolic features to ESCs (high glycolysis, low mitochondrial respiration).[8,71,72] This data suggests that metabolic reprogramming, similar to its requirement for tumorigenesis, represents a roadblock that somatic cells must overcome to acquire pluripotency. Consistent with this idea, promotion of glycolysis improves iPSC reprogramming, whereas its inhibition impairs the conversion of somatic cells to iPSCs.[71,73 74]

At the molecular level, this metabolic reprogramming is associated with an increase in the expression of several glycolysis-regulating enzymes in PSCs, such as hexokinase and LDH-A.[72] Moreover, PDK1 and UCP2 expression is upregulated in PSCs limiting pyruvate entry into the mitochondria, thus reducing oxidative phosphorylation.[61,62,68] However, the precise mechanism driving the expression of these genes in PSCs is poorly understood. Notably, among the targets of the stemness factor OCT4 are several metabolic genes that could control the balance between glycolysis (pyruvate carboxylase, hexokinase-1) and oxidative metabolism (NDUFA3, ATP5D and ATP5f1).[75-77] Furthermore, the reprogramming factors MYC and Lin28 stimulate glycolysis,[78] suggesting that metabolic reprogramming could be a part of the transcriptional network regulated by these factors to promote pluripotency. Finally, HIF proteins have also been associated with the regulation of stemness. HIF-2a regulates the expression of OCT4, and

stabilization of HIF proteins by hypoxia or chemical activators increases reprogramming efficiency.[73,74,79]

Amino acid catabolism is also rewired in PSCs, and plays a key role in maintaining their pluripotent state. mESC growth is absolutely dependent on threonine and, consistent with this, threonine dehydrogenase (TDH), the enzyme controlling the rate-limiting reaction in the metabolism of this amino acid, is highly expressed in mESCs.[80] TDH expression is also upregulated in early blastocysts and after iPSCs reprogramming, and disappears upon differentiation, highlighting the dependence of pluripotency on threonine metabolism.[9,80] However, human cells do not encode a functional TDH and, therefore, it is currently unclear how significant this pathway is in humans. Threonine breakdown generates glycine, which is decarboxylated by GLDC to produce 1-carbon equivalents to fuel the folate cycle. Importantly, as observed in some cancer cells, GLDC expression is also upregulated in PSCs compared to differentiated cells.[9]

B. Metabolism in adult stem cells

Adult tissues contain a small number of cells with self-renewal and differentiating potential, which are essential to maintain tissue homeostasis. Unlike proliferative PSCs, most of these adult stem cells are quiescent, including long-term hematopoietic stem cells (LT-HSCs), mesenchymal stem cells (MSCs), neural stem cells (NSCs), epidermal stem cells and muscle satellite stem cells. Due to their quiescent state, these adult stem cells have a very characteristic metabolic program, which has been extensively studied in LT-HSC. Metabolism in these cells is skewed towards glycolysis, a phenotype that was first associated to the hypoxic environment of the bone marrow niche where they reside.[81,82] However, this metabolic reprogramming also limits the production of mitochondrial ROS, which can induce either differentiation or apoptosis of these

cells.[83] This suggests that increased glycolysis in LT-HSC may be a requirement of their differentiation state rather than an adaptation to the environment. In line with this, it has been shown that the HSC transcription factor MEIS1 regulates the expression of HIF1a, which drives this metabolic reprograming.[81] In addition, several PDK isoforms, which limit the flux of pyruvate into the mitochondria, have been reported to control quiescence of LT-HSCs.[81,84,85] Interestingly, myeloid progenitors derived from HSC also rely on glycolysis, although it has been proposed that they use it to feed anabolic pathways, in agreement with their high rate of proliferation.[9]

