Suppression of Deacetylase SIRT1 Mediates Tumor-Suppressive NOTCH Response and Offers a Novel Treatment Option in Metastatic Ewing Sarcoma


Abstract

The developmental receptor NOTCH plays an important role in various human cancers as a consequence of oncogenic mutations. Here we describe a novel mechanism of NOTCH-induced tumor suppression involving modulation of the deacetylase SIRT1, providing a rationale for the use of SIRT1 inhibitors to treat cancers where this mechanism is inactivated because of SIRT1 overexpression. In Ewing sarcoma cells, NOTCH signaling is abrogated by the driver oncogene EWS-FLI1. Restoration of NOTCH signaling caused growth arrest due to activation of the NOTCH effector HEY1, directly suppressing SIRT1 and thereby activating p53. This mechanism of tumor suppression was validated in Ewing sarcoma cells, B-cell tumors, and human keratinocytes where NOTCH activation of the NOTCH effector HEY1, directly suppressing SIRT1 and thereby activating p53. This mechanism of tumor suppression was validated in Ewing sarcoma cells, B-cell tumors, and human keratinocytes where NOTCH dysregulation has been implicated pathogenically. Notably, the SIRT1/2 inhibitor Tenovin-6 killed Ewing sarcoma cells in vitro and prohibited tumor growth and spread in an established xenograft model in zebrafish. Using immunohistochemistry to analyze primary tissue specimens, we found that high SIRT1 expression was associated with Ewing sarcoma metastasis and poor prognosis. Our findings suggest a mechanistic rationale for the use of SIRT1 inhibitors being developed to treat metastatic disease in patients with Ewing sarcoma. Cancer Res; 74(22): 1–11. ©2014 AACR.

Introduction

NOTCH signaling is an evolutionary conserved pathway involved in tissue patterning and cell specification during normal development. It is initiated following interaction of a cell surface expressed ligand (JAG1, JAG2, DLL1, 3 and 4) with a transmembrane monomeric NOTCH receptor (NOTCH1–4). Binding of the ligand is followed by two successive proteolytic cleavage steps catalyzed by TNFα-converting enzyme and the presenilin-γ secretase complex that release the NOTCH intracellular domain (NICD) to the cytoplasm. Upon translocation to the nucleus, NICD activates the transcription factor CSL. The amplitude and duration of the NOTCH response are regulated by acetylation of NICD on specific lysine residues (1). Only few CSL targets are known, most prominently the HES and HEY family of transcriptional repressors. In many mammalian cell types, the NOTCH pathway enhances stem cell potential and suppresses differentiation, whereas in others, it exerts an opposite role suppressing tumor development (2). Oncogenic NOTCH pathway activation by mutation occurs in many cancers including T-cell leukemia and a variety of solid tumors including breast, colorectal, ovarian, and non–small cell lung cancers. Accordingly, a number of pharmacologic NOTCH inhibitors are currently in early clinical development. However, the consequences of activated NOTCH signaling are cell-type–specific and there is a growing list of tissues and neoplasms in which NOTCH activation has a tumor-suppressive effect, including keratinocites, tumors of the prostate, liver, skin, lung, gastrointestinal stromal tumors, a wide range of B-cell malignancies, and in Ewing sarcoma (3). Currently, the mechanisms of tumor-suppressive NOTCH signaling remain unknown.

Ewing sarcoma pathogenesis is driven by the chimeric ETS oncogene EWS-FLI1. EWS-FLI1 acts as an aberrant oncogenic transcription factor with both activating and repressive gene regulatory properties (4). We have previously...
reported that EWS-FLI1 represses JAG1 expression keeping NOTCH signaling off (5). We found that silencing of EWS-FLI1 results in activation of tumor cell autonomous NOTCH signaling, leading to a strong transcriptional induction of HEY1, which was paralleled by activation of the tumor suppressor TP53 and consequently upregulation of the cell-cycle inhibitor p21 and cell-cycle arrest (3). We showed that HEY1 was sufficient to elicit a TP53 response in Ewing sarcoma cell lines, but the mechanism of TP53 activation remained unknown.

We here demonstrate that knockdown of EWS-FLI1 and induction of HEY1 result in TP53 acetylation. Carboxy terminal acetylation by p300 was demonstrated to be essential for TP53 transcriptional activity (6). Consequently, factors that lead to deacetylation of TP53 interfere with TP53 stress response (7). We now report SIRT1 as a HEY1-repressed deacetylase preventing TP53 acetylation downstream of suppressed NOTCH signaling in Ewing sarcoma and other tissues in which NOTCH acts tumor suppressive.

