Method

Improved mass spectrometry-based activity assay reveals oxidative and metabolic stress as sirtuin-1 regulators

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ABSTRACT

Sirtuin-1 (SirT1) catalyzes NAD⁺-dependent protein lysine deacetylation and is a critical regulator of energy and lipid metabolism, mitochondrial biogenesis, apoptosis, and senescence. Activation of SirT1 mitigates metabolic perturbations associated with diabetes and obesity. Pharmacologic molecules, cellular redox, and nutritional states can regulate SirT1 activity.

Technical barriers against measuring endogenous SirT1 activity have limited characterization of SirT1 in disease and its activation by small molecules. Herein, we developed a relative quantitative mass spectrometry-based technique for measuring endogenous SirT1 activity (RAMSSAY/RelAtive Mass Spectrometry Sirt1 Activity assaY) in cell and tissue homogenates using a biotin-labeled, acetylated p53-derived peptide as a substrate. We demonstrate that oxidative and metabolic stress diminish SirT1 activity in the hepatic cell line HepG2. Moreover, pharmacologic molecules including nicotinamide and EX-527 attenuate SirT1 activity; purported activators of SirT1, the polyphenol S17834, the polyphenol resveratrol, or the non-polyphenolic Sirtris compound SRT1720, failed to activate endogenous SirT1 significantly. Furthermore, we provide evidence that feeding a high fat high sucrose diet (HFHS) to mice inhibits endogenous SirT1 activity in mouse liver.

In summary, we introduce a robust, specific and sensitive mass spectrometry-based assay for detecting and quantifying endogenous SirT1 activity using a biotin-labeled peptide in cell and tissue lysates. With this assay, we determine how pharmacologic molecules and metabolic and oxidative stress regulate endogenous SirT1 activity. The assay may also be adapted for other sirtuin isoforms.

1. Introduction

The mammalian sirtuin family consists of seven highly conserved class III NAD⁺-dependent deacetylases and ADP ribosyltransferases. Sirtuins are located in distinct cellular compartments, controlling important metabolic and signaling pathways [1,2]. The most prominent member sirtuin-1 (SirT1) activates processes relevant to caloric restriction and fasting [3–5]. Overexpression or activation of SirT1 [6,7] effectively alleviates metabolic and cardiovascular complications in animal models of metabolic diseases including type-2 diabetes and obesity [8–12]. Molecular targets of SirT1 include transcription factors that control essential cellular stress response pathways which improve metabolic functions such as mitochondrial biogenesis and fatty acid utilization [9,13–17].

Abbreviations: BSA, bovine serum albumin; CysNO, S-nitrosocysteine; B6J, C57BL/6J mouse strain; DBC-1, deleted in breast cancer 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; HEK-293, human embryonic kidney cell-293; HepG2, human hepatocellular carcinoma cell line; HFHS, high fat high sucrose diet; HPGH, high palmitate high glucose medium; HPLC, high performance liquid chromatography; IAM, iodoacetamide; IgG, immunoglobulin G; IP, immuno-precipitation; LacZ, beta-galactosidase; NAD⁺, nicotinamide adenine dinucleotide; ND, chow diet; MS, mass spectrometry; p53, tumor suppressor p53; RONS, reactive oxygen and nitrogen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SirBACO, Sirtuin-1 Bacterial Artificial Chromosome Overexpressor; SirT1, Sirtuin-1

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The first non-histone SirT1 substrate identified was p53. SirT1 deacetylates the C-terminal Lys382 of p53, repressing its transcriptional activity, and consequently inhibits apoptosis and cell cycle arrest. Subsequently, numerous other targets have been characterized, including transcription factors, co-activators, histones and non-nuclear proteins [18–21]. Assessment of the acetylation status of these proteins, including p53 and histone H3, using antibody-based analytical methods are standard measures of SirT1 activity in cells and tissues [11,12,22].

SirT1 activity is affected by NAD⁺ co-substrate availability [23,24], oligomerization status [25], association with regulatory proteins [26,27], and post-translational modifications [22,28–33] including cysteine thiol oxidation. During the catalytic cycle, SirT1 deacetylase target proteins, converting NAD⁺ into nicotinamide and O-acetyl ADP-ribose [16,17,34]. Thus, primary assays use radioisotope labeled substrates including 14C-NAD⁺, 14C-acetylated p53 or 3H-acetylated histones [16,35–37]. More recent methods, however, employ a two-step fluorometric assays such as Fluor de Lys [38–43] or high-performance liquid chromatography (HPLC) [44–47] to detect deacetylated peptide levels. The fluorometric assays rely on substrate deacetylation because this gives trypsin access to cleave off the quenched fluorophore. The developed fluorescence is proportional to SirT1 activity. These assays are reliable for measuring the activity of purified SirT1, but generate variable results in cell and tissue samples, due to lack of sensitivity and specificity. To address these limitations, we used a biotin-labeled acetylated p53 peptide to develop a relative quantitative mass spectrometry-based method, which is specific and sensitive. This method allows differential quantification of the peak intensities of deacetylated-p53 vs. acetylated-p53 to measure in vivo SirT1 activity. Because custom-synthesized peptide substrates are commercially available, our strategy can also be applied for analysis of other siruin isoforms and peptide substrates. Employing this method, we investigated the impact of polyphenolic (S17834, resveratrol) or non-polyphenolic (SRT1720, EX-527) compounds, cellular redox potential (H₂O₂, CysNO, GSSG), and nutritional state (HPHG, high fat high sucrose diet) on SirT1 activity, and consequently inhibits apoptosis and cell cycle arrest.

