The c-MYC oncoprotein, the NAMPT enzyme, the SIRT1-inhibitor DBC1, and the SIRT1 deacetylase form a positive feedback loop

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Silent information regulator 1 (SIRT1) represents an NAD+-dependent deacetylase that inhibits proapoptotic factors including p53. Here we determined whether SIRT1 is downstream of the prototypic c-MYC oncogene, which is activated in the majority of tumors. Elevated expression of c-MYC in human colorectal cancer correlated with increased SIRT1 protein levels. Activation of a conditional c-MYC allele induced increased levels of SIRT1 protein, NAD+, and nicotinamide-phosphoribosyltransferase (NAMPT) mRNA in several cell types. This increase in SIRT1 required the induction of the NAMPT gene by c-MYC. NAMPT is the rate-limiting enzyme of the NAD+ salvage pathway and enhances SIRT1 activity by increasing the amount of NAD+. c-MYC also contributed to SIRT1 activation by sequestering the SIRT1 inhibitor deleted in breast cancer 1 (DBC1) from the SIRT1 protein. In primary human fibroblasts previously immortalized by introduction of c-MYC, down-regulation of SIRT1 induced senescence and apoptosis. In various cell lines inactivation of SIRT1 by RNA interference, chemical inhibitors, or ectopic DBC1 enhanced c-MYC-induced apoptosis. Furthermore, SIRT1 directly bound to and deacetylated c-MYC. Enforced SIRT1 expression increased and depletion/inhibition of SIRT1 reduced c-MYC stability. Depletion/inhibition of SIRT1 correlated with reduced lysine 63-linked polyubiquitination of c-Myc, which presumably destabilizes c-MYC by supporting degradative lysine 48-linked polyubiquitination. Moreover, SIRT1 enhanced the transcriptional activity of c-MYC. Taken together, these results show that c-MYC activates SIRT1, which in turn promotes c-MYC function. Furthermore, SIRT1 suppressed cellular senescence in cells with deregulated c-MYC expression and also inhibited c-MYC-induced apoptosis. Constitutive activation of this positive feedback loop may contribute to the development and maintenance of tumors in the context of deregulated c-MYC.

tumor metabolism | immortalization | p53 | tumor suppression | acetylation

The protein product of the proto-oncogene c-MYC is at the center of a transcription factor network that regulates cellular proliferation, replicative potential, cell–cell competition, cell size, differentiation, metabolism, and apoptosis (1–3). Expression of c-MYC is induced rapidly by diverse mitogens and is down-regulated during differentiation. Deregression of c-MYC activity has been implicated in the genesis of the majority of human cancers, and its inhibition represents a possible alternative to current cancer treatments (4, 5). The oncogenic activation of c-MYC often is caused by constitutive expression of c-MYC resulting from mutations in upstream regulators, such as the components of the adenomatous polyposis coli (APC)–β-catenin–TCF4 pathway, or genomic alterations, such as amplifications and translocations. In addition, the turnover rate of the c-MYC protein often is affected in tumors. Although several E3 ubiquitin ligases and signaling pathways have been reported to regulate ubiquitination and degradation of c-MYC, additional mechanisms likely contribute to this phenomenon. Activation of the c-MYC proto-oncogene antagonizes replicative and Ras-induced senescence and is sufficient for cellular immortalization (6–9). Furthermore, elevated levels of c-MYC may induce replication stress and reactive metabolites that elicit apoptosis or premature senescence through p53-dependent or -independent pathways (10–13).

c-MYC directly induces the human telomerase reverse transcriptase (hTERT) gene, which encodes the catalytic subunit of telomerase (7). However, hTERT expression may prolong the replicative lifespan of cells to only a limited extent (8). Therefore, we hypothesized that c-MYC may regulate other factors that antagonize cellular senescence and mediate cellular immortalization.

The human silent information regulator 1 (SIRT1) gene encodes an NAD+-dependent protein deacetylase, which is involved in epigenetic silencing, heterochromatin formation, regulation of metabolism, DNA repair, and cellular stress responses. These functions are mediated by deacetylation of histones, transcription factors, chromatin-modifying enzymes, and other nuclear proteins (14, 15). Recently, the NAD+ salvage pathway and its rate-limiting enzyme, nicotinamide phosphoribosyltransferase (NAMPT), have been implicated in the activation of SIRT1 (16). In contrast, the deleted in breast cancer 1 (DBC1) gene product negatively regulates SIRT1 activity through binding to its active site and thereby inhibiting SIRT1–substrate interaction (17, 18). Moreover, DBC1 was shown to be involved in the induction of apoptosis in response to TNF-α (19). In yeast, Drosophila, and Caenorhabditis elegans, ectopic expression of SIR2, the orthologue of SIRT1, extends lifespan (20). However, these effects recently were shown to depend on the genetic background in Drosophila and C. elegans (21). In addition, SIRT1 extends the replicative lifespan of human cells (22), an effect that can be attributed, at least in part, to the SIRT1-mediated deacetylation and inhibition of p53 (23–25). Furthermore, other proapoptotic factors such as Foxo transcription factors, Smad7, Ku70, p73, and poly(ADP-ribose) polymerase 1 (PARP1).

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are negatively regulated by SIRT1 (15, 26). In summary, these properties of SIRT1 led us to hypothesize that SIRT1 may play a role downstream of c-MYC.

Here we report that c-MYC activates the SIRT1 enzyme, which critically contributes to suppression of senescence in cells with deregulated c-MYC and suppression of c-MYC–induced apoptosis. Furthermore, we could delineate two mechanisms involving NAMPT and DBC1 by which c-MYC enhances SIRT1 activity. In addition, we identified a positive feedback loop between c-MYC and SIRT1, supporting an important role for SIRT1 in c-MYC–driven tumorigenesis.

Results

Posttranscriptional Activation of SIRT1 by c-MYC. Here we sought to determine whether SIRT1 represents an effector of the c-MYC oncoprotein. In line with this hypothesis, SIRT1 protein expression increased as early as 9 h after activation of a conditional c-Myc-estrogen receptor (ER) fusion protein in c-myc–deficient RAT1 fibroblasts or of a conditional c-MYC allele in the P493-6 B-cell line (Fig. 1, A and SI Appendix, Fig. S1 A and B). SIRT1 mRNA was not affected by c-MYC activation in these cells, whereas known c-MYC target genes were clearly induced (Fig. 1, B and C). SIRT1 mRNA levels were not affected by acute activation of c-MYC in two other cellular systems tested (SI Appendix, Fig. S1C). Furthermore, down-regulation of c-MYC expression by an inducible shRNA directed against c-MYC was followed by a decrease in SIRT1 protein in the colorectal cancer cell line LS-174T (Fig. 1D). When c-MYC expression was induced by restimulation with serum, SIRT1 levels increased in RAT1 fibroblasts but not in c-myc–deficient RAT1 cells (Fig. 1E). Again, the SIRT1 mRNA was unaffected by experimental modulation of c-MYC activity in LS-174T and RAT1 fibroblasts (SI Appendix, Fig. S1 C and D). Taken together, these results demonstrate that c-MYC activation is sufficient for posttranscriptional induction of SIRT1 protein and is necessary for the increase in SIRT1 protein after mitogenic stimulation.

To validate whether the abundance of SIRT1 protein is regulated at the posttranscriptional level, MCF-7– and human diploid fibroblast (HDF)-derived cell lines constitutively expressing a SIRT1-GFP fusion protein or GFP under control of an LTR promoter were analyzed. SIRT1-GFP protein expression was down-regulated after serum starvation in both cell types (Fig. 1F and SI Appendix, Fig. S1E). After readdition of serum, which generally is accompanied by an increase in c-MYC expression, SIRT1-GFP but not GFP expression was induced. Moreover, inhibition of proteasomal degradation induced SIRT1 expression in c-myc–deficient RAT1 fibroblasts, whereas in wild-type c-myc RAT1 cells the SIRT1 level was affected only marginally by MG132 treatment (Fig. 1G). Taken together, these findings suggest that the c-MYC–mediated increase in the SIRT1 protein may be caused by inhibition of the proteasomal degradation of SIRT1.