Sirtuins are an evolutionary highly conserved protein family homologous to yeast silent information regulator 2 (sir2) and link cellular metabolism to tissue homeostasis and differentiation. Sirtuins have recently attracted considerable interest due to their role in inflammation, protection from neurodegenerative diseases, organismal longevity, and their emerging importance for cancer (8). Of the seven members of this family in man, SIRT1, 3, 6, and 7 localize mainly to the nucleus, whereas SIRT2 resides exclusively in the cytoplasm and SIRT4 and 5 in mitochondria. SIRT1, 2, 3, and 5 catalyze NAD+-dependent deacetylation of targets, whereas SIRT4 and 6 mediate ADP-ribosylation of protein substrates. SIRT1 has originally been identified as a class III histone deacetylase removing histone H1K26, H3K9, H3K56, and H4K16 acetylation marks, thus facilitating heterochromatin formation. In addition, it plays a role in the repression of euchromatic gene regulation as part of a corepressor complex with the demethylase LSD1 and associated proteins. Among others, this complex binds to CSL and represses genes regulated by the NOTCH signaling pathway including HEY1 (9). In addition, SIRT1 can modulate NIDC activity by deacetylation as demonstrated in endothelial cells (1). Gene regulation by SIRT1 is also affected by deacetylation of other transcription factors including AR, FOXO, E2F1, HIC1, BCL6, NF-kB, and notably TP53 (for review, see ref. 10). SIRT1 physically interacts with TP53 and deacetylates Lys382, thus reducing the transcriptional activity of TP53. Knockdown of SIRT1 allows for TP53 acetylation, which is an indispensable prerequisite for the destabilization of the TP53–MDM2 interaction, inducing transcriptional activity and enabling TP53 stress response (12).

We here report that modulation of SIRT1 by HEY1 provides a feedback mechanism that couples NOTCH signaling to TP53 activation in tissues in which NOTCH activity acts tumor suppressive. We demonstrate that in Ewing sarcoma, SIRT1 expression is specifically confined to metastases and that pharmacologic inhibition of SIRT1 activity efficiently kills SIRT1-positive Ewing sarcoma cells in vitro and prohibits growth and migration of tumor cells in vivo.

**Materials and Methods**

**Cell lines, transfections, and drug treatments**

Cell lines of this study are summarized in Supplementary Table S4A. All cell lines were verified by short tandem repeat (STR) analysis and are routinely subjected to mycoplasma and SMRV testing in regular intervals. Ewing sarcoma cell lines and keratinocytes were transfected with Lipofectamine Plus reagent (Invitrogen) and subjected to puromycin selection (1 μg/mL) the next day. Ninety-six hours post-transfection, puromycin-selected cells were harvested, washed, and subjected to RNA and protein extraction. "697" and Nalm-6 were transfected by electroporation (Gene Pulser XCell, Bio-Rad) using commercial electroporation buffers from Bio-Rad. Tenovin-6 (Tnv-6; DundeeCell) and MG132 (Alexis, Biochem) were used at concentrations and time periods indicated in the figures. Plasmids and siRNA targeting sequences used in the study have previously been published and are listed in Supplementary Table S4B and S4C.

**Proliferation and viability assays**

Cell viability was assessed using the colorimetric MTT metabolic activity assay. Briefly, Ewing sarcoma cells (2 × 10⁴ cells per well) were cultured in 96-well plates at 37°C and exposed to varying concentrations of Tnv-6. Solvent (DMSO)-treated cells served as a negative control group. Seventy two hours after treatment, 20 μL of MTT solution (Sigma-Aldrich; 5 mg/mL in PBS) was then added and incubated for another 3 hours. Half-maximal inhibitory concentration (IC₅₀) values were calculated by using Spotfire (TIBCO; duplicate analysis). All experiments were performed at least 3 times in quadruplicates.

**Western blot analyses and immunoprecipitations**

For immunoblot analysis, total proteins were resolved by 8.5% or 10% SDS-PAGE and processed according to standard protocols. Antibodies in the study are listed in Supplementary Table S4D. For monitoring antibody binding, blots were incubated for 1 hour with either horseradish peroxidase–coupled secondary antibody for chemiluminescent detection with Super Signal West Pico chemiluminescent substrate (Pierce Biotechnology, THP) or with fluorescent dye–coupled secondary antibodies (DyLight 649 and DyLight 800, Pierce Biotechnology, THP) for detection by the Odyssey infrared imager (LI-COR Biosciences). TP53 was precipitated with either CM1 or D01 antibodies and probed with either acetylation-specific or D01 antibodies.