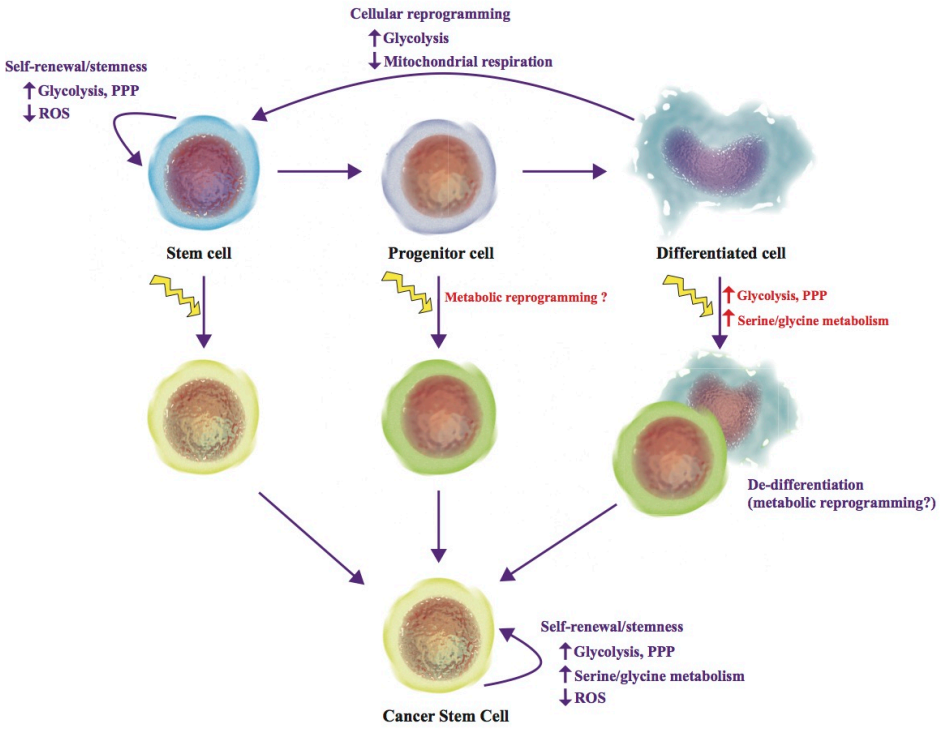
Similar to LT-HSCs, MSCs and NSCs also need to keep ROS levels low in their hypoxic niches to maintain the quiescent state.[86,87] Again, this is accompanied by increased glycolysis and reduced oxidative phosphorylation,[86,87] suggesting that glucose metabolism reprogramming plays a relevant role in preserving the quiescent state and the long-term self-renewal activity of adult stem cells.

Less is known about the metabolic requirements of highly proliferative adult stem cells. The intestinal epithelium is rapidly regenerated by the activity of the highly proliferative intestinal stem cells (ISCs). Although the metabolic requirements of these cells have not been directly addressed, there is some data suggesting that ISCs might also rely on glycolysis. By measuring NADH levels in intestinal crypts, Stringari et al. found a metabolic gradient along the crypt axis, being glycolysis more prominent at the base of the crypts, where ISCs reside.[88] Furthermore, intestinal regeneration after injury is regulated by the fructose-2,6-bisphosphatase TIGAR, which diverts glucose metabolism into the PPP.[89] Importantly, this increased PPP provides NADPH for antioxidant function and ribose-5-phosphate for nucleotide synthesis, both essential for the rapid proliferation of the intestinal epithelium.[89] Since intestinal regeneration is driven by ISCs, these

results suggest that low levels of ROS and activation of anaplerotic pathways might be required for proper ISCs function. Although these two studies suggest that diverting glucose metabolism may be important in ISCs, whether such metabolic adaptation is driving these cells rather than representing a secondary adaptation remains to be tested.

IV. METABOLIC REPROGRAMMING IN CANCER STEM CELLS AS A DRIVER OF TUMORIGENESIS

Data generated during the last decades has demonstrated that tumors stem from accumulation of mutations in a single normal cell, the cancer cell of origin, which will give rise to the whole tumor. Among tumor cells, only a small fraction retain tumor initiating capability, being responsible of metastatic dissemination and tumor relapse. Since these CSCs have stem cell properties, it has been proposed that they could share a common stemness program with stem cells. As described above, stem cell metabolism and cancer cell metabolism are remarkably similar, and recent data indicates that metabolic reprogramming regulates CSCs function and



tumorigenesis. Together, these data suggest that metabolic reprogramming might be a key factor controlling the acquisition of stem cell properties and the genesis of CSCs (Figure 2).