To determine whether the regulation of SIRT1 by c-MYC also occurs in vivo, we analyzed colorectal cancer specimens, which generally show high c-MYC expression because of mutational activation in vivo, we analyzed colorectal cancer specimens, which generally show high c-MYC expression because of mutational activation of the APC–β-catenin pathway and/or K-Ras or B-Raf mutations (27–29). In primary colorectal cancer biopsies derived from 15 patients, carcinoma cells with high c-MYC expression consistently showed elevated SIRT1 expression, whereas adjacent normal colon crypts displayed barely detectable expression of SIRT1 and c-MYC (Fig. 1H and SI Appendix, Fig. S2). Therefore, the expression of SIRT1 may be regulated by c-MYC in normal and malignant tissues.
**Fig. 2.** c-MYC activates SIRT1: effects on p53 and mediation by NAMPT. (A) Cells were transfected with plasmids encoding the indicated proteins and treated with etoposide (20 μM) for 7 h (HCT-116 cells; Left) or 10 Gy γ-irradiation (MCF-7 cells; Center and Right) and fixed after 4 h. Expression of ectopic MYC-HA and a c-MYC mutant with the transactivation domain replaced by the repressive Sin3 domain of MAD1 (MADMYC-HA) was detected with an anti-HA antibody (Center and Right), and SIRT1-GFP expression was detected by GFP fluorescence (Left). Acetylation (ac) of p53K382 was detected by indirect immunofluorescence. Arrows indicate the positions of cells positive for ectopic proteins (SIRT1-GFP, c-MYC, or MADMYC-HA). (B) P493-6 c-MYC tetracycline-off cells were kept in the absence or presence of tetracycline. After 72 h cell lysates were subjected to immunoblot analysis of the indicated proteins. As documented by β-actin detection, different amounts of protein were loaded to adjust for comparable p53 levels in all samples. (C) NADH and (D) NAD+ concentrations were determined, as described previously (85), in RAl1 myc−/− fibroblasts stably expressing c-Myc-ER. After serum starvation for 48 h in 0.1% FBS, c-Myc-ER was activated for 24 h by addition of 300 nM 4-OHT. Cell lysates were subjected to the cycling reaction (for details see SI Appendix, SI Methods). The graphs show mean values ± SD of three independent experiments. Results of the individual measurements are given in SI Appendix, Table S1. (E) Mean values of NAD+/NADH ratios of MYC OFF (−4-OHT) and MYC ON (+4-OHT) are given with SDs. Data were taken from the analysis shown in C and D and SI Appendix, Table S1. (F) Schematic representation of the NAD+ salvage pathway. NAD+ is generated by NAMPT-mediated conversion of NAM to NMN, which is converted to NAD+ by the enzymes nicotinamide/nicotinic acid mononucleotide adenyltransferase (NMAT) 1–3. (G) Schematic representation of human, mouse, and rat NAMPT promoter regions. The transcription start site is indicated by “+1”. E-box positions (gray squares) and E-box motifs conserved between species are indicated. qPCR amplicons are indicated by pairs of arrows. (H) ChIP analysis of c-MYC binding to the NAMPT promoter in serum (ser)-stimulated MCF-7 cells. After starvation with 0.1% FBS for 48 h, half of the cells were restimulated with 10% (vol/vol) FBS for 12 h. The assay was performed in triplicate using a polyclonal anti-MYc antibody and rabbit IgGs as control. c-MYC enrichment at E-boxes was determined by qPCR with primer pairs flanking E-boxes (see G). The DNA input was normalized with a genomic amplicon devoid of E-boxes. (I) qPCR analysis of NAMPT mRNA expression upon c-MYC activation in P493-6 cells. RNA was isolated 12 and 24 h after removal of tetracycline. (J) qPCR analysis of NAMPT mRNA expression upon DOX (1 μg/mL)-mediated induction of a conditional c-MYC allele for the indicated periods in the MCF-7 cell line JIMMR1 (86). RNA samples for the 0 (noninduced) and 12-h time points were harvested simultaneously. Analyses were performed in triplicates. (K) Induction of NAMPT protein upon activation of c-MYC-ER in HDF. Cells were serum starved for 48 h and then stimulated with 4-OHT for the indicated times, lysed simultaneously, and subjected to Western blot analysis. β-actin served as loading control. (L) Induction of SIRT1 by c-Myc is dependent on NAMPT. HDF-Myc-ER cells were transfected with the indicated siRNAs and then starved as in K and treated with 4-OHT for 48 h. Cells were lysed simultaneously and analyzed by Western blotting for the expression of the indicated proteins. β-actin served as loading control.

**c-MYC Induces SIRT1 Deacetylase Activity.** SIRT1 is known to deacetylate p53 at lysine residue 382 (K382), thereby attenuating p53 activity and affects critical downstream targets such as p53.

**Induction of NAMPT by c-MYC Mediates SIRT1 Activation.** Because the deacetylase activity of SIRT1 depends on NAD+, we hypothesized that the increased SIRT1 activity induced by c-MYC may be caused by c-MYC–mediated changes in metabolism affecting the NAD+/NADH ratio. Indeed, c-MYC activation provoked an increase in NAD+ and a decrease in the NADH levels, resulting in a more than fourfold increase in the NAD+/NADH ratio (Fig. 2 C–E and SI Appendix, Table S1). Activation of either glycolysis or the NAD+ salvage pathway, which is the primary means of cellular NAD+ regeneration (30), may induce such changes. Interestingly, the addition of FK866 (31), a specific inhibitor of NAMPT, which is the rate-limiting enzyme of the salvage pathway, resulted in a pronounced decrease in NAD+ before and after c-MYC activation, whereas chemical inhibition of glycolysis did not affect the NAD+ levels significantly (SI Appendix, Fig. S4 A and B) but reduced NADH by ∼50% when it was applied for 18 h. As shown previously (32), inhibition of glycolysis by 2-deoxyglucose resulted in an increase of the NAD+/NADH ratio, arguing against a major contribution of glycolysis to the overall NAD+ levels and to SIRT1 activation. Because the inhibition of NAMPT activity had such pronounced effects on NAD+, our data suggest that the contribution of the salvage pathways to the NAD+ pool in the cell exceeds the amount of NAD+ generated by lactate dehydrogenase-A (which is induced by c-MYC) during glycolysis. We therefore hypothe-
sized that these changes could result from a c-MYC–induced activation of NAMPT that promotes the conversion of NAM to NAD⁺ via nicotinamide mononucleotide (NMN) (Fig. 2F). Analysis of the NAMPT promoter sequence revealed three conserved, noncanonical E-boxes in the vicinity of the transcriptional start site (Fig. 2G), two of which are CACCGG motifs. Binding of c-MYC–induced c-MYC motifs in promoter regions of other genes has been reported previously (33). ChiP analysis revealed binding of endogenous c-MYC to a region encompassing the noncanonical E-boxes in the NAMPT promoter. Occupancy by c-MYC was enhanced after mitogenic stimulation by serum, which is known to activate expression of c-MYC. The E-box located ∼2.5 kb upstream of the transcriptional start site did not display occupation by c-MYC (Fig. 2H). In addition, inspection of publicly available ChiP-Seq analyses of genome-wide c-MYC binding confirmed the presence of c-MYC at the NAMPT promoter coinciding with histone modifications indicating transcriptional activity (SI Appendix, Fig. S5A and B). Upon c-MYC activation, increased expression of NAMPT mRNA was observed in the B-cell line P493-6 and in MCF-7 cells (Fig. 3A and J). The NAMPT protein level also increased after activation of c-MYC-ER in HDF (Fig. 2A). Down-regulation of NAMPT expression by siRNAs largely prevented an increase in SIRT1 after c-MYC activation (Fig. 2L). Taken together, these results establish the NAMPT gene as a mediator of SIRT1 activation by c-MYC.

**c-MYC-DBC1 Association Facilitates Activation of SIRT1.** We previously identified the DBC1 protein as a c-MYC–interacting protein (34). Interestingly, the functionally important MYC Box-II domain is essential for the c-MYC–DBC1 interaction. Furthermore, DBC1 recently was shown to inhibit the SIRT1 enzyme by binding to its active site (17, 18). This observation raised the question whether DBC1 may play a role in the regulation of SIRT1 by c-MYC. Indeed, SIRT1- and DBC1-GST fusion proteins were found to associate directly with recombinant c-MYC in vitro, suggesting that these interactions may affect each other in vivo (Fig. 3A). When increasing amounts of ectopic c-MYC were expressed in HEK-293 cells, the amount of endogenous DBC1 bound to ectopic SIRT1 gradually decreased (Fig. 3B). Because the amount of c-MYC associated with SIRT1 did not increase proportionally to the amount of expressed c-MYC, c-MYC presumably sequesters DBC1 away from SIRT1 rather than competing with DBC1 for binding to SIRT1 (Fig. 3B). It is conceivable that a competition between DBC1 and MYC for binding to SIRT1 may occur preferentially when c-MYC is expressed at lower or intermediate levels. In summary, these observations show that, in addition to affecting the NAD⁺/NADH ratio, c-MYC may contribute to the activation of SIRT1 by binding directly to the SIRT1 inhibitor DBC1 and preventing its interaction with SIRT1.

