

# Cancer-Specific Functions of SIRT1 Enable Human Epithelial Cancer Cell Growth and Survival

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## Abstract

**SIRT1 is a conserved NAD-dependent deacetylase that regulates life span in accord with nutritional provision. In mammalian cells, SIRT1 also down-regulates stress-induced p53 and FoxO pathways for apoptosis, thus favoring survival under stress. The functioning of SIRT1 under normal, non-stressed conditions of cell growth is unknown. Here we have asked if SIRT1 has the capacity to influence cell viability in the absence of applied stress. For this purpose we used synthetic small interfering RNA to silence *SIRT1* gene expression by RNA interference (RNAi). We show that the process of RNAi, by itself, does not affect cell growth and is not sufficient to activate a cellular stress response (indicated by lack of activation of endogenous p53). We also show that, in the absence of applied stress, SIRT1 silencing induces growth arrest and/or apoptosis in human epithelial cancer cells. In contrast, normal human epithelial cells and normal human diploid fibroblasts seem to be refractory to SIRT1 silencing. Combined gene knockout with RNAi cosilencing experiments indicate that SIRT1 and Bcl-2 may suppress separable apoptotic pathways in the same cell lineage and that the SIRT1-regulated pathway is independent of p53, Bax, and caspase-2. Alternatively, SIRT1 may suppress apoptosis downstream from these apoptotic factors. In either case, we show that FoxO4 (but not FoxO3) is required as proapoptotic mediator. We further identify caspase-3 and caspase-7 as downstream executioners of SIRT1/FoxO4-regulated apoptosis. Our work identifies SIRT1 as a novel target for selective killing of cancer versus noncancer epithelial cells.** (Cancer Res 2005; 65(22): 10457-63)

## Introduction

SIRT1 is the human orthologue of SIR2, a conserved NAD-dependent protein deacetylase that regulates life span in accord with nutritional provision and under conditions of stress. SIR2 has been well characterized in yeast and in *Caenorhabditis elegans* (1–3). Histone deacetylation by SIR2 in yeast extends life span and results in selective silencing of mating-type genes and ribosomal DNA (4, 5). A shift from anaerobic to aerobic respiration up-regulates SIR2 deacetylase activity by increasing the NAD/NADH ratio (2, 6). In mammalian cells, SIR2 is represented by seven homologues (SIRT1–7), of which SIRT1 is closest to the yeast gene. Mammalian SIRT1 is emerging as a key

regulator of cell survival in the face of cellular stresses which otherwise trigger apoptotic pathways via activation of p53 and/or FoxO transcription factors.

The p53 protein, a key tumor suppressor, is activated and acetylated in response to various types of stress. SIRT1 can deacetylate p53 protein and it is proposed that deacetylation by SIRT1 reduces p53-dependent apoptosis in response to stress (7, 8). A second stress response system in mammalian cells is controlled by the FoxO family of forkhead (or winged helix) transcription factors (9). Mammalian cells express four conserved *FoxO* genes, namely *FoxO1*, *FoxO3*, *FoxO4*, and *FoxO6*. SIRT1 interacts with and deacetylates FoxO3 and FoxO4, and this interaction is enhanced in response to stress and may thus favor cell cycle arrest over apoptosis (10, 11).

Remarkable similarities of function exist between p53 and FoxO proteins (9, 12). For example, they share the ability to promote apoptosis, induce cell cycle arrest, and support the repair of damaged DNA. The commonality between p53 and FoxO extends to include nuclear/cytoplasmic shuttling, interaction with 14.3.3 proteins, proteosomal degradation following ubiquitinylation, and extensive posttranslational modification including multiple site-specific phosphorylations. Both proteins also interact with the coactivator cAMP-responsive element binding protein binding protein (CBP)/p300 and are acetylated, and this seems important for their activation in response to stress (13, 14). The discovery that SIRT1 is able to complex with, deacetylate, and, hence, down-regulate both p53 and FoxO is exciting because it identifies SIRT1 as an integrator of cellular pathways activated in response to diverse types of stress (e.g., nutritional starvation/FoxO, reactive oxygen species/FoxO/p53, and UV-induced DNA damage/p53).

The functioning of SIRT1 in mammalian cells has largely been studied in cell culture systems under conditions of applied stress and in the presence of SIRT1 antagonists, such as nicotinamide, and SIRT1 agonists, such as resveratrol. However, although very useful, it should be remembered that these biochemical agents are not selective for SIRT1 and are expected to interfere with other NAD-dependent systems in cells, including other SIRT family members. Many published studies also use enforced expression of exogenous genes in the cultured cells, including dominant-negative mutants of SIRT1. Although enforced high-level expression of exogenous genes is likely to be registered as abnormal by the mammalian cell (a particularly important consideration when studying homeostatic and stress response systems), these experimental approaches have produced valuable insights into the functioning of mammalian SIRT1 under conditions of experimentally applied cellular stress. Cellular stress is inflicted by a variety of methods, including serum withdrawal from the cell culture medium, nutritional depletion or starvation, and/or drug administration and/or exposure to oxidative and genotoxic stress (1–3, 6–8, 10, 11, 15–19).