2. Materials and methods

2.1. Reagents, materials, and antibodies

S17834 (6,8-diallyl-5,7-dihydroyx-2-(2-allyl-3-hydroxyl-4-methoxyphenyl)1-H-benzo (b)pyran-4-one) and SRT1720 (N-(2-[3-(piperazine-1-yl)methyl]imidazo [1,2-b] [1,3]thiazol-6-yl)phenyl)-2-quinoxaline-carboxamide), EX-527 (6-chloro-2,3,4,9-tetrahydro-1-H-carbazole-1-carboxamide), were obtained from the Institut de Recherche Servier (Suresnes, France). The following antibodies were used: anti-Flag M2 (Sigma, St. Louis, MO; F1804), anti-Sirtuin-1 (Abcam, Cambridge, MA; ab110304), anti-GAPDH (Cell Signaling Technology, Danvers, MA; #2118). Anti-Flag M2 Affinity Gel was purchased from Sigma Aldrich, catalog number: A2220. Avidin agarose (cat # PI29200), streptavidin agarose (cat # 20347) and streptavidin magnetic beads (cat # 88816) were obtained from Thermo Fisher Scientific, Waltham, MA. Biotin-labeled Ac-Lys382-p53 peptide with a 6-carbon linker (cat # 65045) was synthesized by AnaSpec, San Jose, CA. Zeba™ spin desalting columns (40K MWCO, 87767), Lipofectamine™ and cell culture media were bought from Life Technologies (Grand Island, NY).

2.2. Cell culture

HepG2 cells (ATCC, Manassas, VA) were maintained in Dulbecco’s Modified Eagle Medium containing 10% fetal bovine serum and penicillin/streptomycin (Gibco, Grand Island, NY). Transfected cells were either incubated in control medium containing 5 mM glucose and 0.67% bovine serum albumin (BSA, fatty acid free, Sigma-Aldrich St. Louis, MO) or medium supplemented with high palmitate (0.4 mM palmitic acid and 0.67% BSA) and high glucose (25 mM glucose, referred to as HPHG) for 16 h.

2.3. Experimental animals

Male SirT1 Bacterial Artificial Chromosome Overexpressor (SirBACO) mice with C57BL6/NJ genetic background were obtained from Dr. Wei Gu, (Columbia University, NY). A cohort of 2-month-old male SirBACO mice and WT littersmates were fed control or high fat and high sucrose diet (HFHS: 35.5% fat representing 60% calories, 16.4% sucrose) ad libitum for ten months (D09071702 and D09071703) to investigate the effects of metabolic stress. Mice were housed in rooms with 12-h light/dark cycle in groups of 3–4, whenever possible. The Institutional Animal Care and Use Committee at Boston University School of Medicine approved the animal protocol. Mice were euthanized after ten months on the diet and livers were perfused, excised, snap-frozen, and stored in liquid nitrogen or at −80°C for later analysis.

2.4. Homogenization and protein extraction of mouse liver

Homogenization and extraction of individual liver samples were carried out in NP-40 lysis buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, and a protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany).

2.5. Preparation of S-nitrosocysteine

S-nitrosocysteine (Cys-NO) stock solutions were freshly prepared by mixing equimolar amounts of L-cysteine and NaNO₂ under acidic conditions (0.25 M HCl) in the presence of 0.1 mM diethylenetriamine pentaacetaete (DTPA). The concentration of Cys-NO in solution was measured spectrophotometrically at 334 nm, ε₃₂₄ = 900 M⁻¹ cm⁻¹ and adjusted to 500 mM. Dilutions of Cys-NO were prepared in HEN buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine) immediately before conducting experiments.

2.6. SirT1 activity measurement using p53-targeted mass spectrometry (RAMSSAY)

HepG2 cells (approx. 1.3 × 10⁵ cells) were infected with 0.16 × 10^⁶ pfu WT SirT1 or dominant negative H355A mutant SirT1 (HA) adenovirus for 48 h. Cell or tissue lysates (800 μg of protein prepared in 500 μl NP-40 lysis buffer) were precleared with 5 μl streptavidin magnetic beads for 1 h at 4 °C on a rotator. Precleared lysates were incubated with biotin-labeled acetylated p53 peptide (Biotin-LC-KKG-QSTSRRHK(K)-LMFKEETG; 10 μM final concentration) and 100 μM NAD⁺ for 30 min at 37°C. Streptavidin beads (10 μl of a 1:1 slurry) were added and incubated for 1 h at room temperature. Beads were washed three times with 300 μl PBS, three times with 300 μl Tris buffer (25 mM, pH 7.4), and three times with 300 μl of ddH₂O. Biotin-tagged peptides were either eluted into 100 μl 25 mM Tris buffer with 5 mM biotin pH 8.0 at RT or into 100 μl ddH₂O with 5 mM DTT heated at 90°C for 5 min. The supernatant was dried down in a SpeedVac™ concentrator (Savant, Thermo Fisher Scientific), suspended in 15 μl ddH₂O, and then desalted with a C18 ZipTip™ (EMD Millipore, Billerica, MA) before mass spectrometry analysis. The acetylated and deacetylated p53 peptides were analyzed with an UltrafluxExtreme MALDI-TOF/TOF MS (Bruker Daltonics, Billerica, MA) using α-cyano-4-hydroxycinnamic acid as matrix. The MALDI-TOF mass spectra were summed from 2000 laser shots for good signal intensity with a resolution of 10,000 fwhm and mass accuracy <10 ppm at m/z 400. Concentration changes of the acetylated and deacetylated p53 were calculated by determining the difference in relative peak intensities observed for the [M + H]⁺ signal corresponding to each.
2.7. Statistical analysis