**Reporter gene assays**

For promoter activity analysis, TC252 Ewing sarcoma cells were plated at an initial concentration of 10⁴ cells/mL into 24-well plates. Cells were transiently transfected with 50 ng of each SIRT1–firefly reporter construct plus 100 ng of pSport HEY1 and pSuper empty vector, using Lipofectamine (Invitrogen). Luciferase reporter assays were then performed using Renilla as internal control. All plasmids used are listed in Supplementary Table S4B.
Gene expression analysis
Changes in gene expression profiles upon knockdown of EWS-FLI1 were followed on Affymetrix HGU133A arrays (Affymetrix, Inc.) as previously reported (3).

Quantitative real-time RT-PCR
Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) and quantitative analysis by TaqMan reverse transcriptase PCR (qRT-PCR) was performed as previously described (3). The relative expression levels of the genes assessed were calculated by the $2^{-\Delta\Delta C_T}$ method.

Immunohistochemistry on tissue microarrays and formalin-fixed, paraffin-embedded tumor sections
Three different Ewing sarcoma tissue microarray (TMA) series were used in this study: one (TMA1) previously described (13) containing 280 samples (277 Ewing sarcoma and three unclassified samples from Rizzoli Institute, Bologna, Italy); one (TMA2) containing 112 de-identified Ewing sarcoma samples from the University of Michigan (Ann Arbor, MI); and one previously described TMA from St. Jude Children’s Hospital containing primary tumor samples with complete clinical follow-up from 43 patients (Supplementary Table S2; ref. 14). In addition, formalin-fixed, paraffin-embedded (FFPE) tissue blocks from 14 paired primary tumor samples with complete clinical follow-up from 43 patients (Supplementary Table S2; ref. 14). In addition, formalin-fixed, paraffin-embedded (FFPE) tissue blocks from 14 paired primary Ewing sarcoma tumor and corresponding metastasis tissues were obtained from the University College London (see also Supplementary Materials).

Tissue processing, antibody staining (Supplementary Table S4C), and interpretation of staining results were performed as previously described (13). Two pathologists blind to the origin and identity of cases independently evaluated the percentage and intensity of stained cells (I. Machado, A. Llombart-Bosch). Statistical differences in staining patterns were evaluated using the $2 \times 2$ Fisher exact (2-tailed) probability test.

Zebrafish embryo xenotransplantation and in vivo Tn5-6 treatment
TGI(tki:EGFP) zebrafish were handled compliant to local animal welfare regulations and maintained according to standard protocols (www.ZFIN.org). Two-day-old zebrafish embryos were anaesthetized with 0.001% tricaine (Sigma-Aldrich). TC252 and A673 stably expressing mCherry were loaded as a single-cell suspension in 2% polyvinylpyrrolidone-40 (Sigma-Aldrich) into 1.0 mm OD × 0.78 mm ID borosilicate needles (Harvard Apparatus). Circa 500 cells were injected into the yolk, using a Pneumatic PicoPump (World Precision Instruments, Inc.; 10–20 psi, 100–400 ms). Larvae were maintained at 34°C. One day after implantation, six embryos were placed well in a 24-well plate, in a volume of 1 mL. Instant Ocean eggwater supplemented with either DMSO or Tn5-6, with daily refreshing of the solutions. After 3 days of treatment, embryos were fixed overnight (O/N) in 4% paraformaldehyde at 4°C. Embryos were imaged in glass-bottom 96-well plates using a NIKON3 confocal microscope (4× lenses). Image processing was performed with ImageJ 1.43 (NIH, Bethesda, MD), ImagePro Analyzer 7.0 (Media Cybernetics) analysis was performed as described previously (15).

Results
Identification of candidate genes mediating HEY1 induced TP53 stabilization downstream of EWS-FLI1
We previously showed that the NOTCH effector HEY1 stabilizes and activates TP53 in Ewing sarcoma upon silencing of EWS-FLI1, but the mechanism behind remained unknown (3). Because TP53 stability is largely regulated by posttranslational modifications (PTM), we hypothesized that HEY1 represses a TP53 interacting protein involved in the destabilization of TP53. We considered either a repression of the MDM2/MDM4 ubiquitin ligase complex (Supplementary Results and Supplementary Fig. S1) or of an enzyme involved in destabilizing posttranslational TP53 modification.