**SIRT1 Suppresses c-MYC–Induced Apoptosis.** Next we investigated whether SIRT1 inactivation affects apoptosis induced by acute activation of conditional c-MYC alleles. Indeed, apoptosis induced by c-MYC–ER was augmented substantially by the addition of NAM in RAT1A cells (Fig. 4A). Also, when ectopic c-MYC was expressed in U2OS cells, a pronounced increase in apoptosis was observed when SIRT1 was down-regulated simultaneously by induction of a microRNA (miRNA) directed against its 3′-UTR (Fig. 4B). In these cells, ectopic c-MYC combined with down-regulation of SIRT1 enhanced acetylation of p53 at K382 (Fig. 4C), accompanied by an up-regulation of p53 and its targets PUMA and p21. Therefore, as also documented in P493-6 cells (Fig. 2B), c-MYC–induced SIRT1 deacetylates p53 and thereby attenuates the transcriptional activity of p53 after c-MYC activation, resulting in decreased expression of proapoptotic genes and thereby presumably attenuating c-MYC–induced apoptosis.

In Fig. 3B we show that enforced expression of c-MYC sequesters the SIRT1 inhibitor DBC1 from SIRT1, possibly supporting SIRT1 activity. Therefore, we investigated whether modulation of DBC1 expression has an effect on c-MYC–induced apoptosis. Indeed, ectopic expression of DBC1 increased c-MYC–induced apoptosis and decreased proliferation, whereas suppression of DBC1 by a DBC1-specific miRNA resulted in decreased apoptosis and increased proliferation in U2OS cells (Fig. 4D and E). Similarly, c-MYC-ER activation resulted in less apoptosis in human fibroblasts when DBC1 was down-regulated by specific siRNAs (SI Appendix, Fig. S6). Interestingly, in the presence of DBC1-specific siRNAs, c-MYC-ER activation resulted in increased expression of the c-MYC target gene product CDK4 (Fig. 4F), a result that is in line with increased proliferation. Therefore, the degree of DBC1-mediated inhibition of SIRT1 may be a critical determinant of the outcome of c-MYC activation.

The cells used in the apoptosis assays described above (RAT1-Myc-ER, U2OS, HDF-c-MYC-ER) express wild-type p53, as documented by sequence analysis and/or by increased p53 accumulation upon etoposide treatment or c-MYC activation (SI Appendix, Fig. S7A and B). To determine whether the antiapoptotic and protective effects of SIRT1 also may be mediated by deacetylation of SIRT1 substrates other than p53, human U937 monoblast cells, which express mutant p53, were analyzed. The SIRT1-specific inhibitor EX527 (35) or sirtinol, which is a synthetic small-molecule inhibitor of SIRT1 and SIRT2 (36), induced apoptosis in U937 cells stably expressing a v-myc gene but not in parental U937 cells (Fig. 4G). As shown for EX527, this effect resulted in reduced proliferation (Fig. 4H and I). Therefore, at least some of the protective effects of SIRT1 observed after c-MYC activation may be mediated via SIRT1 substrates other than p53.

**Role of SIRT1 in c-MYC-Immortalized HDF.** HDFs immortalized by retroviral introduction of constitutive expression of c-MYC (6) showed increased expression of SIRT1 protein (Fig. 5A). We next asked whether c-MYC–induced SIRT1 activity is required to maintain these cells. Only in c-MYC–immortalized but not in primary HDFs down-regulation of SIRT1 by siRNAs induced a pronounced increase in cell size and senescence-associated β-galactosidase activity at pH 6, two markers of cellular senescence (Fig. 5B). Furthermore, inactivation of SIRT1 resulted in an increased fraction of apoptotic cells in c-MYC–immortalized but not of hTERT-immortalized or primary HDFs (Fig. 5C).
Similarly, the addition of sirolin resulted in an increase of apoptosis only in c-MYC–immortalized cells (Fig. 5 D). Taken together, these results suggest that SIRT1 plays a role in the suppression of apoptosis and cellular senescence in cells previously immortalized by c-MYC. Therefore, increased SIRT1 activity may be relevant for the long-term survival and expansion of cancer cells exhibiting deregulation of c-MYC.

**c-MYC Is an SIRT1 Substrate.** As mentioned above, we detected a direct interaction of recombinant or ectopic c-MYC and SIRT1 proteins (Fig. 3 A and B). Coimmunoprecipitation analysis of endogenous proteins confirmed that this interaction also occurs between endogenous c-MYC and SIRT1 (Fig. 6 A). When c-MYC was acetylated by cAMP-response element binding protein (CBP) in vitro and then was exposed to recombinant SIRT1, efficient deacetylation of c-MYC was observed that was dependent on the presence of NAD+ and was blocked by NAM (Fig. 6 B). Treatment of cells with NAM resulted in hyperacetylation of c-MYC, whereas treatment with trichostatin A (TSA), an inhibitor of histone deacetylases, had no effect on c-MYC acetylation.
SIRT1 Stabilizes c-MYC. Acetylation of the c-MYC protein also regulates its ubiquitination and thereby affects the rate of proteasomal degradation (37, 38). In line with a role of SIRT1-mediated deacetylation in the regulation of c-MYC degradation, the c-MYC protein levels in subconfluent sirt1−/− mouse embryo fibroblasts (MEFs) were reduced compared with subconfluent sirt1+/+ MEFs (Fig. 7A), whereas c-myc mRNA expression remained unchanged (Fig. 7B). Accordingly, c-MYC target genes also were expressed at higher levels in the sirt1+/+ MEFs than in SIRT1-deficient MEFs (Fig. 7B). As shown by [35S]methionine pulse-chase analysis and densitometric quantification, ectopic expression of SIRT1 increased the half-life of endogenous c-MYC from 25 to 40 min in MCF-7 cells (Fig. 7C). Unexpectedly, ectopic expression of SIRT1 in combination with cycloheximide (CHX) treatment resulted in increased c-MYC turnover (SI Appendix, Fig. S8A). Therefore, CHX treatment combined with ectopic SIRT1 expression presumably causes a nonphysiological degradation of c-MYC, whereas SIRT1 expression alone increases the half-life of c-MYC. These conclusions also were supported by experiments in which miRNA- or shRNA-mediated down-regulation of SIRT1 reduced the half-life of endogenous c-MYC from 32 to 23 min in U2OS cells (Fig. 7D and SI Appendix, Fig. S8B). Moreover, administration of tenovin-6, a recently described SIRT1 inhibitor (39), reduced the c-MYC half-life from 34 to 25 min in HCT-116 cells (Fig. 7E). As expected, c-MYC had slightly varying half-lives in the different cell lines. Taken together, these results suggest that the half-life of c-MYC is increased by SIRT1-mediated deacetylation.