A. The cancer cell of origin and the CSC theory

About forty years ago, Hamburger and Salmon discovered that only a small subset of multiple myeloma cells were capable of clonogenic growth, which they named CSCs.[90,91] Twenty years later, the laboratory of John E. Dick experimentally demonstrated the existence of CSCs in a human cancer.[92,93] They found that only a small subpopulation of $CD34^{+}CD38^{-}$ leukemia cells had the ability to self-renew and differentiate, suggesting that these cells could be leukemia-initiating cells. Based on these studies, later work demonstrated that other human cancers also follow the CSC model, including colon cancer, pancreatic cancer, breast cancer, brain tumors and ovarian cancer.[94] The CSC theory predicts that tumors, like normal tissues, are hierarchically organized, where a small population of stem-like cells (CSCs), with self-renewal and differentiation potential, is responsible for the propagation of the tumor. This is in agreement with the observation that an ES cell-like signature is associated with less differentiated and aggressive tumors,[95] consistent with CSCs being responsible of most metastases.

But where do these CSCs come from? Since these cells are the only ones with the ability to generate a tumor, one possibility is that these CSCs derive from the original healthy cell hit by the transformation event(s), which will eventually give rise to the tumor (Figure 2). Moreover, this cancer cell of origin must have stem cell properties to give rise to the whole tumor, including CSCs. Thus, it has been hypothesized that normal stem cells can often represent the cancer cell of origin, in which activation of oncogenes or loss of tumor suppressors initiate the transformation program required for tumorigenesis.[96] This is the case, for instance, of intestinal tumors in mice, where activation of the Wnt pathway in the crypt intestinal stem cells leads to

adenoma formation, while activation of this pathway in differentiated intestinal epithelial cells only induces slow growing benign tumors at low frequency.[97,98] Importantly, an intestinal stem cell signature defines cancer stem cells in human colorectal cancer, suggesting that intestinal stem cells could also be the cell of origin in this type of cancer.[99]

However, the picture is a little more complex, since in most adult tissues stem cells differentiate into several committed progenitors, which give rise to the different terminally differentiated cells that form the tissue. Lineage tracing experiments have confirmed that transformation of distinct progenitors within a tissue can account for the different cancer subtypes arising from that particular tissue. This is the case for skin tumors, medulloblastomas, breast cancer, leukemias and lung cancer.[96,100]

The fact that the cancer cell of origin intrinsically has stem cell properties does not absolutely imply that it derives from a normal stem cell. Somatic cells can be reprogrammed into iPS cells by activation of a genetic and epigenetic program induced by four transcription factors,[101] proving that a terminally differentiated cell can acquire stem cell properties. Similarly, activation of an oncogenic program in a differentiated cell could confer stem cell properties to this cell, thus becoming a CSC (Figure 2). Indeed, overexpression of c-MYC in adult epithelial cells activates an ESC-like transcriptional program promoting the generation of CSCs.[102] In line with this, activation of the NF-kB pathway in intestinal epithelial cells induces their dedifferentiation and the acquisition of intestinal stem cell properties and, therefore, tumor initiating potential.[103] This implies that some of the mechanisms controlling stemness in normal stem cells could also be important for the generation of CSCs. Importantly, it has recently been shown that the same transcription factors used for reprogramming to pluripotency drive tumor initiation *in vivo*.[104,105]