HEY1 was ectopically expressed in TC252 cells and genome-wide gene expression changes were compared with the EWS-FLI1 signature previously obtained by knockdown in five different Ewing sarcoma cell lines (TC252, WE68, STA-ET-1, STA-ET-7.2, SK-N-MC; ref. 4). Of 45 genes with relevance to TP53 modification (16–18), five fulfilled the criterion of being significantly suppressed in response to both HEY1 expression in TC252 and EWS-FLI1 silencing in all five tested Ewing sarcoma cell lines (Supplementary Table S1): **PRKDC**, encoding DNA-dependent protein kinase; **YEATS4**, whose product GAS41 is involved in dephosphorylation of TP53 Ser366 (19); **PPA1**, stabilizing MDM4 and dephosphorylating TP53 at Ser15 (20, 21); **PPP2R5C**, a component of protein phosphatase 2A dephosphorylating TP53 at various residues including Thr55, Ser37, and Ser16 (22–24), and **SIRT1**, a type III deacetylase known to target histone and non-histone proteins including TP53. While suppression of PRKDC is expected to perturb the DNA damage checkpoint (25), HEY1 mediated suppression of GAS41, or protein phosphatases 1 and 2, and specifically of the deacetylase SIRT1 has the potential to lead to TP53 stabilization and activation. We observed a marked upregulation of SIRT1 RNA expression in 59 primary Ewing sarcoma samples as compared with 89 normal tissues (Fig. 1A) and acetylation of TP53 in response to EWS-FLI1 knockdown and ectopic HEY1 expression in the Ewing sarcoma cell line TC252 (Fig. 1B). Because acetylation and deacetylation play a central role in the regulation of the TP53 pathway (16), we therefore focused our further analysis on SIRT1.

SIRT1 mediates TP53 stabilization by HEY1 downstream of NOTCH and of EWS-FLI1 silencing
Ectopic HEY1 consistently reduced SIRT1 expression leading to TP53 stabilization and consequently to the induction of CDKN1A expression in wild-type TP53 cell types, for which NOTCH activation has been reported to act tumor suppressive. This is demonstrated for three Ewing sarcoma cell lines (TC252, VH64, and WE68; Fig. 1C), the B-ALL cell line 697, the B-cell lymphoma cell line Nalm-6, and primary human keratinocytes lacking endogenous HEY1 expression (Supplementary Fig. S2). SIRT1 modulation is dependent on the concentration of ectopic HEY1 as demonstrated in HEK293 cells carrying doxycycline-inducible Flag-tagged HEY1, where it was completely reversible upon release from doxycycline-dependent HEY1 induction (Supplementary Fig. S3A). Conversely,
knockdown of endogenous HEY1 in the osteosarcoma cell line U2OS by siRNA increased SIRT1 accompanied by TP53 modulation (Supplementary Fig. S3B). These results indicate that the mechanism of TP53 activation by HEY1-mediated SIRT1 suppression is not restricted to Ewing sarcoma but also operative in other tissues and tumors.

Figure 1. HEY1 induces TP53 acetylation via direct modulation of deacetylase SIRT1 upon silencing of EWS-FLI1. A, SIRT1 RNA expression in 59 primary Ewing sarcoma (ES) samples as compared with 89 human tissues from the Novartis gene expression atlas. The box and whiskers plot shows the log2 expression levels on Affymetrix arrays as described (4). The box contains data within the upper and lower quartile; the median of the data is shown as horizontal line within the box; the whiskers extend at maximum to 1.5 × IQR (interquartile distance = distance between upper and lower quartile); more extreme data points (outliers) are depicted as circles. The difference between the groups is highly significant (P = 2.2e-16, Wilcoxon test). See also Supplementary Table S1. B, immunoblot of immunopurified TP53. TC252 cells were transfected with control empty vector, EWS-FLI1 directed shRNA (EF30), HEY1 expression plasmid, or, for positive control, a p300 expression construct. Cells were lysed and TP53 was immunoprecipitated using anti-TP53 antibody. Equal amounts of immunopurified TP53 were probed with pan-acetylation antibody and for control of equal TP53 loading, DO-1. C, modulation of SIRT1 and induction of TP53 and CDKN1A by ectopic HEY1 expression in three Ewing sarcoma cell lines (TC252, VH64, and WE68). Con, empty vector control transfection. D, NICD1 downregulates SIRT1 and induces TP53 protein expression in TC252 cells. E, knockdown of EWS-FLI1 modulates SIRT1 expression in a HEY1-dependent way. TC252 cells were transfected with either control scrambled shRNA (Co) or EWS-FLI1 targeting shRNA EF30 in the presence of either nontargeting siRNA control (siCo) or HEY1-specific siRNA. Because of the unavailability of sensitive HEY1-specific antibodies, HEY1 expression was monitored by real-time quantitative RT-PCR (mean values ±SEM of three determinations), whereas EWS-FLI1, SIRT1, and β-actin were followed on the protein level. F, HEY1 suppresses the SIRT1 promoter by binding to an E-box element. Firefly luciferase reporter assays were conducted in TC252 cells upon cotransfection of the indicated reporter vectors carrying a SIRT1 promoter fragment with the E-box in either wild-type or mutant configuration (39) with either empty vector control or HEY1 expression plasmid. Mean ±SEM of three independent experiments is shown. * indicates significant (P < 0.011) by 1-sample t test. n.s., not significant (P > 0.05).
Consistent with HEY1 acting downstream of activated NOTCH signaling, ectopic NICD1 expression also induced SIRT1 downregulation and TP53 stabilization (Fig. 1D). To confirm dependence of EWS-FLI1–dependent SIRT1 regulation on HEY1 in Ewing sarcoma cells, we performed EWS-FLI1 knockdown in the absence and presence of siRNA to HEY1. Silencing of EWS-FLI1 by shRNA resulted in a marked induction of HEY1 and a strong downregulation of SIRT1, which was completely rescued upon concomitant inhibition of HEY1 expression (Fig. 1E).