The functional capacity of SIRT1 under normal, nonstressed cell growth conditions is unknown. Here, we have asked if SIRT1 has

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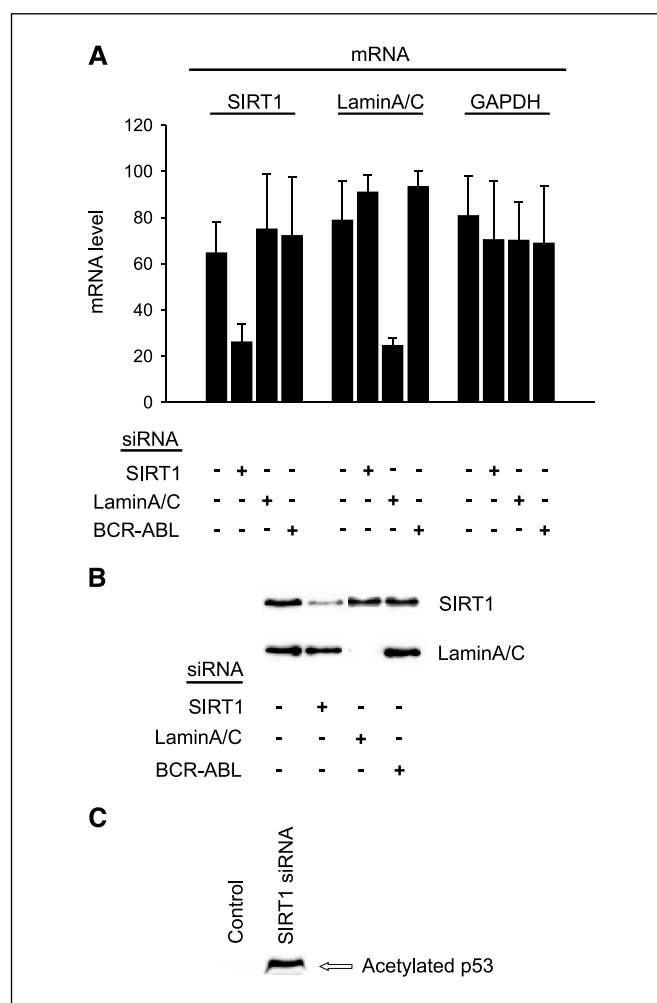
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the capacity to exert a constitutive influence on mammalian cell survival (i.e., Does SIRT1 influence cell survival in the absence of applied stress?). For this purpose, we used synthetic small interfering RNA (siRNA) to silence gene expression by RNA interference (RNAi; ref. 20).

## Materials and Methods

**Cell lines and transfections.** Epithelial cancer cells lines used in this work were derived from human colorectal carcinomas: HCT116 isogenic clones (p53+/+ and p53-/-; Bax+/- and Bax-/-; and p21+/+ and p21-/-); LoVo (p53 wild type); DLD1, SW48, and HT29 (all p53 mutant); from human breast carcinomas: MCF-7 and HTB-126; or from human cervical cancer (SiHa). Noncancer human cell lines were epithelial ARPE-19 and HTB-125 cells and fibroblastic normal primary diploid fibroblasts (NDF). ARPE-19, HTB-126, and HTB-125 cells were obtained from American Type Culture Collection (Manassas, VA) and were cultured as recommended by the suppliers. Cells were subcultured and transfected as described (21, 22) using synthetic, high-performance liquid chromatography-purified siRNAs (MWG, Ebersberg, Germany) formulated into liposomes (OligofectAMINE, Life Technologies, Carlsbad, CA). Controls included liposomes formulated in the absence of siRNA. siRNA concentration was 0.58  $\mu$ g per  $1.5 \times 10^5$  cells per well. At this concentration, synthetic siRNA does not induce an IFN response in mammalian cells (23). The final volume of culture medium was 1.5 mL per well. Cells were monitored by microscopy at 24, 48, and 72 hours and harvested for biochemical analyses at 48 and 72 hours posttransfection.

**Small interfering RNA sequences and quantitation of messenger RNAs.** Two independent siRNA sequences were used to silence SIRT1 expression. Sequence 1: sense 5'-ACUUUGCUGUAACCCUGUA(dTdT)-3', antisense 5'-UACAGGGUACAGCAAAGU(dTdT)-3'. Sequence 2: sense 5'-AGAGUUGCCACCCACACCU(dTdT)-3', antisense 5'-AGGUGUGGGUGGCAACUCU(dTdT)-3'. Two independent siRNA sequences were also used to silence FoxO4. Sequence 1: sense 5'-UCUCACCUCUCCAUUCC(dTdT)-3', antisense 5'-GGAAUGGGAAGAGGUGAGA(dTdT)-3'. Sequence 2: sense 5'-AGAAGCCGAUAUGUGGACC(dTdT)-3', antisense 5'-GGUCCACUAUCG-GCUUCU(dTdT)-3'. Unless otherwise stated, data shown was generated using SIRT1 sequence 1 and FoxO4 sequence 2. FoxO3 siRNA sequence was as follows: sense 5'-GCACAGAGUUGGAUGAAGU(dTdT)-3', antisense 5'-ACUUCAUCCAACUCUGUGC(dTdT)-3'. The sequences for Bcl-2 siRNA, caspase-2, and lamin A/C siRNA were as published (21). mRNA levels were quantitated by quantitative PCR using the MJ DNA Engine Opticon system, Qiagen SYBR Green PCR kit plus Qiagen Sensiscript Reverse Transcriptase. SIRT1-specific primers 5'-TCAGTGTTCATGTTCCCTTGC-3' and 5'-AATCTGCTCCTTGGCACTCT-3' were used in the thermal cycle: 50°C for 30 minutes, 94°C for 15 minutes, followed by 35 cycles of 94°C for 45 seconds, 57°C for 45 seconds, 72°C for 1 minute, then 75°C for 15 seconds before the plate was read. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers 5'-CGGAGTCAACGGATTGTTCGTAT-3' and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' were used with 50°C for 30 minutes, 94°C for 15 minutes, then 28 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, followed by 75°C for 15 seconds before plates were read. For lamin A/C, primers 5'-AAGCAGCGTGAGTTTGAGAGC-3' and 5'-AGGGTGAACCTTGGTGGGAAC-3' were used in the thermal cycle: 50°C for 30 minutes, 94°C for 15 minutes, then 30 cycles of 94°C for 45 seconds, 58°C for 45 seconds, 72°C for 1 minute, followed by 80°C for 15 seconds before the plates were read. Bcl-2 primers were as published (21). FoxO4 primers 5'-AGTCTGAGGTGCTGCGGAG-3' and 5'-GGTGGTGGCGTATCAGAGG-TG-3' were used in the thermal cycle: 50°C for 30 minutes, 94°C for 15 minutes, then 50 cycles of 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 1 minute, followed by 75°C for 15 seconds before plates were read. All the PCR quantitations were repeated thrice. Selectivity of each of the siRNAs was routinely established by comparison with negative controls for siRNA transfection (BCR-ABL siRNA) and for the process of