Statistical analysis was performed using Prism 5.0 (GraphPad Software). Means were compared between two groups by one-way ANOVA or multiple comparisons two-way ANOVA analysis with Bonferroni’s post-test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. The principle of the relative quantitative mass spectrometry-based activity assay (RAMSSAY) using a biotin-tagged p53 peptide

We have selected matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS due to its wide availability, high sample throughput, relative ease of use, and tolerance to all classes of samples. Acetylated lysine 382 of the tumor suppressor p53 is a well-characterized SirT1 target. Therefore, we selected a readily acetylated peptide corresponding to amino acid residues 372–389 of p53 as a SirT1 substrate. Biotin, covalently attached to the N-terminus of the peptide, enables highly efficient enrichment and cleanup for MS analysis via streptavidin-avidin supports [48,49] (Fig. 1A). Because of the ease of custom peptide synthesis, the assay is adaptable to different peptide substrates for testing of newly discovered protein acetylation sites or other protein deacetylases. We selected streptavidin magnetic beads for fast sample preparation and tested various elution conditions. Mass spectrometry [50,51] is somewhat incompatible with detergents or chaotropic salts, which are required to denature and disrupt the highly stable biotin-streptavidin complex. Several reports showed that water heated above 70 °C efficiently disrupts the complexes [52]. With our assay, a combination of water and DTT released more biotinylated peptide than pure water (Supplemental Figs. 1A and B), but the recovery rate of biotinylated peptide remained below 10%. Competition with free biotin [53–55] in Tris buffer followed by desalting of the peptide with C18 Zip-tips resulted in a 50% higher recovery rate, so we used this protocol in all subsequent experiments (Supplemental Fig. 2 A and B). We detected acetylated and deacetylated peptide peaks with the expected mass shift of 42 Da and differentially quantified the abundances of these peaks in the same mass spectrum (Fig. 1B). The acetylated and non-acetylated peptides had comparable ionization and detection efficiencies. To determine SirT1 activity, we calculated the ratio of acetylated to deacetylated peptide peak intensities. Since the substrate concentration is known, the computation of specific activities is possible.

3.2. RAMSSAY is specific for SirT1 activity

SirT1 activity requires the cofactor NAD⁺ (nicotinamide adenine dinucleotide) to deacetylate lysine residues of target proteins. The enzymatic reaction catalyzed by SirT1 produces the deacetylated substrate, nicotinamide (NAM) and O-acetyl-ADP-ribose (OAADPr) [56,57]. NAM acts as a competitive feedback inhibitor by binding to a

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Fig. 1. Principle of RAMSSAY. A) SirT1 activity was calculated by the peak intensity ratio of deacetylated (p53) versus acetylated p53 peptide substrate (Ac-p53). The [M + H]⁺ peaks of the deacetylated (m/z 2430.4) and acetylated (m/z 2472.4) p53 peptide are detected at isotopic resolution (cluster of peaks is derived from the natural abundance distribution of carbon 13 in the peptide). The height of the monoisotopic (first) peak in each cluster annotated by the dotted line was used for the calculation. B) The right panel shows typical examples of RAMSSAY with HepG2 cell lysates to measure endogenous (top and middle) or overexpressed SirT1 activity (bottom). Nicotinamide (10 mM; middle) inhibited SirT1 activity and prevented deacetylation of the peptide.
NAD+ hydrolysis [45]. EX-527 is a SirT1 specific deacetylase activity, in HepG2 cells (1.3 × 10^5 cells). Cell lysates overexpressing FLAG-SirT1 or dominant negative FLAG-SirT1 H355A were incubated HepG2 cells overexpressing WT SirT1 with CysNO overnight. Increasing CysNO concentrations (100–800 μM) did not alter SirT1 protein expression (Fig. 3A and B, and Supplemental Fig. 6). However, our assay measured concentration-dependent SirT1 inhibition with an IC₅₀ of ~222 μM. Using endogenous acetylated p53 as a surrogate marker of SirT1 activity, we observed comparable results (Supplemental Fig. 7).