B. Metabolic properties of CSCs

As discussed before, metabolic reprogramming appears to be an essential feature of cancer cells and, in some cases, can drive tumorigenesis. This predicts that tumor-initiating cells would be the original cells acquiring the metabolic requirements for tumor initiation. If this is true, then metabolic reprogramming in CSCs should have a prominent role in regulating tumor formation (Figure 2). However, little is known about the metabolic properties of CSCs. Previous work had demonstrated that metformin, a widely used antidiabetic drug that targets cellular metabolism by inhibiting the electron transport chain (complex I), selectively killed breast cancer CSCs.[106] Although the precise mechanism underlying this phenotype was unknown, this result suggested that CSCs could have unique metabolic properties. Following this idea, later experiments in glioblastoma have shed light on the metabolic nature of CSCs. Glioblastoma is the most common and malignant primary brain tumor in adults. A small sub-population of glioblastoma stem cells (GSCs) has been found to be responsible for tumor initiation, therapy resistance and tumor recurrence.[107] Despite some controversy about the energy source of GSCs, accumulating data strongly indicates that increased glycolysis regulates tumor initiation of GSCs.[108-111] Inhibition of the glycolytic pathway induces apoptosis of GSCs,[112,113] and the expression of some glycolytic enzymes is upregulated in GSCs compared to their parental cells.[111] Furthermore, an unbiased RNAi screen comprising the complete human kinome and phosphatome has identified the glycolytic enzymes PDK1, PKM2 and, most prominently, PFKFB4, as key factors promoting the survival of brain CSCs.[114] In line with this, inhibition of HK2 by miR-143 in rat glioblastoma inhibits glycolysis and decreases stemness while promoting differentiation of GSCs.[115]

Besides glioblastoma, metabolic reprogramming in CSCs has recently emerged as a driver of other cancer types. As mentioned before, glycine metabolism has been found to be a hallmark of non-small cell lung cancer tumor initiating cells.[41] Genome-wide transcriptome analysis of

$CD166^+$ tumor cells, a cell population enriched for tumor initiating cells, showed that one of the most represented pathways is the glycine, serine and threonine metabolism pathway. Moreover, the expression of several enzymes regulating glycine/serine metabolism, and especially GLDC, are upregulated in lung tumor initiating cells. Importantly, enforced expression of GLDC promotes tumorigenesis in non-transformed cells by increasing glycine, glucose and pyrimidine metabolism.[41] Human leukemia stem cells (LSCs) also exhibit a unique metabolic phenotype. Similar to LT-HSC, functionally defined LSCs are characterized by a "ROS-low" state, probably needed to maintain their quiescent state. However, unlike HSCs, these cells do not use glycolysis for energy production but rather rely on oxidative phosphorylation.[116] The reason for such differences, however, remains unclear.

The epithelial-mesenchymal transition (EMT) is a well known regulator of the CSC phenotype.[117] Overexpression of EMT transcription factors drives the acquisition of CSC properties in otherwise non-stem cancer cells, and it is associated with more aggressive tumors and metastasis. The EMT transcription factor SNAIL has recently been found to be a repressor of the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBP1) in basal-like breast cancer. Repression of FBP1 induces glycolysis and suppresses oxidative metabolism and ROS production in basal-like breast cancer cell lines and, importantly, this metabolic reprogramming increases CSCs and tumorigenesis in this type of cancer by enhancing the interaction of β-catenin with T-cell factor.[118] Thus, it appears that a critical regulator of the genesis of CSCs also controls a metabolic switch that favors the acquisition of CSCs properties and tumor formation. In line with this, knockdown of the metabolic enzyme ATP-citrate lyase (ACL), which connects glucose metabolism to lipid synthesis and is required for tumor growth,[119,120] suppresses cancer stemness *in vitro* by affecting *SNAIL* expression and function.[121] Together, these findings suggest

that EMT and metabolic reprogramming might be interconnected processes regulating the acquisition of CSCs properties in some types of cancer.