**Figure 1.** Selective silencing of SIRT1 in HCT116 cells. **A**, effects of SIRT1 siRNA, BCR-ABL siRNA, and lamin A/C siRNA on relative levels of mRNAs encoding SIRT1, lamin A/C, and GAPDH as indicated. Cells were harvested 48 hours after siRNA transfections and mRNA levels were determined by quantitative PCR (see Materials and Methods). **B**, immunoblots to show protein levels for SIRT1 and lamin A/C in cells harvested 48 hours posttransfection with siRNAs as indicated. **C**, immunoblot for acetylated p53 48 hours posttransfection with SIRT1 siRNA compared with mock-transfected controls (as indicated). Actin was used as internal reference for gel loading and protein levels were quantitated by densitometry (see Materials and Methods and text).

RNAi (lamin A/C siRNA) and by assessment of target and nontarget mRNA levels by quantitative PCR (e.g., see Results and Discussion; Fig. 1).

**Immunoblotting.** For immunoblotting, equivalent numbers of the transfected cells were prepared as described previously (21). Actin was used as loading reference control throughout. Antibodies were anti-SIRT1 (H-300, Santa Cruz, Santa Cruz, CA); anti-p53 (DO-1, Santa Cruz); anti-lamin A/C (636, Santa Cruz); anti-cleaved caspase-3, caspase-6, caspase-7, caspase-8, and caspase-9 (Cell Signalling); anti-actin (MAB1501, Chemicon, Temecula, CA); anti-phosphoserine-15 p53 [p-p53 (Ser<sup>15</sup>)-R, Santa Cruz]; anti-acetylated-p53 (Lys<sup>382</sup>, Cell Signalling); and anti-HDM2 (antibody 4B2; ref. 24). Visualization of bound antibodies was by enhanced chemiluminescence (Roche, Basel, Switzerland). Quantitation was by spot densitometry of signals within the linear range.

**Determination of apoptosis.** Apoptotic cells were identified by fluorescence-activated cell sorting (FACS) using Annexin V-Fluos (Roche) following the protocol of the manufacturer. Apoptosis was verified in some experiments by DNA laddering using the Suicide-Track DNA ladder isolation kit (Oncogene, San Diego, CA) and by detection of activated caspases (see text).

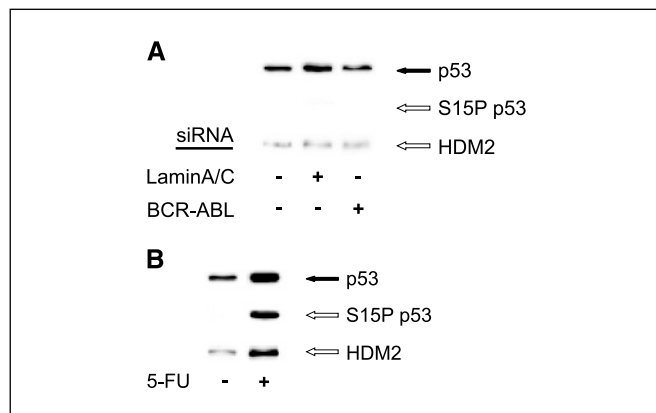
## Results and Discussion

Initial studies used p53<sup>+/+</sup> HCT116 cells. This is a human colorectal cancer cell line in which isogenic clones have been generated by the Vogelstein Laboratory with gene knockouts for p53, Bax, and p21 (25, 26). These isogenic clones are invaluable for the dissection of apoptotic pathways, particularly when used in combination with gene silencing by RNAi (21). It is important to note that we use early-passage HCT116 paired clones that exhibit comparable cell growth and proliferation characteristics (27).