3.4. Oxidative and metabolic stress inhibit SirT1 activity in HepG2 cells

Through changes in intracellular NAD⁺ concentration, protein interactions, or post-translational modification, oxidative stress affects SirT1 activity [27,65–68]. We previously showed that S-nitrosoglutathione (GSNO) inhibits SirT1 in vitro with an IC₅₀ of 69 μM [68] by reversibly modifying several cysteines of SirT1. Removal of these modifications by chemical reduction with DTT fully restores SirT1 activity. Exposure of cells to S-nitrosocysteine (CysNO) or hydrogen peroxide also inhibits SirT1 through reversible cysteine oxidation [22]. Moreover, we demonstrated that mutant SirT1 with three cysteine-to-serine substitutions (C61S, C318S, and C613S) maintains full enzyme activity when exposed to oxidative or metabolic stress. Here, we incubated HepG2 cells overexpressing WT SirT1 with CysNO overnight. Increasing CysNO concentrations (100–800 μM) did not alter SirT1 protein expression (Fig. 3A and B, and Supplemental Fig. 6). However, our assay measured concentration-dependent SirT1 inhibition with an IC₅₀ of ~222 μM. Using endogenous acetylated p53 as a surrogate marker of SirT1 activity, we observed comparable results (Supplemental Fig. 7).

Obesity, metabolic syndrome, and type-2 diabetes are associated with oxidative stress, leading to SirT1 inhibition [69,70]. We previously reported results obtained using an in vitro model which mimics metabolic stress and increases oxidants [22] in cultured cells by exposing them to high palmitate high glucose (HPHG). Similarly, loading cells with permeable GSSG diethyl ester mimics oxidative stress by disturbing the intracellular ratio of reduced-to-oxidized glutathione (GSH:GSSG) [71,72]. HepG2 cells exposed overnight to hydrogen peroxide, GSSG diethyl ester, or HPHG showed 70–80% diminished SirT1 activity without changing protein expression (Fig. 3C and D, and Supplemental Fig. 8).

3.5. Detection of SirT1 activity in hepatic lysates

SirT1 bacterial artificial chromosome overexpressor (SirBACO) mice [73] overexpress SirT1 up to 2-fold in the liver, compared to WT mice (Fig. 4A and B). Because of high non-specific deacetylase activity in hepatic lysates, the Fluor-de-Lys assay failed to detect changes in SirT1 activity in SirBACO mice (data not shown), leading investigators to use Western blot-based methods to measure SirT1 activation in these mice [74]. Our MS-based assay, however, measured an ~2-fold increase in SirT1 activity, consistent with SirT1 protein expression levels in SirBACO mice (Fig. 4A and Supplemental Fig. 9).

We next investigated the nutritional effects on liver SirT1 activity in mice fed regular chow (ND) or HFHS for ten months. HFHS increased SirT1 protein expression in both WT and SirBACO mice (Fig. 4C and D). Although SirT1 expression in HFHS-fed WT mice increased to levels comparable to SirBACO mice, the activity dropped to ~50% of the level observed in ND WT mice. As expected, SirT1 activity was markedly higher in ND-fed SirBACO mice compared to ND-fed WT controls, and HFHS significantly decreased SirT1 activity in both (Fig. 4C and D, and Supplemental Fig. 10). The data are in accordance with our previous findings [11,12] that HFHS inhibits SirT1 by oxidative inactivation.

4. Discussion

We developed a specific and sensitive mass spectrometry-based assay that reliably measures SirT1 activity in cell lysates. The assay measures concentration-dependent SirT1 inhibition with an IC₅₀ of ~222 μM. Using endogenous acetylated p53 as a surrogate marker of SirT1 activity, we observed comparable results (Supplemental Fig. 7).
assay to measure endogenous SirT1 activity. The assay offers significant improvement over current methods, because it avoids potential artifacts associated with methods that indirectly detect SirT1 activity, including Western blots and fluorophore-containing peptide-based measurements [36,47,75]. Because of the higher sensitivity and specificity of the RAMSSAY method, we can now reliably determine SirT1 activity in cell and tissue lysates [44-46].

To measure SirT1 deacetylase activity in this assay, we used a synthetic p53-derived peptide that was acetylated and biotin-tagged for efficient recovery from lysates. To overcome recognized technical limitations to the efficient recovery of biotinylated peptides, we developed this assay by investigating different elution conditions. Streptavidin/avidin coated supports are highly selective to retrieve biotinylated molecules from lysates. However, the non-covalent bond between biotin and streptavidin/avidin with a $K_D$ of $\sim 10^{-15}$ M hampers complex dissociation [52,76]. Bioengineered streptavidin/avidin such as nitrated CaptAvidin [77], deglycosylated neutravidin, or monomeric avidin [78] have lower binding affinities for biotin and require less stringent conditions for elution. Furthermore, most detergents in lysis buffers interfere with mass spectrometry [54], so their use should be minimized if not avoided. Elution with free biotin followed by ZipTip desalting yielded the best results.

We used known SirT1 inhibitors to verify the specificity of our assay. EX-527 ($IC_{50} = 38$ nM) specifically inhibits SirT1 deacetylase activity and is more potent than nicotinamide (NAM; $IC_{50} = 40-50$ $\mu$M) [40,42,58]. Both EX-527 and nicotinamide inhibited deacetylation of the peptide substrate in HepG2 cells. Overexpression of dominant-negative inactive mutant SirT1 (SirT1 H355A) had a similar effect and almost erased SirT1 activity (98% vs. control; Fig. 2A and B). The results of these experiments confirm that our assay measures specifically SirT1 deacetylase activity in HepG2 cells.