SIRT1 Promotes Lysine-63-Linked Polyubiquitination of c-MYC. Because acetylation is known to affect the ubiquitination of lysine residues, we tested whether SIRT1-mediated deacetylation modulates ubiquitination of c-MYC. The net amount of polyubiquitinated c-MYC protein was reduced in HCT-116 cells ectopically expressing wild-type c-MYC and HA-tagged ubiquitin after treatment with tenovin 6 (Fig. 8A), despite increased turnover of c-MYC (Fig. 7E). c-MYC is known to be conjugated with both lysine-48 (K48)- and lysine-63 (K63)-linked polyubiquitin chains (40) that mediate proteolytic and non-proteolytic functions, respectively (41). To investigate what types of ubiquitin ligation are affected by SIRT1 inhibition, cells were transfected with HA-tagged K48R- or K63R-ubiquitin mutants. A reduction of polyubiquitinated c-MYC in response to tenovin-6 was observed with the K48R ubiquitin mutant, as with wild-type ubiquitin, but not with the K63R mutant (Fig. 8A). This result suggested that the decrease in c-MYC half-life observed after SIRT1 inhibition may be caused by reduced K63 polyubiquitination, which normally could stabilize c-MYC by competing with K48-linked degradative ubiquitination of lysine residues. Also, after miRNA-mediated reduction of SIRT1 expression in U2OS cells, the total amount of c-MYC–conjugated polyubiquitin as well as non–K48-linked polyubiquitin chains decreased when equal amounts of immunoprecipitated c-MYC were analyzed (SI Appendix, Fig. S8C). Furthermore, mono, bi-, and polyvalences of K63-linked ubiquitin molecules conjugated to c-MYC were diminished as a result of the SIRT1 knockdown (SI Appendix, Fig. S8C). To identify the involved critical lysine residues of c-MYC, a mutant with six lysines (K298, K317, K323, K326, K341, and K365) changed to arginine (Myc-K6R), previously shown to be defective in homologous to E6-AP carboxy-terminus H9 (HectH9)-mediated K63-linked ubiquitination (40), was analyzed. Interestingly, polyubiquitination of the Myc-K6R mutant was reduced after SIRT1 inhibition by Tenovin-6 in a manner similar to wild-type c-MYC (Fig. 8B and SI Appendix, Fig. S8D). The same pattern of polyubiquitination of Myc-K6R and of wild-type c-MYC also was obtained using K48R and K63R ubiquitin mutants in response to Tenovin-6. Therefore, the six lysines mutated in Myc-K6R presumably are not targeted by SIRT1 for deacetylation. In summary, our results suggest that SIRT1-mediated deacetylation increases conjugation of K63-linked ubiquitin chains to c-MYC; these chains do not support degradation but may lead to stabilization of c-MYC by preventing K48-linked degradative ubiquitination.

SIRT1 Increases Transcriptional Activity of c-MYC. To evaluate the functional consequences of SIRT1-mediated deacetylation and stabilization of c-MYC, we measured the transcriptional activity of
c-MYC in reporter assays (Fig. 8C and SI Appendix, Fig. S8 E and F). Coexpression of SIRT1 resulted in a pronounced increase in the transactivation of an E-box-containing reporter construct by c-MYC, whereas a catalytically inactive SIRT1 mutant did not influence the transactivation of c-MYC, presumably by increasing the abundance of c-MYC via interfering with its degradation. In combination with the effects of c-MYC on SIRT1 described above, c-MYC and SIRT1 therefore seem to be connected by a positive feedback loop involving several factors and mechanisms.

**Discussion**

Our results show that the activation of SIRT1 by c-MYC and the subsequent deacetylation of p53 or other SIRT1 substrates provide a mechanism for promoting c-MYC–induced cellular proliferation by suppressing apoptosis and senescence. These findings suggest that endogenous SIRT1 exerts protumorigenic activities in the context of c-MYC–driven tumor development, at least in part because of the ability to prevent or attenuate cellular senescence and/or c-MYC–induced apoptosis. A number of studies link SIRT1 to cancer-relevant substrates, including p53, E2F1, BAX, hypermethylated in cancer (HIC1), and FOXO3 (15). The deacetylation of these proteins by SIRT1 results in inhibition of apoptosis and senescence, and therefore may favor the growth of cancer cells and thereby contribute to tumor growth and progression.
We provide evidence that c-MYC regulates SIRT1 activity by at least two mechanisms: (i) by induction of the NAMPT gene, leading to an increase in the SIRT1 cofactor NAD^+, and (ii) via sequestration of the SIRT1 inhibitor DBC1. This dual regulation results in an increased amount and activation of the SIRT1 protein. The molecular details of SIRT1 stabilization are still unknown, although it has been shown that augmented activity of SIRT1 is accompanied by an increase in the amount of SIRT1 protein (22, 50). In line with this observation, many enzymes accumulate upon activation because they are engaged in stabilizing complexes when binding to substrates (51). In the case of SIRT1, stabilization scenario is a conformational tightening of the ternary complex of the Sir2 apoenzyme with NAD^+ and an acetylated substrate which appears to be more compact than the binary complex and therefore less accessible to proteolytic degradation (52).

NAMPT may stimulate SIRT1 activity via two routes (53): Besides enhancing NAD^+ production, NAMPT also reduces the concentration of cellular NAM, the main endogenous SIRT1 inhibitor. The NAMPT-mediated activation of SIRT1 has been implicated in the regulation of differentiation, stress response, and metabolism and in the promoting the extension of the cellular life span (22, 30). SIRT1 accomplishes these roles by deacetylating of substrates, such as the p53 and PARP proteins, and by protection from PARP-induced and/or p53-dependent cell death (22, 26, 30). Therefore, c-MYC-induced NAMPT expression may contribute to cell proliferation in part by activating SIRT1 and inhibiting various proapoptotic factors. Intriguingly, activation of lymphocytes, which is associated with elevated c-MYC levels, leads to enhanced NAMPT expression (54). Moreover, the subcellular localization of NAMPT was shown to be regulated in a cell cycle-dependent manner (55), indicative of an additional mode of regulation by c-MYC. Furthermore, increased NAMPT expression in tumors correlates with cancer progression and bad prognosis (56, 57). These oncogenic properties of NAMPT are consistent with its induction by c-MYC. NAMPT inhibitors are being tested currently in clinical cancer trials (58). In mouse tumors inhibition of NAMPT affects not only NAD^+ levels but also glycolysis (59). Therefore, in addition to inducing glycolytic enzymes, c-MYC also may enhance glycolysis via the activation of NAMPT and the salvage pathway.

Recently, DBC1 was identified as a negative regulator of SIRT1 (17, 18, 60), but how and in which physiological context the DBC1–SIRT1 interaction is regulated remained elusive. The results shown here suggest that the elevated or constitutive c-MYC expression found in tumor cells may interfere with the DBC1-mediated inhibition of SIRT1 via sequestration of DBC1 by c-MYC, resulting in increased SIRT1 activity and ultimately in elevated c-MYC levels and activity. However, our experiments do not rule out the possibility that the observed effect also may be caused by c-MYC interfering with the inhibitory effect of DBC1 on other enzymes, such as histone deacetylase 3 and SuvH3-9 (61, 62). Intriguingly, a decrease in the interaction between DBC1 and SIRT1 has been found recently in mammary carcinoma cell lines (63), which are known to display elevated c-MYC expression and c-MYC amplifications.

We showed that c-MYC–activated SIRT1 feeds back to c-MYC by deacetylating c-MYC, in turn increasing the stability of endogenous c-MYC. Deacetylation through SIRT1 and inhibiting various proapoptotic factors. Intriguingly, activation of lymphocytes, which is associated with elevated c-MYC levels, leads to enhanced NAMPT expression (54). Moreover, the subcellular localization of NAMPT was shown to be regulated in a cell cycle-dependent manner (55), indicative of an additional mode of regulation by c-MYC. Furthermore, increased NAMPT expression in tumors correlates with cancer progression and bad prognosis (56, 57). These oncogenic properties of NAMPT are consistent with its induction by c-MYC. NAMPT inhibitors are being tested currently in clinical cancer trials (58). In mouse tumors inhibition of NAMPT affects not only NAD^+ levels but also glycolysis (59). Therefore, in addition to inducing glycolytic enzymes, c-MYC also may enhance glycolysis via the activation of NAMPT and the salvage pathway.

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We showed that c-MYC–activated SIRT1 feeds back to c-MYC by deacetylating c-MYC, in turn increasing the stability of endogenous c-MYC. Deacetylation through SIRT1 presumably renders target lysines available for K63-mediated polyubiquitination. It was shown previously that K63-linked ubiquitination of c-MYC is mediated by HectH9 and recruits cofactors such as p300 and thereby enhances c-MYC’s transcriptional activity (40). In accordance with our results, K63-linked ubiquitination mediated by HectH9 has been shown to lead to increased c-MYC function (41). K63-linked ubiquitin chains usually do not promote proteasomal degradation but may result in protein stabilization by replacing degradative K48-linked chains, as recently shown for the c-JUN coactivator RACO-1 (64). The typical half-life of c-MYC is less than 30 min (65, 66), but in cancer c-MYC exhibits an extended half-life (67–69). In some cancers, these high c-MYC levels have been associated with impaired c-MYC turnover through Fbw7/Cdc4 mutation (70). However, so far Fbw7/CDC4 is the only E3 ligase of c-MYC that has been shown to be a target for muta-