HEY proteins are known to preferentially bind to class B E-box sequences (26, 27). The SIRT1 promoter region contains a (GACGTG) motif at −373 base pairs from the transcriptional start site (Fig. 1F). In reporter gene assays with a 562 base pair SIRT1 promoter fragment driving luciferase expression in TC252 cells, HEY1 reduced the activity more than 3-fold. This transcriptional repressive effect was completely lost upon mutation of the E-box element (Fig. 1F). Together with published chromatin immunoprecipitation sequencing data from HEK293 embryonal kidney cells (26), K562 myeloid leukemia, and HepG2 hepatocellular carcinoma cells (ENCODE; ref. 28) that demonstrated HEY1 binding to the SIRT1 promoter at about 300 nucleotides, these results confirm SIRT1 as a direct HEY1 target gene.

**Inhibition of SIRT1 leads to Ewing sarcoma cell death in vitro**

Modulation of SIRT1 in Ewing sarcoma cells was sufficient to induce acetylation of TP53 and activation of TP53 target genes such as CDKN1A (Fig. 2A), as also observed upon HEY1 expression (Fig. 1C). In fact, ectopic expression of increasing amounts of SIRT1 in the presence of HEY1 was able to abolish HEY1-induced TP53 acetylation already at the lowest levels (Fig. 2B). These results suggest that SIRT1 expression in Ewing sarcoma is involved in functional impairment of TP53 activity, which can be restored by activation of the NOTCH signaling pathway through HEY1 induction upon knockdown of EWS-FLI1.

We next tested for the impact of SIRT1 modulation on cell fate of Ewing sarcoma cells. Knockdown of SIRT1 but not of SIRT2 induced TP53 acetylation and cell death in TC252 cells (Fig. 3A). This result suggested that SIRT1 expression may serve as a promising pharmacologic target in Ewing sarcoma. We therefore tested the sensitivity of Ewing sarcoma cell lines to the small-molecule SIRT1/2 inhibitor Tnv-6 (29). As shown in Fig. 3B, Tnv-6 killed Ewing sarcoma cell lines with IC_{50} values between 0.8 and 8.0 µmol/L. The lowest IC_{50} values were found in cell lines with wild-type TP53, which also expressed the highest levels of SIRT1, consistent with the induction of acetylated TP53 and of CDKN1A as a marker of TP53 transcriptional activation (Fig. 3C). Dependence of Tnv-6–induced cell death on TP53 was best demonstrated by rescue of cell viability without any change in SIRT1 expression levels if TP53 was knocked down before Tnv-6 treatment in wild-type TP53 TC252 cells (Fig. 3D). Cell death induction by Tnv-6 was dependent on SIRT1 but not on SIRT2, as SIRT2 knockdown failed to rescue TC252 cell death induced by drug treatment (Fig. 3E). Together, these results indicate that Ewing sarcoma cells are highly sensitive to Tnv-6 treatment and that the level of sensitivity depends on the presence or absence of intact TP53 and SIRT1.