Polyphenols [13] and structurally unrelated Sirtrins compounds [9] are believed to stimulate SirT1 activity directly. Small molecule SirT1 activators such as SRT1720 improve insulin sensitivity, inhibit atherogenesis, increase mitochondrial content, and prolong survival of obese mice [62-64,79-81]. Recent reports demonstrated that pharmacological activation of SirT1 by these small molecules might falsely indicate indirect SirT1 activation in vitro due to an assay artifact [47]. Several studies have shown that resveratrol and SRT1720 increase SirT1 activity only when assayed with peptide substrates containing a covalently attached fluorophore [36,47,75]. On the contrary, and consistent with other reports [36,75,82], both SirT1 activators failed to increase deacetylase activity in HepG2 cells when measured with our assay (Supplemental Figs. 4A and B). The polyphenol S17834 developed by Servier showed a trend of increased endogenous SirT1 activity. Regulation of SirT1 activity by polyphenols largely depends on the cell type [83,84]. In many cases, indirect factors including changes in SirT1 expression, protein interactions, post-translational modifications or antioxidant effects of polyphenols influence deacetylase activity. Thus, measurements of SirT1 activity, expression level, and downstream targets are pivotal to thoroughly characterizing the pharmacodynamics of small molecule SirT1 activators.

Nutritional status, inflammation, stress response, and tissue damage are closely associated with changes in cellular redox homeostasis and SirT1 activity. Conflicting effects on SirT1 expression and activity have been reported in some cell and mouse models [85]. Cells challenged by oxidative or genotoxic stress have significantly decreased SirT1 expression and activity [66,86-88]. Other reports, including our studies, showed unchanged SirT1 expression levels while observed SirT1 activity decreased due to oxidative post-translational modifications or protein-protein interaction [11,12,22,27,69,89]. Here we similarly reported unaltered SirT1 expression in HepG2 cells exposed to oxidative
or metabolic stress in association with decreased activity (Fig. 3A–D). In contrast, increased SirT1 expression has been reported for fasting and caloric restriction [3,89,90], although oxidants increased in muscle, liver, and heart of fasted mice [3,19,89] and aged monkey hearts [91]. Consistent with the fact that SirT1 activity does not always correlate with its expression levels, we found that HFHS diet upregulated SirT1 expression in mouse livers while decreasing its activity (Fig. 4C and D). Also, SirBACO mice, an in vivo model of SirT1 overexpression, showed decreased SirT1 activity after feeding despite even greater increased hepatic SirT1 expression (Fig. 4A–D).

The assay described in this manuscript only measures SirT1 enzyme deacetylase activity and its modulation by post-translational modifications. However, the biology of SirT1 is far more complex and requires additional control experiments including measuring the intracellular NAD+:NADH ratio [43,56], a co-substrate that may become limiting in pathophysiology including aging, ischemia, and metabolic disease [57,92]. SirT1 is the largest member of the sirtuin family and its N- and C-terminal unstructured regions interact with various proteins that function as scaffolds [93], enzyme modulators (aros), or transcription factors [94]. Thus, surrogate markers of SirT1 activity including acetylated-histone H3 or acetylated p53 are excellent endogenous indicators of SirT1 activity. In cardiac myocytes and other cells, SirT1 may translocate from the cytoplasm to the nucleus [95,96]. Thus, intracellular localization could be another factor affecting SirT1 biology.

We established a new specific assay to measure endogenous SirT1 activity in cell and tissue lysates (Fig. 1). Our assay protocol can be used as a template for the testing of a variety of custom synthesized substrate peptides, in addition to the p53 peptide employed by us. In this example reported here, putative small molecule SirT1 activators RSV, SRT1720, or S17834 failed to increase SirT1 deacetylase activity. We confirmed that metabolic and oxidative stress in cells and mice markedly inhibited SirT1 activity despite increased protein expression. Our study underlines the importance of sensitive and specific methods to measure SirT1 activity, in addition to SirT1 protein expression, in order to reliably elucidate the biological function of SirT1, particularly in the settings of metabolic and oxidative stresses, and, most importantly, for drug development of SirT1 activators.

Author disclosure statement

No competing financial interests exist.

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Fig. 4. Endogenous SirT1 activity in liver of WT and SirBACO mice fed normal and high fat high sucrose diet. A) SirT1 activity was measured in liver homogenates from WT (n = 4) and SirBACO mice (n = 4). p53/Ac-p53 peak intensity ratios were calculated from mass spectra shown in Supplemental Fig. 8. Data are presented as means ± SD of N = 4 and were analyzed with unpaired t-test (**p < 0.05). B) Western blot analysis of SirT1 expression levels in liver from WT and SirBACO mice. The ratio of SirT1 to GAPDH between the blots denotes the results of the densitometric analysis. C) SirT1 activity was measured in WT (n = 3) and SirBACO mice (n = 3) fed normal chow (ND) or HFHS diet. p53/Ac-p53 peak intensity ratios were calculated from mass spectra shown in Supplemental Fig. 9. Data are presented as means ± SD of N = 3 and were analyzed with one-way ANOVA followed by Bonferroni’s post-test (*p < 0.05, ***p < 0.001). D) Western blot analysis of SirT1 expression levels in mouse liver. The ratio of SirT1 to GAPDH between the blots denotes the results of the densitometric analysis.
Appendix A. Supplementary data

Supplementary text can be found at https://doi.org/10.1016/j.rexd.2019.101150.