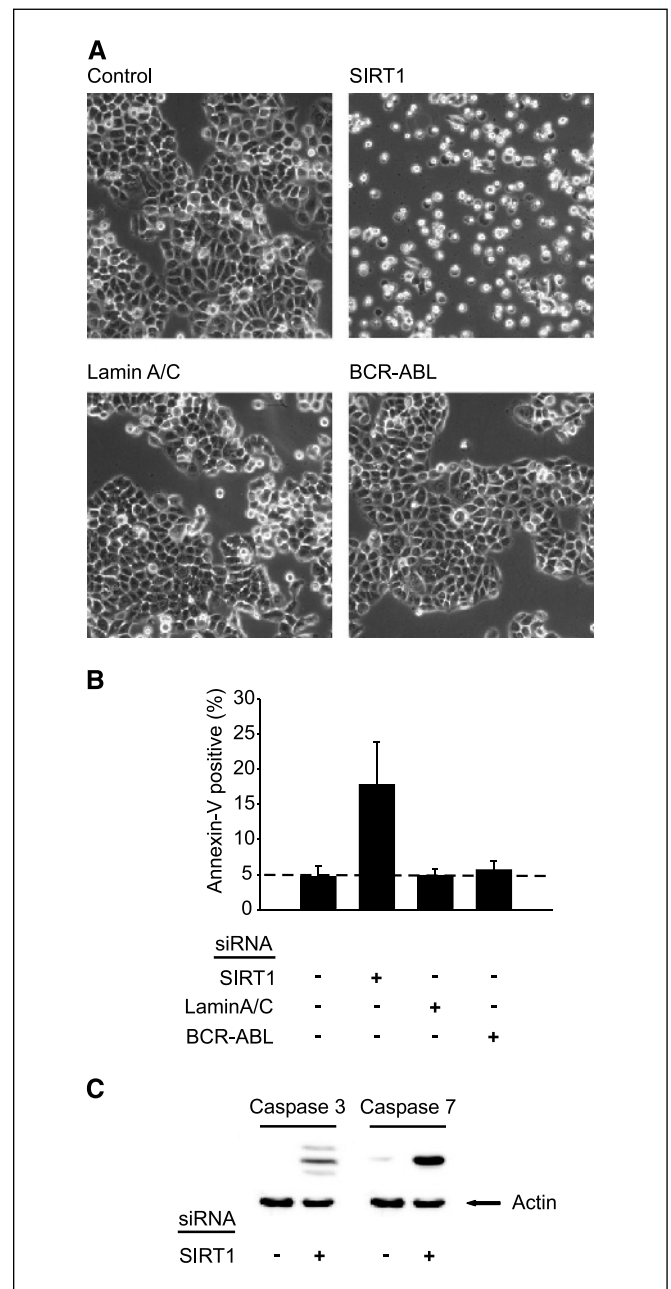
**Selective silencing of SIRT1 by RNA interference.** Transfection with SIRT1 siRNA (Materials and Methods; transfection efficiency ~80%) caused ~60% reduction in SIRT1 mRNA levels accompanied by ~80% reduction in SIRT1 protein at 48 hours posttransfection (Fig. 1A and B). Control transfections included lamin A/C siRNA, effective for RNA interference with loss of lamin A/C mRNA and lamin A/C protein (Fig. 1A and B). The process of RNAi induced by lamin A/C siRNA did not cause activation of endogenous cellular p53 as indicated by lack of p53 accumulation, serine 15 phosphorylation, or up-regulation of the p53 target gene *HDM2* (Fig. 2). Silencing of lamin A/C was also without any visible detrimental effect on the transfected cells (compare lamin A/C with untreated control cells; Fig. 3A) and this is consistent with previous results (21). Another control was BCR-ABL siRNA (Fig. 1A and B), which has no known target in human epithelial cells. Neither lamin A/C or BCR-ABL control siRNAs affected SIRT1 expression (Fig. 1A and B). Conversely, SIRT1 siRNA was without effect upon lamin A/C mRNA/protein or GAPDH mRNA levels (Fig. 1A and B). Thus, SIRT1 siRNA selectively silences SIRT1 expression. This was confirmed using two independent SIRT1 siRNA sequences (Materials and Methods).

SIRT1 can deacetylate p53 protein (see Introduction) and we reasoned that knockdown of SIRT1 protein levels, as evident in Fig. 1B, might result in the accumulation of acetylated p53. Indeed, this proved to be the case (Fig. 1C). Thus, we show that p53 acetylation/deacetylation can operate in the absence of applied stress and seems to be under constitutive regulation by SIRT1.

**SIRT1 silencing induces apoptosis.** Unexpectedly, SIRT1 silencing resulted in massive apoptosis of human p53<sup>+/+</sup> HCT116 colorectal cancer cells (Fig. 3A and B). Indeed, by 48 hours



**Figure 2.** Induction of RNAi per se does not initiate a p53 stress response. *A*, immunoblots to show lack of activation of endogenous p53 (as indicated by no detectable serine 15 phosphorylation nor up-regulation of the p53 target gene *HDM2*) in HCT116 cells mock transfected or transfected with control BCR-ABL or lamin A/C siRNAs. *B*, positive control showing activation of p53 in response to genotoxic stress in HCT116 cells treated for 24 hours with 375  $\mu$ M/L 5-fluorouracil.



**Figure 3.** Silencing of SIRT1 induces apoptosis in HCT116 cells. Effects of SIRT1, BCR-ABL, and lamin A/C siRNAs on HCT116 cells as visualized 48 hours posttransfection by phase contrast microscopy (*A*) and Annexin V labeling (*B*) to confirm early apoptotic cell membrane changes (see Materials and Methods). Background levels of Annexin V labeling were ~5% (dashed line). *C*, immunoblots show detection of activated cleavage products of caspase-3 and caspase-7 at 48 hours following siRNA transfections. Actin loading control was diluted 1:10 relative to the caspase blots.

posttransfection, most cells seemed to be dying (Fig. 3A). Apoptosis was not attributable to RNAi per se because it was only observed in those cells treated with SIRT1 siRNA and not in the RNAi controls treated with lamin A/C siRNA (Fig. 3A and B). Because these results were obtained under normal conditions of cell culture, we conclude that SIRT1 constitutively inhibits apoptosis in p53<sup>+/+</sup> HCT116 cells.