Fig. 8. SIRT1 regulates ubiquitination and activity of c-MYC. (A) HCT-116 cells were transiently transfected with wild-type c-MYC together with the indicated HA-tagged ubiquitin constructs. Forty hours after transfection, cells were treated with DMSO or the SIRT1 inhibitor tenovin-6 (10 μM) for 8 h followed by immunoprecipitation of c-MYC and immunoblot detection of HA-ubiquitin using a monoclonal HA-specific antibody (12CA5). Immunoprecipitated c-MYC was detected using a monoclonal c-MYC-specific antibody (C33). (B) Densitometric analysis of c-MYC ubiquitination. As in A, HCT-116 cells coexpressing wild-type c-MYC or the Myc-K8R were cotransfected with wild-type ubiquitin or mutant ubiquitin (K48R-Ub, K63R-Ub) constructs. The signal intensities of the blots were quantitated with a CCD camera. (C) HEK-293 cells were cotransfected with the M4-min-tk–luc reporter construct (38) with four MYC/Max binding sites and expression plasmids encoding c-MYC, c-MYC-K323R, HA-SIRT1, and HA-SIRT1-H363Y. Mean values of three independent experiments performed in duplicate are shown. Western blot analysis of the indicated proteins detected in reporter assay lysates is shown in the lower panel. (D) qPCR analysis of the c-MYC target gene DKC 11 h after activation of a tetracycline-regulatable c-MYC allele in P493-6 B cells stably expressing pNCo-SIRT1 or the vector backbone pNCo as a control. Error bars represent SD of biological triplicates.
tion al inactivation, albeit with low frequency. Therefore, the increased half-life of c-MYC resulting from SIRT1-mediated deacetylation may explain the elevated c-MYC protein levels found in certain tumor types and may contribute to tumor formation. Our results are in contradiction to recently published work by You et al. (42), because we did not observe a direct induction of SIRT1 mRNA expression by c-MYC or a negative feedback inhibition of c-MYC by SIRT1. In our study, we used multiple experimental systems and analyses that generally are accepted as required for the confirmation of direct regulation by c-MYC in the field (71, 72). However, none of the different systems used here revealed an induction of SIRT1 mRNA following c-MYC activation. Furthermore, the published microarray or serial analysis of gene expression studies investigating transcriptional regulation by c-MYC did not reveal a significant mRNA induction of SIRT1 by c-MYC (73, 74). However, this result does not exclude the possibility that c-MYC could affect SIRT1 mRNA expression indirectly under certain conditions. Another result conflicting with the results reported by Yuan et al. (42) is the influence of SIRT1 on c-MYC stability. Although Yuan et al. found that enforced SIRT1 expression increases the rate of c-MYC turnover, our results suggest that SIRT1 stabilizes c-MYC. This discrepancy likely arises from the different experimental approaches. Furthermore, the half-lives of c-MYC reported by Yuan et al. in the absence of SIRT1 deviate substantially from those reported in the literature (ca. 3 h vs. 20–40 min (65, 66)), whereas our results are well within the range of previously published data (67–69). When we compared half-life measurements of c-MYC in the presence of ectopic SIRT1 expression, we found that results obtained after CHX treatment oppose those obtained by SS pulse-chase experiments. Therefore, we suspect that CHX treatment in this context causes an artificial degradation of c-MYC. Although Yuan et al. (42) consistently used CHX treatment and found a negative effect of SIRT1 on c-MYC half-life, our SS pulse-chase analyses show that SIRT1 increases the half-life of c-MYC. The latter results are consistent with the increased steady-state levels and transcriptional activity of c-MYC we observed in response to increased SIRT1 expression and with the reduced c-MYC stability caused by knockdown, enzymatic inhibition, or deletion of SIRT1. Furthermore, in our hands mutation of K323 in c-MYC did not affect SIRT1-mediated changes in c-MYC stability and c-MYC–driven transcription. Yuan et al. (42) found that mutation of K323 influences c-MYC stability and transactivation. The reason for these discrepancies will have to be resolved in the future.

Our results support a tumor-promoting function of SIRT1 in the context of c-MYC activation. Other studies also have provided evidence for a protumorigenic function of SIRT1 (39, 45, 75–77). For example, the tumor suppressor HIC1, which is epigenetically silenced in cancer, functions as an inhibitor of SIRT1 expression (75). Furthermore, SIRT1 inhibitors have antitumor activity in vivo (39, 45). Nonetheless, recent mouse tumor models argue for a tumor-suppressive role of SIRT1 (78). Since these mouse models did not represent c-MYC–induced tumors, it will be important to analyze the role of SIRT1 in MYC-driven tumor models and to clarify this point in the future. It should be mentioned, however, that at present there is no evidence for genetic and epigenetic alterations affecting SIRT1 in cancer. Therefore, the SIRT1 gene itself does not seem to represent a proto-oncogene or a tumor-suppressor gene. Only recently it has been shown, that SIRT1 has species-specific functions that also are dependent on the genetic background (21, 79). Therefore, it seems that SIRT1 is involved in oncogenic or tumor-suppressive signaling in a species-, tumor type-, and context-dependent manner. In the future comparative analysis of mouse and human SIRT1 functions may reveal explanations for the ambivalent role of SIRT1 in the pathogenesis of human and mouse tumors.

Taken together, the positive feedback loop linking c-MYC, NAMPT, DBC1, and SIRT1 described here suggests that therapeutic inhibition of NAMPT or SIRT1 enzymatic activities may be a suitable approach to sensitize human cancer cells with deregulated expression of c-MYC, regardless of their p53 status.

**Methods**

**Cell Culture and Conditional Systems.** P493-6 cells, RAT1 (TGR-1), and c-Myc−/−RAT1 (HO15.19) fibroblasts were maintained as described previously (74). U2OS cells, MCF-7 cells, MCF-7 cells with an inducible c-MYC allele (PJMMR1), MEFs (sirt−/−, +/+), HDF cells, and c-MYC-immortalized HDF cells were kept in DMEM containing 10% (vol/vol) FBS. c-Myc−−/−RAT1 (subclone HO15.19) stably expressing Myc-ER were kept in phenol-red free DMEM supplemented with 8% (vol/vol) FBS. HDF-Myc-ER (immortalized by hTERT) cells were grown in phenol-red free DMEM and 10% (vol/vol) FBS. To down-regulate endogenous c-MYC in the PJMMR1 (MCF-7) cell line, the antiestrogen ICI 182,781 (1 μM) was added to the cells for 60 h before activation of DOX-inducible c-MYC.

**RNA Interference.** To generate pEHi vectors targeting DBC1 or SIRT1, the specific hairpins from the pSM2c library (80) were subcloned into the pEHi vector as described previously (81). siRNAs were transfected at a final concentration of 5–40 nM using the fast-forward protocol (Qiagen). Sequences of shRNAs, miRNAs, and siRNA oligonucleotides are available on request.

**Western Blot Analysis.** Cells were lysed in RIPA buffer (50 mM Tris HCl (pH 7.4), 1% Nonidet P-40, 0.1% SDS, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, and protease inhibitor mixture (Complete Mini; Roche). To detect p53 acetylation, NAM (5 mM) and TSA (1 μM) were added to the lysis buffer. The signals were obtained with enhanced chemiluminescence reagent (Perkin-Elmer) and recorded with a 440CF imaging system (Kodak). Antibodies used are given in SI Appendix, SI Methods. All other methods, further details, and associated references are provided online in SI Appendix, SI Methods.

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Supporting Information Appendices

Supporting Information Corrected January 16, 2012

The c-MYC oncoprotein, the NAMPT enzyme, the SIRT1-inhibitor DBC1, and the SIRT1 deacetylase form a positive feedback loop

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Supplementary Methods

Cell culture and treatments

HEK293 Flp-In™ T-REx™ cells (Invitrogen) were stably transfected with pcDNA5/FRT/TO. HCT116 cells were kept in McCoy's medium with 10% (vol/vol) FBS. LS-174T were cultured in RPMI-1640 with 5% (vol/vol) FBS and human U937 (myc6 clone expressing the OK10 v-myc gene and parental GTB) monoblasts in RPMI-1640 with 10% (vol/vol) FBS. 293 cells were grown in DMEM medium supplemented with 5% (vol/vol) FBS. Nicotinamide (NAM), 2-deoxyglucose (DG) and trichostatin A (TSA) were purchased from Sigma, Sirtinol from Alexis Biochemicals, EX527 from Tocris Bioscience and FK866 from Axon Medchem. Tenovin-6 was a kind gift from Sonia Lain, University of Dundee.