References

1. M.C. Haigis, D.A. Sinclair, Mammalian sirtuins: biological insights and disease

2. S. Michan, D. Sinclair, Sirtuins in mammals: insights into their biological function,

3. H.Y. Cohen, C. Miller, K.J. Bitterman, N.R. Wall, B. Hekking, B. Kessler,

4. J.C. Milne, P.D. Lambert, S. Schenk, D.P. Carney, J.J. Smith, D.J. Gagne, L. Jin,

5. O. Medvedik, D.A. Sinclair, J.M. Olefsky, M.R. Jirousek, P.J. Elliott, C.H. Westphal,

6. J. Beta, X. Li, DYRK1A and DYRK3 promote cell survival under stress, Cell 107

7. S. Bajpai, C.A. Reinhart-King, I. Mattagajasingh, C.-S. Kim, A. Naqvi, T. Yamamori, T.A. Ho

8. P. J. Elliott, M. Jirousek, Sirtuins: novel targets for metabolic disease, Curr. Opin.

9. J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu,

10. M. Potente, M. Robinson, M.C. Motta, E. van Aeen, A. Czopik, A.D. Steele, H.

11. N. Reim, N. Schneider, T. Weightman, N. Grunert, T. Stumvoll, P. Zimny-Arndt,

12. J. DeRicco, K. Kasuno, K. Irani, SIRT1 promotes endothelium-dependent vascular

13. D. Wegener, C. Hildmann, A. Schwienhorst, Recent progress in the development of

14. P.A. Marcotte, P.L. Richardson, P.R. Richardson, J. Guo, L.W. Barrett, N. Xu,

15. J.E. Kim, J. Chen, Z. Lou, DBC1 is a negative regulator of SIRT1, Nature 451 (2008)

16. L. Seidah, J. Elie, M. Parent, J. Dessy, K. Jones, G. Labrousse, C. Carpentier,

17. T. McDonagh, J. Hixon, P.S. DiStefano, R. Curtis, A.D. Napper, Microplate

18. T. McDonagh, J. Hixon, P.S. DiStefano, R. Curtis, A.D. Napper, Microplate

19. D. Shao, et al.

20. X. Guo, J.G. Williams, T.T. Schug, X. Li, DYRK1A and DYRK3 promote cell survival

21. S.P. Gygi, D.A. Sinclair, F.W. Alt, M.E. Greenberg, Stress-dependent regulation of

22. M. Jung, A. Schwienhorst, Phosphorylation regulates SIRT1 function, PLoS One 3 (2008)

23. K. Deursen, G.J. Gores, J. Chen, Z. Lou, E.N. Chini, Deleted in breast cancer

24. T. McDonagh, J. Hixon, P.S. DiStefano, R. Curtis, A.D. Napper, Microplate

25. T. McDonagh, J. Hixon, P.S. DiStefano, R. Curtis, A.D. Napper, Microplate

26. J.S. Smith, C.B. Brachmann, I. Celic, M.A. Kenna, S. Muhammad, V.J. Starai,

27. J. Biomol. Screen 8 (2003) 89

28. S.-R. Yang, J. Wright, M. Bauter, K. Seweryniak, A. Kode, I. Rahman, Sirtuin reg-

29. B. Heltweg, M. Jung, A homogeneous nonisotopic histone deacetylase activity

30. B. Heltweg, F. Dequiedt, E. Verdin, M. Jung, Nonisotopic substrate for assaying both

31. M. Linguat, A. Schwienhorst, J. Menez, E. Verdin, A. Schwienhorst, A. Schwienhorst,

32. J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu,

33. J. B. Yoon, H. Kang, J. Park, E. Kang, C. Lee, M. Park, J. Yoon, Y. Kim, C.

34. B. Heltweg, F. Dequiedt, E. Verdin, M. Jung, Nonisotopic substrate for assaying both

35. B. Heltweg, F. Dequiedt, E. Verdin, M. Jung, Nonisotopic substrate for assaying both

36. J. Auwerx, Resveratrol improves mitochondrial function and protects against me-

37. T. McDonagh, J. Hixon, P.S. DiStefano, R. Curtis, A.D. Napper, Microplate

38. T. McDonagh, J. Hixon, P.S. DiStefano, R. Curtis, A.D. Napper, Microplate

39. B. Heltweg, M. Jung, A homogeneous nonisotopic histone deacetylase activity

40. P.A. Marcotte, P.L. Richardson, P.R. Richardson, J. Guo, L.W. Barrett, N. Xu,

41. D. Wegener, C. Hildmann, A. Schwienhorst, Recent progress in the development of

42. M.M.M. Bachschmid, S. Schildknecht, R. Matsui, R. Zee, D. Hauesler, R.A. Cohen,

43. J.S. Smith, C.B. Brachmann, I. Celic, M.A. Kenna, S. Muhammad, V.J. Starai,

44. K. Ho

45. J.C. Milne, P.D. Lambert, S. Schenk, D.P. Carney, J.J. Smith, D.J. Gagne, L. Jin,