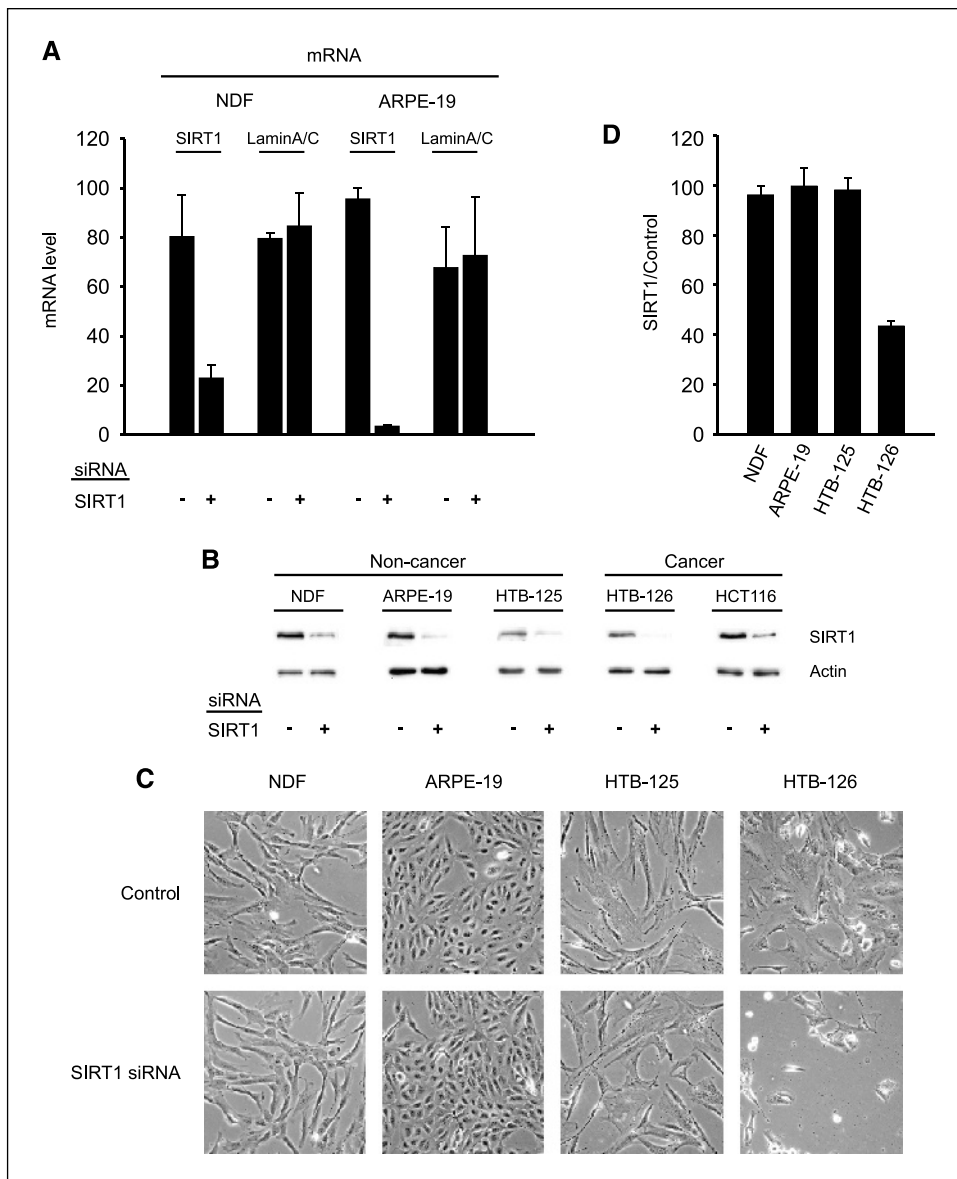
**Apoptosis involves caspase-3 and caspase-7.** Downstream executioners of apoptosis induced by SIRT1 silencing were identified as caspase-3 and caspase-7 and activated cleavage

products are shown in Fig. 3C. No activation cleavage was detectable in the controls (Fig. 3C; data not shown). Overall, these results indicate that SIRT1 constitutively suppresses apoptosis in HCT116 cells via pathway(s) involving caspase-3 and caspase-7.

**Apoptosis is observed in various cancer cell lines.** HCT116 cells are epithelial cells derived from a human colorectal carcinoma. To test the generality of induction of apoptosis in response to SIRT1 silencing, we screened a range of human colorectal epithelial cancer cell lines (six individual clones of HCT116 and cell lines LoVo, DLD-1, SW48, and HT29), two human epithelial cell lines derived from carcinomas of the breast (MCF-7 and HTB-126), and a line of human cervical cancer cells (SiHa). In all cases the control transfections (BCR-ABL siRNA and lamin A/C siRNA) had no apparent effect on cell growth or viability. In contrast, cells transfected with SIRT1 siRNA either entered apoptosis (colorectal and cervical cancer cells; data not shown) or ceased growth (mammary cancer cells; see, e.g., Fig. 4C and D: cell line HTB-126). Future studies will explore these apparent cell type differences in response to SIRT1 silencing.