**The SIRT1/2 inhibitor Tnv-6 inhibits Ewing sarcoma growth and spread in a zebrafish xenotransplantation model**

Because inhibition of SIRT1 was able to kill Ewing sarcoma cell lines, we tested Tnv-6 for tumor-inhibitory activity in an established xenotransplantation model in zebrafish embryos (30). Two cell lines with high and low SIRT1 expression and distinct Tnv-6 in vitro sensitivity (Fig. 3B), the wild-type TP53 cell line TC252 and the TP53-mutant cell line A673, were fluorescently labeled with mCherry and injected into the yolk sac of flI:Egfp transgenic zebrafish embryos. Implanted embryos were either treated with solvent (DMSO) or 6 µmol/L Tnv-6 for 4 days, and growth and spread of tumor...
cells through the embryo body were monitored by confocal microscopy (Fig. 4A). Figure 4B combines the results for all embryos injected in three independent replica experiments in a scatter plot that visualizes the distance and direction of migration for each object (cluster of tumor cells) for each embryo. As quantified in Fig. 4C, Tvn-6 not only significantly reduced tumor burden \((P < 0.0001)\) but also significantly inhibited migration of TC252 cells \((P < 0.0001)\) but not of A673 cells. These data show that pharmacologic inhibition of SIRT1 interferes with Ewing sarcoma growth and migration \(in vivo\).
Figure 4. Effect of Tnv-6 treatment on Ewing sarcoma cells in zebrafish embryos. Five hundred cells of either wild-type TP53, high SIRT1 expressing TC252, or TP53 mutant low SIRT1 A673 cell lines were implanted into the yolk sac of the indicated numbers of Tg/fl1:EGFP transgenic zebrafish embryos 2 days after fertilization, and embryos were either control (DMSO) or Tnv-6 (6 µmol/L) treated from day 1 to 4 after implantation. A, representative picture and quantitative analysis of TC252 implanted fish embryos. Algorithms use the green channel to find the outline of the Tg/fl1:EGFP embryo and estimate the site of implantation (X). Using the mCherry signal from ES cells, all tumor cell foci (objects) are outlined and numbered. To determine tumor burden per embryo, the number of objects was multiplied by the average size of objects. The distance of migration away from the site of implantation was determined per object and cumulative results for all embryos from three independent experiments are presented in scatter dot plots in B. Here, each color represents one embryo per group, each dot one cluster of tumor cells. Site of implantation: (x, y = 0, 0). C, cumulative results of tumor burden and migration of TC252 and A673 cells in embryos after three days of drug treatment, 4 days postinjection, normalized against DMSO. Shown are means ± SEM of three independent experiments with a total of 214 and 126 embryos for TC252 and A673, respectively. Statistical significance of results was analyzed by unpaired t test. n.s., no significant difference.

SIRT1 expression in Ewing sarcoma primary tumors and metastases

To validate SIRT1 expression in primary tumors, we screened 392 paraffin-embedded, formalin-fixed Ewing sarcoma samples on two series of TMAs (TMA1 and TMA2; Supplementary Fig. S4) for SIRT1 positivity by immunohistochemistry (IHC). Cases were scored semiquantitatively, and five groups were formed according to the percentage and intensity of mainly nuclear stained cells. Cases were scored as noninformative (necrosis, scant material, or artifact), negative (mild < 5%), 1+ (mild staining in 5%–10% of cells), 2+ (moderate staining in 10%–50% of cells), or 3+ (strong staining in >50% of cells). Two hundred ninety-eight samples gave informative results, of which 272 samples and 26 samples were obtained from primary tumors and metastases, respectively. For 248 cases, information on disease extent was available (Supplementary Fig. S4). Figure 5A provides examples for the distinct staining patterns obtained. One hundred thirty-one (48%) primary tumors tested negative and 54 (20%) showed only mild staining, whereas 65 (24%) and 22 (8%) displayed moderate and strong nuclear SIRT1 staining, respectively (Fig. 5B).

Focusing on primary tumors from patients with localized disease 109 (51%), 41 (19%), 53 (25%), and 11 (5%) showed no, mild, moderate, and strong staining. Corresponding numbers obtained for primary tumors from patients who presented with metastases at diagnosis were 12 (35%), 10 (29%), 5 (15%), and 7 (21%), suggesting a nonsignificant tendency toward higher positivity in patients with primary disseminated disease (P = 0.550). The site of metastasis was known for the informative primary tumors from patients with metastatic disease on
TMA1 in 29 cases (Supplementary Table S2). Here, primary tumors associated with lung metastasis and primary tumors from patients with bone metastases tested positive in 50% and 40%, respectively.