46. O. Medvedik, D.A. Sinclair, J.M. Olefsky, M.R. Jirousek, P.J. Elliott, C.H. Westphal,

47. D. Shao, F.L. Berridge, J.S. Nicholls, A. Schwartz, A. Humphrey, J. DeBord,

48. S. Bajpai, C.A. Reinhart-King, I. Mattagajasingh, C.-S. Kim, A. Naqvi, T. Yamamori, T.A. Ho

49. J.S. Smith, C.B. Brachmann, I. Celic, M.A. Kenna, S. Muhammad, V.J. Starai,

50. J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu,

51. J. B. Yoon, H. Kang, J. Park, E. Kang, C. Lee, M. Park, J. Yoon, Y. Kim, C.

52. J. B. Yoon, H. Kang, J. Park, E. Kang, C. Lee, M. Park, J. Yoon, Y. Kim, C.

53. J. B. Yoon, H. Kang, J. Park, E. Kang, C. Lee, M. Park, J. Yoon, Y. Kim, C.

54. J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu,

55. J. B. Yoon, H. Kang, J. Park, E. Kang, C. Lee, M. Park, J. Yoon, Y. Kim, C.

56. J. B. Yoon, H. Kang, J. Park, E. Kang, C. Lee, M. Park, J. Yoon, Y. Kim, C.

57. J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu,

58. J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu,

59. J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu,

60. J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu,
as novel metabolites derived from the Sir2 family of beta-NAD+ dependent histone/protein deacetylases, J. Biol. Chem. 277 (2002) 18535–18544, https://doi.org/10.1074/jbc.M206712000.

[46] K.G. Tanner, J. Landry, R. Sternglanz, J.M. Demu, silent information regulator 2 family of NAD-dependent histone/protein deacetylase generates a unique product, 1-O-acyl-ADP-ribose, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 14178–14182, https://doi.org/10.1073/pnas.220546697.

[47] M. Pacholer, J.E. Blesasdale, B. Chruny, D. Cunningham, D. Flynn, R.S. Garufolo, D. Griffith, M. Grills, C. Sadek, T. Fedorov, X. Qiu, B. Stockman, V. Tanahashi, A. Varghese, J. Ward, J. Wittka, K. Ahn, SRT210, SRT213, SRT416, and re- sveratrol are not direct activators of SIRT1, J. Biol. Chem. 285 (2010) 8340–8351, https://doi.org/10.1074/jbc.M109.052496.

[48] M. Sethuraman, M.E. McComb, H. Huang, S. Huang, T. Heibeck, C.E. Costello, R.A. Cohen, Isopeptide-coded affinity tag (ICT) approach to reductive proteomics: identification and quantification of oxidant-sensitive cystine thiols in complex protein mixtures, J. Proteome Res. 3 (2004) 1228–1233, https://doi.org/10.1021/pr049877c.

[49] S. Hou, L. Shi, H. Lei, Biotin-streptavidin affinity purification of RNA-protein complexes assembled in vitro, Methods Mol. Biol. 1421 (2016) 23–34, https://doi.org/10.1007/978-1-4939-3591-8_3.

[50] E.K. De La Fuente, C.A. Dawson, L.D. Nelin, R.D. Bongard, T.L. McAuliffe, T.J. Verbeuren, R.A. Cohen, Polyphenols stimulate AMP-activated protein kinase, inhibitors of the deacetylase SIRT1, J. Med. Chem. 48 (2005) 8045–8052, https://doi.org/10.1021/jm049862f.

[51] A. Holmberg, A. Blomstergren, O. Nord, M. Lukacs, J. Lundeberg, M. Uhlén, The A.W. Brändli, R.G. Parton, K. Simons, Transcytosis in MDCK cells: identification of biochemical mechanisms, Diabetes 59 (2010) 1006–1012, https://doi.org/10.2337/db10-0321.

[52] M.P. Merker, Biotinylation of membrane proteins accessible via the pulmonary route/protein mixtures, J. Proteome Res. 3 (2004) 1228–1233, https://doi.org/10.1021/pr049877c.

[53] M. Schöttmuller, P. Pfeifer, C. Wohlfarth, E. Grego, S. Händler, T. Prozorovski, U. Schulze-Topphoff, D. Kübel, M. Greulich, M. Rostovtsiauskaja, From sirtuin to human biology: an update, J. Biol. Chem. 287 (2012) 44244–44252, https://doi.org/10.1074/jbc.M112.407268.

[54] J. Yuan, K. Loo, L. Liu, S. Rawat, Regulation of SIRT1 activity by genotoxic stress, Genes Dev. 26 (2012) 791–796, https://doi.org/10.1101/gad.218482.1112.