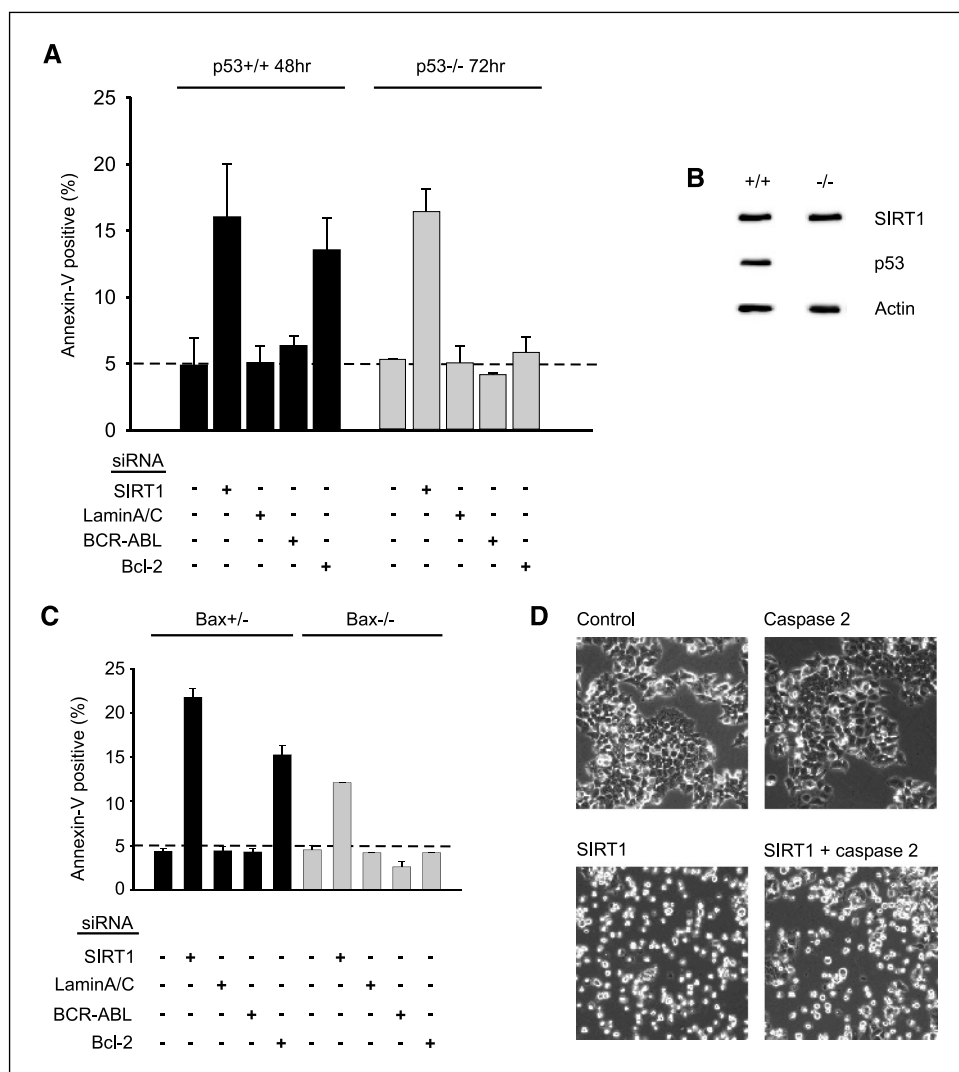
**Noncancer cells are refractory to SIRT1 silencing.** We next asked if SIRT1 silencing also induces apoptosis or growth arrest in human cell lines of normal, noncancerous origin. For this purpose, we used two normal human epithelial cell lines (ARPE-19 and HTB-125) and NDFs. ARPE-19 is a line of normal human pigmented retinal epithelial cells (28), and HTB-125 is a line of human epithelial cells derived from normal mammary tissue peripheral to an infiltrating ductal carcinoma (which was the source of the HTB-126 cancer cell line; see above; ref. 29). HTB-125 and HTB-126 thus represent paired normal and cancer cell lines derived from the same tissue.

Importantly, all three noncancer cell lines showed good transfection efficiencies, and SIRT1 silencing was at least equivalent to that obtained for HCT116 epithelial cancer cells (Fig. 4A and B). Remarkably, however, SIRT1 silencing had no apparent effect on the cell growth or viability of the noncancer cells up to 7 days posttransfection. (Figure 4C shows images of noncancer cells at 48 hours posttransfection and also includes images of the HTB-126 cancer cells for comparison with normal



**Figure 4.** Noncancer cells are refractory to SIRT1 silencing. *A*, mRNA levels for SIRT1 and lamin A/C in NDFs and ARPE-19 epithelial cells following transfections with SIRT1 siRNA. *B*, protein levels of SIRT1 in cells harvested 48 hours posttransfection with siRNAs as indicated. Actin loading control is also shown. Equivalent blots for HTB-126 mammary cancer cells and HCT116 colorectal cancer cells are included for ease of comparison of the noncancer cells with the cancer cells. *C*, phase contrast images of NDFs, normal retinal epithelial cells (ARPE-19), and normal mammary epithelial cells (HTB-125) 48 hours posttransfection with SIRT1. The far right-hand panel shows the effects of SIRT1 silencing on HTB-126 mammary carcinoma cells that are directly comparable with the adjacent panel of HTB-125 normal mammary epithelial cells derived from adjacent tissue (see text). *D*, proliferation of SIRT1-silenced cells relative to controls as determined by cell counting of noncancer cells (NDF, ARPE-19, and HTB-125) and of cancer cells (HTB-126) 48 hours posttransfection.

**Figure 5.** Apoptosis induced by SIRT1 silencing in HCT116 cancer cells is independent of p53, Bax, and caspase-2. Isogenic clones of HCT116 p53+/+ and p53-/- cells were cultured in parallel and transfected with SIRT1 siRNA, lamin A/C siRNA, BCR-ABL siRNA, or Bcl-2 siRNA as indicated. **A**, apoptotic cells determined by Annexin V labeling at 48 hours (p53+/+ cells) or 72 hours (p53-/- cells) posttransfection with siRNAs as indicated. Note that although apoptotic p53-/- cells were detected at 48 hours (~11% Annexin V positive), the maximum response was at 72 hours (~18% Annexin V positive), as shown here. **B**, equivalent levels of SIRT1 protein are evident in p53+/+ and p53-/- cells; actin loading control is shown. **C**, apoptotic, Annexin V-positive cells in isogenic clones of HCT116 Bax+/- and Bax-/- cells 48 hours following transfection with siRNAs as indicated. **D**, phase contrast images showing lack of rescue from SIRT1-induced apoptosis in HCT116 p53+/+ cells following cotransfection with caspase-2 siRNA.



HTB-125 epithelial cells from the same tissue). Cell counts confirmed that SIRT1 silencing did not affect the growth of the noncancer cells relative to the controls (compare noncancer cells NDF, ARPE-19, and HTB-125 with the HTB 126 cancer cell line; Fig. 4D). Consistent with these observations, SIRT1 silencing had no effect on the cell cycle profiles of noncancer cells, as determined by BrdUrd labeling and FACS analysis, whereas the growth arrested cancer cells showed ~45% G<sub>1</sub> accumulation under identical conditions (data not shown).