Strikingly, this tendency turned into a significant difference \((P < 0.001)\) when focusing on 26 informative metastasis-derived samples (Fig. 5B, last column). Here, two thirds of metastases showed SIRT1 expression in more than 10% of cells (8 moderately positive, 9 highly positive), whereas four samples tested negative and five metastases showed SIRT1 positivity in less than 10% of cells (Fig. 5B).

To validate our finding of increased SIRT1 positivity in Ewing sarcoma metastases, we tested an independent series of 14 paired tissue samples for SIRT1 expression by IHC (Table 1, cases 1–14). Including 4 paired samples available from TMA1 and TMA2 (Table 1, cases 15–18), we found lung metastases to score positive in 88% and bone marrow metastases in 55% of cases similar to the frequency in the primary tumors of these metastatic patients (61%), a frequency twice as high as observed on the TMAs for the primary tumors of patients with localized disease. These results suggest that Ewing sarcoma metastases, specifically lung metastases are predominantly highly positive for SIRT1 by IHC, and that this positivity can frequently already be observed in the corresponding primary tumors at diagnosis.

Because this finding may imply a prognostic relevance of SIRT1 positivity, we independently tested a third previously published cohort of 43 patients with Ewing sarcoma with complete clinical follow-up for a median of 154 months for SIRT1 expression by IHC (TMA3; Supplementary Fig. S4; ref. 14). Six of 32 localized tumors (19%) and 6 of 11 tumors from patients with metastatic disease at diagnosis (55%) tested highly or moderately SIRT1 positive. Intriguingly, 5-year survival probability for the 12 SIRT1-positive patients was 25% as compared with 70% for the 31 SIRT1-negative patients.
Discussion

The NOTCH signaling pathway is well known for its complexity. Its role in cancer has been extensively studied in the context of oncogenic NOTCH mutations but little was known about its tumor-suppressive mechanisms. We here report on the mechanism by which HEY1 downstream of activated NOTCH induces TP53 and demonstrate that this mechanism is operative not only in Ewing sarcoma but also in other malignancies and tissues for which NOTCH activity has been linked to tumor suppression. We find that HEY1 consistently suppresses SIRT1 in Ewing sarcoma, B-ALL, and B-cell lymphoma cell lines and primary human keratinocytes, leading to TP53 acetylation, stabilization, and transcriptional activation. SIRT1 has previously been described to be involved in the epigenetic transcriptional repression of the HEY1 promoter. In the absence of NOTCH activation, SIRT1 forms a corepressor complex with LSD1, CoREST1, and CtBP1, leading to concerted repressive histone H4K16 deacetylation and H3K4 demethylation at the HEY1 promoter. Because we find SIRT1 (shown here) and also LSD1 (31) to be consistently expressed in Ewing sarcoma, and concomitantly HEY1 to be completely turned off (5), this mechanism is likely operative in Ewing sarcoma. Upon activation and nuclear translocation of NICD and binding to the NOTCH regulated transcription factor CSL, this corepressor complex is replaced by mastermind-like (MAML) and co-activators, including the histone lysine acetyltransferase p300/CPB, leading to transcriptional activation (9). In addition, NICD stability has been reported to be prolonged by acetylation, which is modulated by SIRT1, with negative consequences on duration and amplitude of the NOTCH response (1). Also, SIRT1 represses NOTCH1 transcription from a highly conserved region in the NOTCH1 promoter in endothelial cells (32). By demonstrating suppression of SIRT1 expression by HEY1, our results add an important negative feedback loop to NOTCH-driven gene regulation (Fig. 6). It also adds a putative feedback mechanism of potential prognostic value to the regulation of TP53. Not only does SIRT1 modulate TP53 stability and activity but also TP53 has been demonstrated to suppress SIRT1 expression through transcriptional repression via binding to a TP53 response element in the SIRT1 promoter (33) and posttranscriptional regulation via TP53-activated microRNA hsa-mir-34a (Fig. 6; ref. 34). Interestingly, hsa-mir-34a and two other SIRT1 regulatory microRNAs, hsa-mir-132 and hsa-mir-93, were recently described components of a favorable prognostic signature in Ewing sarcoma (35). Because we find SIRT1 positivity in Ewing sarcoma mainly associated with metastases, it is intriguing to speculate that the TP53/has-mir-34a/SIRT1 gene regulatory module plays a role in the suppression of metastases. In fact, our results in a zebrafish xenotransplantation model demonstrated that inhibition of SIRT1 prohibits Ewing sarcoma cell dissemination. Because the yolk sac, the site of tumor cell injection, is only poorly vascularized (as is visible from the absence of fl:EGFP-positive endothelial cells in Fig. 4), and tumor cells in control-treated animals migrated not only to the tail but also to the head region of the embryos, Tnv-6 likely inhibited active tumor cell migration, consistent with the documented role of SIRT1 in the regulation of cortactin (36). This migration inhibitory effect could not be studied in vitro because of the strong cytotoxic effect of Tnv-6 and of SIRT1 knockdown observed in the Ewing sarcoma cell lines. Cell death induction upon perturbation of SIRT1 expression or activity was mainly due to activation of TP53 and could be rescued upon knockdown of TP53. In fact, sensitivity to pharmacologic SIRT1 inhibition was highest in wild-type TP53 cell lines, which are representative of more than 90% of Ewing sarcoma (37). However, also mutant TP53 cell lines proved sensitive to Tnv-6 treatment, albeit at much higher doses, which is likely due to the broad transcriptional and epigenetic roles of SIRT1 in genome-wide gene regulation as has previously been observed (29). Although it cannot be excluded that part of the in vitro cytotoxic activity of Tnv-6 may be due to a non-SIRT1/non-TP53–related activity such as induction of autophagy as previously observed in CLL (38), it did not play a role in the zebrafish xenotransplantation model. Here, drug activity was dependent on high SIRT1 expression and the presence of wild-type TP53.