Co-immunoprecipitation of endogenous proteins

Co-immunoprecipitation were performed with U2OS cell lysates as described before (1). After pre-blocking, immunoprecipitation was carried out for 5 hours at 4°C, followed by 3 times washing with IP buffer at 4°C.

Cycloheximide chase analysis

Subconfluent HCT116 cells were treated with Tenovin-6 (10 µM) or DMSO for eight hours, followed by cycloheximide (100 µg/ml) treatment and lysis in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.5% NP-40, 1 mM Na3VO4, 2 µg/ml aprotinin, 1 µg/ml PMSF, and 1 µg/ml leupeptin). A c-MYC-specific rabbit polyclonal antibody (N262, sc-764, Santa Cruz) was used for immunoprecipitation, followed by immunoblot analysis using a c-MYC-specific, mouse monoclonal antibody (C33, sc-42, Santa Cruz).

Deacetylation assay

Cellular sirtuin deacetylase activity was determined with a HDAC Fluorescent Activity Assay/Drug discovery Kit (AK-500, BIOMOL). 5 x 10^5 suspension cells (P493-6) were incubated with a synthetic, acetylated substrate coupled to a fluorophore (Fluor-de-Lys: FdL) for 2 and 4 hours. To measure only SIRT1 mediated deacetylation, activity of other HDACs was blocked by TSA (1 µM) during substrate incubation and in buffers. After centrifugation cell pellets were extracted, a developer was added and the deacetylated substrate was measured at 360 nM excitation and 460 nM emission.

Detection of apoptosis by DNA content analysis

For analysis of DNA content by flow cytometry, supernatants were collected and combined with trypsinized and washed cells. Cells were fixed overnight in 70% ethanol on ice, washed and stained with propidium iodide (50 µg/ml), RNase A (0.2 µg/ml) and Triton-X 100 (0.1%) in PBS.

Determination of cellular NAD+/NADH concentration

Intracellular NAD+ and NADH concentrations were determined as described previously (2). Sub-confluent RAT1-Myc-ER cells cultured in one 14 cm² plate were
lyzed in 2 ml of lysis buffer (100 mM NaHCO$_3$, 20 mM Na$_2$CO$_3$, 10 mM nicotinamide and 0.05 % Triton X-100). This neutral buffer allowed the extraction of both NAD$^+$ and NADH in a single procedure. Only NADH is heat stable, therefore half of each lysate was incubated for 30 minutes at 60°C to selectively decompose NAD$^+$. Samples were centrifuged at 16,000g, 4°C for 5 minutes and kept on ice in the dark thereafter. For the cycling reaction, 50 µl of the sample (or the respective standard diluted in lysis buffer) was mixed with 840 µl buffer containing 100 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.5 mM MTT (3-[4.5 dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide), 0.2 mg/ml alcohol dehydrogenase (ADH) and 10 µl of 200 mM phenazine ethosulfate. After 5 minutes incubation at 25°C, 100 µl of 6 M ethanol was added and the sample was centrifuged at 16,000 g for 30 seconds, followed by immediate fluorimetric measurement of the nucleotide concentration in cuvettes: the absorbance at 570 nM was determined in 10 sec intervals for 2 minutes at room temperature using a spectrophotometer (DU 530; Beckman). For each assay a fresh NAD$^+$ and NADH standard solution was prepared. The nucleotides were quantified in the linear range of the respective standard curve. A Lineweaver–Burk plot was applied to determine the enzyme kinetics (plotted 1/dE and log (1/nM)). Aliquots of the standards, which were within the concentration range of cellular nucleotides, were also analyzed after 30 minutes of incubation at 60°C to confirm heat decomposition of NAD$^+$ and integrity of NADH, respectively. The NAD$^+$ concentration was calculated by subtraction of the yield of NADH (heated sample) from the amount of the total NAD$^+$ plus NADH (non-heated sample). The protein concentration of the lysates was determined with Bradford reagent.

For the analyses shown in Figure S4, NAD$^+$ and NADH levels were determined as described above with some modifications. Cell lysates were harvested by scraping cells in lysis buffer, protected from light throughout the assay and immediately frozen at -80°C. Before heating of one aliquot and analysis, lysates were subjected to a short sonication step, centrifuged at 16,000g for 15 min (4°C) and analyzed in duplicates by the cycling reaction in a 96 well format within 20 minutes: 20 µl lysate, 140 µl cycling buffer (0.3 mg/ml ADH) and 40 µl 3M ethanol were used. The value for delta extinction (dE) was obtained from the measurements before, and 15 minutes after incubation in the dark at room temperature. The protein concentration was also determined in duplicates from the non-heated samples by BCA analysis. All chemicals were purchased from Sigma.

**GST pull-down assay and in vitro translation**

For the in vitro translation the manufacturer’s protocol for the TNT Quick coupled transcription/translation system (Promega) was used. Fusion proteins were prepared as previously described (3). For the binding reactions, 5 µg of the GST fusion protein was bound to glutathione–agarose beads and incubated with $^{35}$S-methionine-labeled in vitro transcribed and translated c-MYC protein in binding buffer.

**Immunoblot analysis**

Cells were lysed in RIPA buffer (50 mM Tris-HCL pH 7.4, 1% NP-40, 0.1% SDS, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na$_3$VO$_4$, protease inhibitor cocktail (Complete Mini, Roche)). For detection of p53 acetylation...
nicotinamide (5 mM) and TSA (1 µM) were added to the lysis buffer. The signals were obtained with enhanced chemiluminescence reagent (Perkin Elmer), and recorded with a 440CF imaging system (Kodak) or the LAS3000 imaging system. The following antibodies were used: rabbit polyclonal antibody specific for specific for acetylated lysines, acetylated p53 (Lys 382), SIRT1 (Cell Signaling Technology), rat or mouse monoclonal antibody specific for HA-epitope (Roche, Convance), mouse anti-c-MYC (9E10), monoclonal mouse p21 antibody (Neomarkers), rabbit polyclonal antibody specific for acetylated p53 (Lys 373/382), SIRT1-specific mouse monoclonal antibody clone 2G1/F7 and rabbit polyclonal anti-SIRT1 for mouse and rat SIRT1 (Upstate Biotechnology), rabbit polyclonal anti-β-actin (Sigma), rabbit serum against SIRT1 (kind gift from Roy Frye). Puma, human SIRT1 (C-terminus), and c-MYC (N-terminus) were detected using rabbit monoclonal antibodies (Epitomics). The rabbit polyclonal antibodies specific for CBP (A22), c-Myc (N262, SC764), GFP (sc-8334) and SIRT1 (H300, sc-15404), as well as the mouse monoclonal antibodies directed against p53 (1801, DO1) and α-tubulin (sc-8035) were purchased from Santa Cruz Biotechnology.

**Immunohistochemistry**

15 non-familial colorectal carcinoma cases were selected without bias. None of the patients had undergone cancer therapy before surgical resection of the lesions. The human colorectal cancer specimens were obtained from surgery patients with informed consent. For staining of c-MYC and SIRT1, sections were de-paraffinized and pretreated by microwave (750 W, 2 × 15 min) in TUF target unmasking fluid (DAKO) for c-MYC and in ProTaqs II Antigen-Enhancer (Quartett, Berlin) for SIRT1. Sections were covered with hydrogen peroxide at a final concentration of 7.5% (vol/vol) for 10 minutes and then blocked with serum (Vectastain ABC Kit Universal: PK-6200) incubated with primary antibodies (c-MYC (N-term.) specific rabbit monoclonal (Epitomics: 1472-1)), 1:20 dilution; SIRT1 (C-term.) specific rabbit monoclonal (Epitomics: 1104-1), 1:100 dilution) for 60 min. For detection a biotinylated secondary antibody was used (Vectastain: ABC Kit Elite Universal). Samples were treated with chromogen (AEC Zymed) for 10 min and counterstained with hematoxylin Gill’s Formula (Vector).

**Immunoprecipitation for detection of acetylated c-MYC**

Whole-cell lysates were prepared in F buffer (10 mM Tris-HCl, pH 7.05, 50 mM NaCl, 30 mM Na₄P₂O₇, 50 mM NaF, 5 µM ZnCl₂, 100 µM Na₃VO₄, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 5 units/ml α2-macroglobulin, 2.5 units/ml pepstatin A, 2.5 units/ml leupeptin, 0.15 mM benzamidin). For immunoprecipitation anti-FLAG M2–agarose-slurry (Sigma) was used.