These overall results indicate (a) that the constitutive functioning of SIRT1 differs between cancer and noncancer cells and (b) that SIRT1 enables cancer cell viability, but (c) is nonessential for the viability of noncancer cells. The observation that SIRT1 is dispensable for normal mammalian cell viability is compatible with data obtained for mice with either genetic knockout or targeted mutation of SIRT1. In such mice, the lack of SIRT1 does not prevent embryonic development although it results in a severe birth phenotype consistent with nutritional perturbation (30, 31).

#### Apoptosis induced by SIRT1 silencing is independent of p53.

It is reported that SIRT1 down-regulates stress-induced activation of p53 (7, 8, 17, 18). Accordingly, we next compared the effects of SIRT1 silencing in isogenic clones of p53+/+ and p53-/- HCT116

cells (25). Unexpectedly, both p53+/+ and p53-/- cells displayed apoptosis, albeit with slower kinetics for the p53-/- cells (Fig. 5A). Further confirmation that apoptosis in response to SIRT1 silencing is independent of p53 was evident in the apoptotic response of the SiHa cervical cancer cells. These cells are derived from a human cervical carcinoma positive for human papilloma virus type 16 (HPV16). In SiHa cells, the p53 protein is undetectable due to the presence of HPV16 E6 protein that targets p53 for ubiquitinylation and degradation (32). Silencing SIRT1 in SiHa cells nonetheless induced apoptosis (not shown), albeit with delayed kinetics and similar to that observed for the HCT116 p53-/- cells (see above).

These results indicate that p53 is dispensable for apoptosis of epithelial cancer cells following SIRT1 silencing, but that p53 nonetheless potentiates the process of apoptosis. A recent report, using enforced expression of exogenous constructs, concludes that p53 can regulate SIRT1 expression via two p53 binding sites in the SIRT1 promoter (17). However, under the conditions of the experiments described here, we were unable to discern any p53-related differences in levels of endogenous SIRT1 expression in p53+/+ and p53-/- isogenic cells (Fig. 5B).

**SIRT1 and Bcl-2 regulate separable apoptotic nodes.** In comparing p53+/+ and p53-/- cells, we included parallel cultures

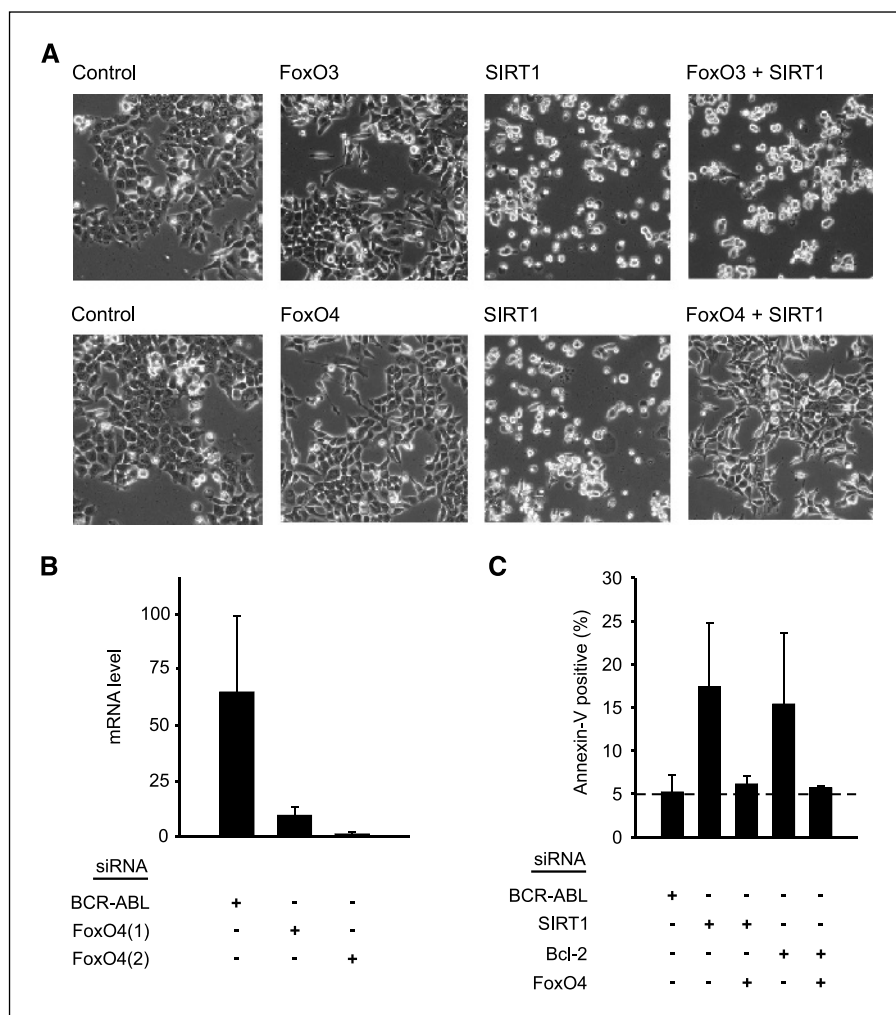
treated with Bcl-2 siRNA. As previously observed (21), good selective knockdown of Bcl-2 protein was obtained (results not shown). Bcl-2 silencing caused apoptosis in p53<sup>+/+</sup> cells but failed to induce apoptosis in p53<sup>-/-</sup> cells, thus demonstrating p53-dependent apoptosis governed by Bcl-2 (Fig. 5A) and confirming our previous evidence for a novel Bcl-2/p53 axis regulating apoptosis in colorectal cancer cells (21).