Table 1. SIRT1 staining results for paired primary tumor and metastasis-derived Ewing sarcoma samples

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<td>18</td>
<td>TMA2</td>
<td>2+</td>
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<tr>
<td>% SIRT1 pos</td>
<td>61%</td>
<td>55%</td>
<td>88%</td>
<td></td>
</tr>
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</table>

NOTE: Moderate (2+) and strong (3+) staining according to Fig. 5A are considered positive and indicated in bold.

*See Supplementary Fig. S4.*

( Supplementary Table S3 and Supplementary Fig. S5). Although these results call for larger prospective studies to validate the potential prognostic importance of SIRT1 in Ewing sarcoma, our combined data strongly imply SIRT1 as a candidate therapeutic target in patients with Ewing sarcoma with metastatic disease.
Our IHC study performed on a total of four independent ES cohorts reproducibly indicated increased SIRT1 expression in metastases, specifically in lung metastases. However, results for the frequency of SIRT1-positive staining in the primary tumors of patients presenting with metastases differed between the series. The UCL and the St. Jude cohorts implied high SIRT1 in metastatic patients, whereas in the Rizzoli and the Michigan cohorts, the frequency of SIRT1-positive primary tumors in localized and metastatic disease were comparable, still with a tendency toward higher numbers of positively staining tumor cells in metastatic cases. It is possible that tumor cell heterogeneity and focal SIRT1 expression might have led to an underestimation of SIRT1-positive cases in the primary tumors of metastatic patients on these TMAs. Also, we cannot exclude that decalcification of bone tumor specimens might have led to false negativity in some cases. Intriguingly, outcome results of the small retrospective St. Jude cohort suggested that not only metastatic patients but also patients with localized but SIRT1-positive tumors had an adverse prognosis. These results need to be confirmed on larger, prospectively collected sample series.

While there is already evidence that Sirtuin activators are clinically well tolerated, early clinical trials with SIRT1 inhibitors have only recently been initiated. Metastases are the main cause of treatment failure and death in cancer. Our findings of high SIRT1 positivity in metastases of patients with Ewing sarcoma and sensitivity of SIRT1-positive tumor cell lines to SIRT1 inhibition in vitro and in vivo may therefore open an exciting new avenue to cancer treatment.

Disclosure of Potential Conflicts of Interest

P. Picci received speakers' bureau honoraria and is a consultant/advisory board member. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: J. Ban, H. Kovar

Development of methodology: J. Ban, A. Fourtouna, V. Berg, E. Snaar-Jagalska, A. Llombart-Bosch

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Ban, D.N.T. Aryee, A. Fourtouna, W. van der Ent, S. Niedan, I. Machado, C. Rodriguez-Galindo, O.M. Tirado, P. Picci, A.M. Flanagan, S.J. Strauss, K. Scotlandi, E.R. Lawlor, A. Llombart-Bosch


Writing, review, and/or revision of the manuscript: A. Fourtouna, E. Snaar-Jagalska, H. Kovar

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Scotlandi, E.R. Lawlor

Other (IHC analysis): I. Machado

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Jozef Ban, Dave N.T. Aryee, Argyro Fourtouna, et al.

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