C. Metabolic reprogramming: driving tumorigenesis from the origin

In 1966, at the meeting of Nobel-Laureates at Lindau, Germany, Otto Warburg proclaimed "the prime cause of cancer is the replacement of the respiration of oxygen in normal body cells by fermentation of sugar". Although a deeper metabolic analysis of cancer cells has ruled out his initial hypothesis of mitochondria being defective in cancer cells, based on the findings discussed in this review, the main idea conveyed by Warburg's work still stands and metabolic reprogramming might be placed at the origin of cancer (Figure 2). An increasing amount of data has demonstrated that the rewiring of metabolism in cancer is required for tumorigenesis and it is not just an adaptation to the faster proliferation exhibited by cancer cells. Importantly, our work, together with recent data generated in other laboratories, strongly indicates that metabolic reprogramming drives tumor initiation.[41,50,53] This implies that the change in metabolism must be present in the cancer cell of origin, either pushing the cell fate towards transformation or creating an appropriate "metabolic state" required for tumorigenesis. Although there is no direct proof of any of these possibilities, several pieces of data suggest that metabolic reprogramming might regulate the genesis of the cancer cell of origin. According to the CSC theory, the cancer cell of origin has, by definition, stem cell properties. As discussed above, the cancer cell of origin might be either a normal stem cell or a differentiated cell that acquires stem cell properties by the oncogenic insult. Regardless, metabolic reprogramming could be involved in the acquisition of this cellular stemness. PSCs and adult stem cells display a strikingly similar metabolic phenotype to cancer cells (see section 2 and 3), suggesting that they could be already primed for tumorigenesis. Moreover, these metabolic adaptations are required to maintain stemness, and a

cancer-like metabolism is required to reprogram a differentiated cell into a stem cell-like state. This dedifferentiation process has been described in some types of cancer (see section 3 and 4A), and therefore, it is tempting to speculate that this metabolic switch can lead to the generation of tumor initiating cells. Finally, CSCs, the direct progeny of this cancer cell of origin, also exhibit many of the metabolic features of stem and cancer cells, and metabolic reprogramming plays a key role in the genesis of these CSCs (see section 4B). Together, these data suggest that metabolic reprogramming might drive tumorigenesis by regulating its inception, that is, the generation of the cancer cell of origin.

V. CONCLUDING REMARKS

Largely ignored from cancer research for many years, there has recently been a renewed appreciation of tumor metabolism as an important aspect of cancer cells. Accordingly, work done over the past few years has revealed many of the metabolic adaptations of cancer cells, as well as some of the molecular determinants controlling this metabolic reprogramming. However, there are still many open questions that remain to be addressed and, very likely, the very next years will bring new findings that will shake the cancer biology field in the same way the oncogene revolution did. Importantly, recent data has demonstrated a driving role for metabolic reprogramming in tumorigenesis in certain types of cancer, but much work has to be done to confirm whether metabolic reprogramming represents a common feature of most human cancers and to fully understand the precise mechanisms involved. In this regard, the idea of metabolic reprogramming driving cancer initiation by controlling the genesis of tumor initiating cells is an attractive possibility with relevant implications in the clinic. Drugs targeting cancer metabolism will be useful not only to avoid tumor growth but also to eradicate tumor-initiating cells, which

will improve the efficacy of current therapies. Therefore, connecting the dots between stem cell metabolism, cancer metabolism and CSCs could finally provide mechanistic evidence on how tumors initiate and propagate, bringing Warburg's original "prime cause of cancer" full circle.

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FIGURE LEGENDS

Figure 1. Main metabolic pathways rewired in cancer cells. Cancer cells exhibit an increase in glucose uptake and metabolism. Glucose-derived carbon is shunted to biosynthetic reactions to build up amino acids, lipis, and nucleotides, which will support cell growth and proliferation. Similarly, glutamine is used to feed the TCA cycle and provides carbon and nitrogen equivalents to sustain anaplerotic reactions.

Figure 2. Metabolic reprogramming at the origin of cancer. CSCs derive from the cancer cell of origin, which can be a stem cell, a progenitor cell or a differentiated cell. Metabolic reprogramming has been shown to regulate stemness in stem cells and to be an important regulator of cellular reprogramming. Moreover, rewiring of some metabolic pathways controls the genesis of CSCs.