**Indirect immunofluorescence analysis**

Cells were grown on glass slides and fixed for 10 minutes in 4% (w/vol) paraformaldehyde. After washing, the cells were permeabilized in PBS with 0.2% Triton X-100 for 15 minutes. After blocking in 100% FBS for 30 minutes, the primary antibodies were added for 1 hour. For detection of acetylated p53 (Lys 382) a rabbit
polyclonal and for detection of the HA-epitope a mouse monoclonal antibody was used. Slides were washed and the respective secondary antibodies were applied for 30 minutes. After a final wash step, slides were embedded in Glycerin/DAPCO and analyzed using an inverted microscope (Axiovert 200M, Zeiss), CCD-camera (Coolsnap HQ, Photometrics), and Metamorph software (Universal Imaging Corporation).

**In vitro c-MYC deacetylation assay**

Purification of His–CBP was performed as described previously (4). Purified MBP–MYC (3 μg) was incubated in 30 μl of HAT buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 10 mM sodium butyrate) with 50 ng of purified His–CBP and 1 μl [14C]acetyl-coenzyme A (55 mCi/mol; NEN) for 1 hour at 30°C. TAP-SIRT1 bound calmodulin-beads were washed in deacetylase buffer (50 mM HEPES pH 7.5, 10 mM MgCl2, 10 mM NaF, 10 mM β-glycerophosphat, 0.2 mM DTT, 25 μM ATP). TAP-purified SIRT1 and recombinant MBP-c-MYC were resuspended in 50 μl SIRT1 deacetylase buffer containing 1 mM NAM (Sigma). The reactions were performed at 37°C for 60 minutes. The acetyl-group of O-acetyl-ADP-ribose was hydrolyzed by adding 15 μl 1 N NaOH at room temperature for 20 minutes, followed by adding 185 μl 0.1 M HCl and 0.16 M acetic acid. Released [3H]-acetate was extracted in 750 μl ethyl acetate and measured with a scintillation counter (Perkin Elmer 1409DSA).

**List of qPCR primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
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<td>5'-CATAGACACGCTGGAACAGG-3'</td>
</tr>
<tr>
<td>SIRT1_hu_rev1</td>
<td>5'-GCAGATGAGGCAAGGTT-3'</td>
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<tr>
<td>SIRT1_hu_frw2</td>
<td>5'-GATTGGCAGATCCTCGAAC-3'</td>
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<td>SIRT1_hu_rev3*</td>
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<tr>
<td>ELF1a_rat_rev</td>
<td>5'-CACGAACAGCAAAACGACCA-3'</td>
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</table>

β-actin primers were used for normalization of mRNA derived from human cell lines, ELF1α primers were used for normalization of mRNA derived from rat cell lines.

* Primer sequences match to the 3'UTR.
Plasmids

Plasmids used were: pVR1012–Gal4–CBP, M4–min-tk–luc (a fusion of four MYC/MAX binding sites (M4) to min–tk luc reporter), hTERT reporter, β-galactosidase expression vector pEQ176, GW-HA-SIRT1, GW-HA-SIRT1H363Y, pGEX5x3-DBC1, pcDNA3-FLAG-c-MYC, pcDNA3-Flag-c-MYC-K323R, pcDNA3-Flag-c-MYC-K323R, pCW7-Ubiquitin, pcW7-K48R-Ubiquitin, pcDNA3-HA-Ubiquitin, pcDNA3-HA-K48R-Ubiquitin, pcDNA3-HA-K63R-Ubiquitin (HA-Ubiquitin constructs were kindly provided by Dr. Aristides Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden), the episomal pRTS (5) vectors pRTS-c-MYC and pRTS-DBC1HA, and the retroviral vectors pBABE-c-MYC-ER, pDON-SIRT1-VSV and pINCO-SIRT1.

RNA interference

For generation of pEMI vectors targeting DBC1 or SIRT1, the respective miRNAs surrounded by miR-30 hairpins derived from the pSM2c library (6) were sub-cloned into the pEMI vector as described previously (7). siRNAs were transfected at 5 - 40 nM final concentration using the fast forward protocol (Qiagen). 6 µl HiPerFect reagent (Qiagen) were used per 500 µl medium in 24 well plates to transfect cells immediately after seeding. Sequences of shRNAs, microRNAs and siRNA oligonucleotides are available on request.

35S-pulse chase analysis

For 35S-methionine in vivo cell labeling, cells were kept in methionine-free RPMI-1640 medium containing 0.15 mCi of 35S-methionine per 5 x 10^6 cells for 35 minutes, followed by chase in RPMI-1640 medium containing cold methionine. Cells were lysed in high stringency AB lysis buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 0.5% NP-40, 0.5% DOC, 0.5% SDS, 0.5% Trasylol, 1 mM EDTA) and subjected to immunoprecipitation with antibodies specific for the proteins of interest. An equal number of TCA-precipitable counts (for 35S-labeled proteins) was used for each sample. Immunoprecipitated proteins were washed and analyzed by SDS-PAGE followed by analysis on a phospho-imager (Fuji).

TAP (tandem-affinity-purification) of SIRT1

HEK293 were stably transfected with a construct expressing a tet-inducible TAP-SIRT1. SIRT1 expression was induced by adding doxycycline and cells lysed after 5 hours in TAP-lyses buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM dithiothreitol (DTT), 1mM EDTA and protease inhibitors). Lysates were cleared by centrifugation at 12,500 rpm for 10 minutes. The supernatants were incubated with IgG-Sepharose (Amersham Biosciences) for two hours at 4°C, washed with lysis buffer and TEV buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol (DTT)). Beads were resuspended in TEV buffer and cleaved with TEV protease for 4 hours. TEV-cleaved proteins were incubated with calmodulin beads and incubated for two hours at 4°C.
Ubiquitination assay

U2OS cells expressing DOX-inducible pEMI vectors encoding a non-silencing or SIRT1-specific microRNA were treated with DOX (0.2 μg/ml). The next day cells were transiently transfected with wild type (wt), or K48R-ubiquitin constructs followed by incubation for 48 hours. Two hours before lysis cells were treated with 50 μM MG115. Cells were harvested in NP-40 lysis buffer (20 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 2 mM EDTA pH 8, 1 mM PMSF, 1 mM Na3VO4, 0.5 mM NaF, 1% SDS, protease inhibitor cocktail (Roche), N-ethyl maleimide). For immunoprecipitations lysates were diluted in 0.1% SDS and incubated with a c-MYC-specific antibody (N262, Santa Cruz). Modified c-MYC proteins were detected using anti-Ub (clone FK2; Affinity) or anti-K63-Ub (clone HWA4C4; Biomol) antibodies.

Subconfluent HCT116 cells were transiently transfected with wild type (wt) or K6R-MYC constructs together with HA-tagged wild type (wt), K48R- or K63R-ubiquitin constructs followed by incubation for 48 hours. Procedures for treatment, lysis and immunoprecipitations were carried out as for U2OS cells. Modified c-MYC proteins were detected using anti-HA (clone 12CA5; Roche) antibodies.
Supplementary Methods References:

Fig. S1. Post-transcriptional induction of SIRT1 after c-MYC activation.
(A) P493-6 cells harboring a tet-repressor-regulated c-MYC allele were kept in the absence (c-MYC on) or presence of tetracycline (c-MYC off) in medium containing 10% (vol/vol) FBS. After 72 hours cell lysates were subjected to immunoblot analysis of the indicated proteins.
(B) After propidium iodide staining, the DNA content of P493-6 cells (treatment as in (A)) was analyzed by flow cytometry. DNA content analysis confirmed a G1-arrest.
(C) qPCR analysis of c-MYC target genes and SIRT1 expression after c-MYC activation. mRNA was extracted at the indicated time points. Treated cells and untreated controls were harvested simultaneously. Fold changes were calculated relative to the untreated controls, which were set to 1 (not depicted). Expression of β-actin was used for normalization. LS-174T colorectal cancer cells were treated with 1 µg/ml DOX to activate the expression of c-MYC-specific shRNA (as in Fig. 1D). Human diploid fibroblasts (HDF) were serum-starved at 0.1% FBS for 48 hours and then re-stimulated with 10% (vol/vol) FBS for 12 hours to induce endogenous c-MYC. P493-6 cells (see A) were treated with tetracycline for 72 hours. RNA was isolated at the indicated time-points after tet-removal. HUVEC endothelial cells were infected with a c-MYC or GFP encoding adenoviral vector (74). Twelve hours after transfection cells were lysed and RNA was analyzed. Expression of c-MYC target genes and SIRT1 was determined as described in Fig.1B,C. SIRT1 was quantified with three different primer pairs (p1,2,3).
(D) H015 (c-myc -/-) and TGR (c-myc +/+ ) RAT1 fibroblasts were analyzed during exponential growth or after serum-starvation and subsequent re-stimulation with 8% (vol/vol) FBS for the indicated periods (as in Fig. 1E). RNA of exponentially growing, serum starved and serum-stimulated cells was isolated at the indicated time-points and analyzed by qPCR with the indicated SIRT1-specific primer pairs (p1, p2) as described in Fig. S1C. Expression of ELF1α was used for normalization. SIRT1 expression of exponentially growing HO15.19 and starved cells was set to one and used as a reference (not depicted).