Bax and caspase-2 are essential mediators of Bcl-2-regulated apoptosis in HCT116 cells (Fig. 5C and D; ref. 21). To ask if Bax is also required for SIRT1-regulated apoptosis, we compared isogenic clones of Bax<sup>+/-</sup> and Bax<sup>-/-</sup> HCT116 cells (26). The results show that apoptosis induced by SIRT1 silencing is independent of Bax and is evident in both the Bax<sup>+/-</sup> and Bax<sup>-/-</sup> cells (Fig. 5C). Furthermore, cosilencing with caspase-2 siRNA, which rescues cells from apoptosis following treatment with Bcl-2 siRNA (21), fails to rescue cells following treatment with SIRT1 siRNA (Fig. 5D). Thus, we show that SIRT1 regulates an apoptotic pathway that is independent of p53, Bax, and caspase-2. This may represent a discrete SIRT1-regulated pathway or, alternatively, reflect a role for SIRT1 downstream of Bcl-2, p53, and Bax. In either case, we conclude that SIRT1 regulates apoptosis independently of Bcl-2 in human HCT116 epithelial cells.

**FoxO4 is an essential mediator of SIRT1 and Bcl-2 apoptotic pathways.** Having shown that SIRT1-regulated apoptosis is independent of p53, we turned our attention to the FoxO family

of proteins, also known to be down-regulated by SIRT1 and capable of inducing apoptosis (10, 11, 17, 19). For example, when mammalian cells are exposed to either oxidative stress or UVC irradiation, SIRT1 and FoxO3 form a complex and this interaction is linked with deacetylation of FoxO3 (10, 11). FoxO4 also complexes with, and is a substrate for, SIRT1 deacetylation when mammalian cells are exposed to oxidative stress (19). FoxO3 and FoxO4 share five putative acetylation sites that are conserved within the FoxO family and their transcriptional activity is governed, in part, by their acetylation status (9). As yet, it is unknown whether SIRT1 deacetylates specific lysine residues on either FoxO3 or FoxO4. Similarly, the possible regulatory effect of SIRT1 upon the differential transcription of FoxO target genes is also unknown: This is a particularly difficult area to explore because multiple transcription factors are regulated by SIRT1 and these transcription factors overlap in their target genes (see Introduction; ref. 9).

Here, we have used RNAi to test the effects of silencing FoxO3 and FoxO4 expression in human colorectal cancer cells (HCT116 p53<sup>+/+</sup>) and have asked if either FoxO protein influences the apoptotic consequences of SIRT1 silencing in the same cells. Both FoxO3 and FoxO4 siRNAs gave selective knockdown of FoxO3 mRNA and FoxO4 mRNA, respectively, and two independent FoxO4 siRNAs showed good knockdown of FoxO4 (>80% reduction in both FoxO3 and FoxO4 mRNAs; see Fig. 6B for FoxO4 mRNA knockdown



**Figure 6.** FoxO4 cosilencing rescues HCT116 p53<sup>+/+</sup> cells from SIRT1 siRNA-induced apoptosis and also from Bcl-2 siRNA-induced apoptosis. **A**, phase contrast images of HCT116 p53<sup>+/+</sup> cells 48 hours posttransfection with either FoxO3 siRNA, FoxO4 siRNA, or SIRT1 siRNA, and cotransfection of FoxO3 siRNA with SIRT1 siRNA, or FoxO4 siRNA with SIRT1 siRNA. **B**, FoxO4 mRNA levels in HCT116 p53<sup>+/+</sup> cells 48 hours posttransfection with BCR-ABL siRNA, FoxO4 siRNA sequence 1, or FoxO4 siRNA sequence 2 (see Materials and Methods). **C**, Annexin V-positive, apoptotic HCT116 p53<sup>+/+</sup> cells were determined 48 hours posttransfection with siRNAs as indicated.

with the two independent FoxO4 siRNAs; Materials and Methods). Individual silencing of FoxO3 or FoxO4 had no apparent effect on cell growth or morphology (Fig. 6A). However, cosilencing experiments revealed that FoxO4 siRNA, but not FoxO3 siRNA, rescues SIRT1 siRNA-treated cells from apoptosis (Fig. 6A) and this was confirmed by Annexin V FACS analysis (Fig. 6C). This indicates that FoxO4 is essential for apoptosis following SIRT1 silencing in HCT116 cancer cells. Furthermore, FoxO4 cosilencing also rescued cells from apoptosis following Bcl-2 silencing (Fig. 6C). Thus, we identify FoxO4 as essential proapoptotic mediator for both SIRT1- and Bcl-2-regulated apoptotic pathways. This indicates that, although SIRT1 and Bcl-2 differ in their requirements for p53, Bax, and caspase-2 (this article; ref. 21), their apoptotic pathways converge in that both require FoxO4 as proapoptotic mediator.

Our results reveal a hitherto unsuspected and fundamental difference between cancer and noncancer cells. This difference is manifest under “nonstress” conditions (in the absence of applied stress) and involves SIRT1 that seems essential for cancer cell viability (see Results and Discussion). In contrast, the viability of noncancerous cells is independent of SIRT1 (Fig. 4), an observation

reinforced by the reported viability of SIRT1<sup>-/-</sup> mouse embryos (30, 31). Our work identifies SIRT1 as a novel target for selective killing of human cancer versus noncancer cells. Killing is independent of p53 and it is predicted that both p53 wild type and p53-deficient cancers may prove susceptible to SIRT1 targeting. Because noncancer cells are refractory to SIRT1 silencing (this work), our results suggest that progress from normal to cancerous cell growth involves a SIRT1-related change in the control of apoptosis. This change is manifest under nonstress conditions and future studies will aim to chart the mechanisms involved in this newly discovered cancer-related process.

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