(E) MCF7 cells stably expressing SIRT1-GFP (left panel) or GFP (right panel) were serum-starved at 0.05% FBS for 48 hours and re-stimulated with 10% (vol/vol) FBS for the indicated periods. Expression of the indicated proteins was determined by Western blot analysis.
**Fig. S2.** Expression of c-MYC and SIRT1 protein in colorectal cancer and normal colonic epithelium. Representative immunohistochemical analyses of sections derived from 3 colorectal biopsies representing colorectal cancer (CRC) or tumor adjacent, normal colonic mucosa with antibodies directed against c-MYC and SIRT1 (magnification: 200x).
**Fig. S3.** Induction of SIRT1 activity by c-MYC
SIRT1 activity was determined in P493-6 cells in the absence (MYC ON) or presence (MYC OFF) of tetracycline. Endogenous c-MYC was down-regulated by serum starvation at 0.1% FBS for 48 hours. To detect SIRT-mediated deacetylation, a synthetic, acetylated substrate (Fluor-de-Lys: FdLys), coupled to a fluorophore was added to cells treated with TSA (1 µM) for the indicated periods. The emission of the deacetylated substrate (given as arbitrary fluorescence units (AFU)) was measured after addition of a developer as described in the HDAC Fluorescent Activity Assay/Drug discovery Kit (AK-500, BIOMOL).
Fig. S4. Effects of NAMPT and glycolysis inhibition on cellular NAD\(^+\) and NADH concentrations. (A) NAD\(^+\) and (B) NADH concentrations were determined as described previously (85); for details see SI Methods) in RAT 1 c-myc \(-/-\) fibroblasts stably expressing c-Myc-ER. After serum-starvation for 48 hours in 0.2% FBS, c-Myc-ER was activated for 18 hours by addition of 4-OHT (500 nM) (+). Ethanol was added to control cells (-). 2-deoxyglucose (2-DG; 10 mM), FK866 (5 nM) or vehicle (DMSO) was added to the cells for 18 hours, concomitantly with 4-OHT or ethanol as indicated. For determination of NAD\(^+\) and NADH, lysates were prepared and subjected to the cycling reaction. The graphs represent mean values +/- SD of 3 independent experiments that were measured in duplicates.
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Fig. S5B; Menssen et al.
Fig. S5. Confirmation of c-MYC occupancy at the NAMPT locus
Public data obtained by ChIP-seq analysis deposited at the ENCODE website was analyzed using the UCSC genome bowser. The identity of the analyzed cell lines is indicated. Peaks represent signals obtained after immunoprecipitation of c-MYC and subsequent sequencing of the co-precipitated DNA. In addition, the distribution of the histone marks H3K4Me1 and H3H4Me3, which are indicative of active promoters and enhancers, is shown in Fig. S5A (upper rows). In B the distribution of H3K27 acetylation, which indicates transcriptional activity, is depicted in the top row. Interestingly, these histone marks overlap with c-MYC binding in the NAMPT promoter region. TSS: transcriptional start site; Human assembly sequences: hg18, March 2006 (NCBI36/hg18); hg19: February 2009 (GRCh37/hg19).
**Fig. S6. Effect of DBC1 knockdown on c-MYC-induced proliferation**

Activation of c-MYC-ER in human diploid fibroblasts (HDF) after transfection of DBC1-specific siRNAs. Triplicates of cells were serum starved for 48 hours, stimulated with 4-OHT and subjected to real-time impedance measurements using an xCELLigence device (Roche). 4-OHT was added 12 hours after cells were seeded. This time point was used for cell index normalization. The cell index represents relative cellular impedance, which generally corresponds to cell numbers. The corresponding Western blot analysis is shown in Figure 4F.
Fig. S7. Analysis of p53 induction.
(A) c-MYC-immortalized HDF were treated with etoposide (20 µM) for the indicated periods and protein expression was determined by Western blot analysis.
(B) RAT1 c-myc−/− (HO15) c-MycER cells were treated with 4-OHT for the indicated periods and protein expression was determined by Western blot analysis.
Fig. S8. Analyses of the effects of SIRT1 on c-MYC ubiquitination and transactivation.

(A) MCF7 cells stably expressing a SIRT-VSV fusion protein (right 4 lanes) or the vector backbone (left 4 lanes) were analyzed. Cells were treated with CHX (cycloheximide) for the indicated periods (minutes) before cell lysates were prepared. c-MYC was immunoprecipitated with a polyclonal c-MYC-specific antibody (N262) and then detected by immunoblot analysis with a monoclonal c-MYC antibody (C33). The densitometric quantification of the remaining protein expressed as percentage of the starting amount is shown in the diagrams (right panel).

(B) MCF7 cells stably expressing a SIRT1-specific shRNA (left 4 lanes) or the vector backbone (pRS; right 4 lanes) were analyzed. Cells were pulse-labeled with 35S-methionine followed by chase in medium containing cold methionine for the indicated periods (minutes). Cell lysates were subjected to immunoprecipitation with anti-MYC antibody (N262) and subjected to SDS-PAGE followed by quantification of the c-MYC-derived signal with a phospho-imager. The densitometric quantification of the remaining protein expressed as percentage of the starting amount is shown in the diagrams (right panel).

(C) U2OS cells co-expressing wild-type ubiquitin (wt-Ub) or mutant ubiquitin (K48R-Ub) and the indicated miRNAs from DOX-inducible pEMI vectors were treated with DOX (0.1 μg/ml). After 72 hours lysates were prepared and subjected to immunoprecipitation of endogenous c-MYC and immunoblot analysis using an anti-Ubiquitin antibody (clone FK2). Right panel: The same analysis as in the left panel was performed using antibodies recognizing K63-linked ubiquitin molecules.

(D) HCT116 cells were transiently transfected with plasmids encoding wild-type c-MYC or the c-MYC-K6R mutant (40), in which the lysines 298, 317, 323, 326, 341 and 355 were changed to arginine (kind gift from Martin Eilers, University of Würzburg, Germany). Cells were co-transfected with the indicated HA-tagged ubiquitin constructs. 40 hours after transfection, cells were treated with DMSO or the SIRT1 inhibitor Tenovin-6 (10 μM) for 8 hours followed by immunoprecipitation of c-MYC and immunoblot detection of HA-ubiquitin using a monoclonal HA-specific antibody (12CA5). Immunoprecipitated c-MYC was detected using a monoclonal c-MYC-specific antibody (C33; lower panel). Results of the densitometric quantification is shown in Fig. 8B.

(E) HEK293 cells were co-transfected with an hTERT–luc reporter construct containing two MYC binding sites and expression plasmids encoding c-MYC, HA-SIRT1 and HA-SIRT1-H363Y. Results of two representative experiments performed in duplicates are shown with mean values and standard deviations.

(F) HEK293 cells were co-transfected with the M4–min-tk–luc reporter construct (38) containing four MYC/MAX binding sites and expression plasmids encoding c-MYC, HA-SIRT1 and HA-SIRT1-H363Y. Results of a representative analysis performed in triplicates are shown with mean values and standard deviations.

(G) HCT116 p53-/- cells were infected with a pRetroSUPER vector encoding SIRT1-specific shRNA (shSIRT1) or the empty vector (pRS). Three shSIRT1-expressing clones and 2 control clones were isolated. Exponentially proliferating cells were subjected to Western blot analysis of the indicated proteins.
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**Table S1.** Determination of cellular NAD⁺ and NADH concentrations after c-MYC activation.

The table provides the raw data of 3 independent measurements of NAD⁺ and NADH concentrations obtained with RAT1 c-myc -/- c-MycER fibroblasts after c-MycER activation by 4-OHT (for details see legend of Fig. 2 and SI Methods).