[55] O. Zitka, S. Skalicková, J. Gumulec, M. Masarik, V. Adam, J. Hubalek, L. Trnkova, J. Kruseova, T. Eckelschlag, R. Kizek, Redox status expressed as GSH/GSSG ratio as a marker for oxidative stress in paediatric tumour patients, Oncol. Lett. 4 (2012) 1247–1253, https://doi.org/10.3892/ol.2012.931.

[56] H. Yamada, T. Araki, N. Endo, K. Yamashita, K. Fukuda, M. Sasada, T. Uchiyama, LPS-induced ROS generation and changes in gluthione level and their relation to the maturation of human monocyte-derived dendritic cells, Life Sci. 78 (2006) 926–933, https://doi.org/10.1016/j.lfs.2005.10.016.

[57] A.S. Banks, N. Kon, C. Knight, M. Matsumoto, R. Gutiérrez-Juárez, L. Rossetti, W. G. Yu, D. Acili, SirT1 gain of function increases energy efficiency and prevents diabetes in mice, Cell Metabol. 8 (2008) 333–341, https://doi.org/10.1016/j.cmet.2008.08.011.

[58] J.L. Fry, L. Al Sayah, R.M. Weisbrod, I. Van Roy, X. Weng, R.A. Cohen, M.M. Bachschmid, F. Seta, Vascular smooth muscle sirtuin-1 protects against diet-induced aortic stiffness, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 774–778, https://doi.org/10.1073/pnas.1116112105.

[59] M. Kaeberlein, T. McDonagh, B. Heltweg, J. Dixon, E.A. Westman, S.D. Caldwell, A. Napper, R. Curtis, P.S. DiSefano, S. Fields, A. Bedalov, B.K. Kennedy, Substrate-specific activation of sirtuins, Science 280 (2005) 17038–17045, https://doi.org/10.1126/science.1106552.

[60] N.M. Green, Avidin, Protein Chem. 29 (1975) 85–153, https://doi.org/10.1126/science.1300471, Accessed date: 1 November 2016.

[61] E. Morag, E.A. Bayer, M. Wilchek, Reversibility of biotin-binding by selective modification of tyrosine in avidin, Biochem. J. 15 (316) (1996) 193–199 (Pt 1), PMID: 8645205; PMCID: PMC1217322, http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1217322, https://doi.org/10.3892/ol.2012.931.

[62] F.S. Facchini, N. Hua, F. Abbassi, G.M. Revain, Insulin resistance as a predictor of age related diseases, J. Clin. Endocrinol. Metab. 86 (2001) 3574–3578, https://doi.org/10.1210/jcem.86.8.7763.

[63] A.E. Civitarese, S. Carling, L.K. Heilbronn, M.H. Bulver, U. Kropf, A. Deutsch, S.R. Smith, E. Ravussin/CALEIRE Pennington Team, Calorie restriction increases muscle mitochondrial biogenesis in healthy humans, PLoS Med. 4 (2007), https://doi.org/10.1371/journal.pmed.0040076.e007.

[64] H. Arai, K. Yamashita, N. Endo, K. Fukuda, M. Sasada, T. Uchiyama, Regulation of SIRT1 activity by genotoxic stress, Nat. Cell Biol. 9 (2007) 1253–1258, https://doi.org/10.1038/nclinics.2012.931.

[65] R.R. Alcendor, S. Gao, P. Zhai, D. Zablocki, E. Holle, X. Yu, B. Tian, T. Wagner, M.E. McComb, C.E. Costello, R.A. Cohen, M.M. Bachschmid, Redox regulation of sirtuin 1 by S-glutathiolation, Antioxidants Redox Signal. 13 (2010) 1023–1032, https://doi.org/10.1089/ars.2009.6295.

[66] S. Hou, L. Shi, H. Lei, Biotin-streptavidin affinity purification of RNA-protein complexes assembled in vitro, Methods Mol. Biol. 1421 (2016) 23–34, https://doi.org/10.1007/978-1-4939-3591-8_3.

[67] E.N. Chini, CD38 as a regulator of cellular NAD+: a novel potential pharmacological target for metabolic conditions, Curr. Pharmaceut. Des. 15 (2009) 57–63 https://doi.org/10.2174/138161209784877246.

[68] R.S. De, G.B. Yoo, V. Petrie, D.C. Jiang, S. Xu, T. Verbeuren, M.E. McComb, C.E. Costello, R.A. Cohen, M.M. Bachschmid, Redox regulation of sirtuin 1 by S-glutathiolation, Antioxidants Redox Signal. 13 (2010) 1023–1032, https://doi.org/10.1089/ars.2009.6295.

[69] C. Olszewska, F.K. Satterberg, C. Uhlén, M. Rostovtsiauskaja, From sirtuin to human biology: an update, J. Biol. Chem. 287 (2012) 44244–44252, https://doi.org/10.1074/jbc.M112.407268.
the heart, Circ. Res. 100 (2007) 1512–1521, https://doi.org/10.1161/01.RES.000027723.65669.64.

[92] S.J. Lin, P.A. Defossez, L. Guarente, Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae, Science 289 (2000) 2126–2128 http://www.ncbi.nlm.nih.gov/pubmed/11000115 , Accessed date: 2 February 2014.

[93] B.J. North, B.L. Marshall, M.T. Borra, J.M. Denu, E. Verdin, The human Sir2 ortholog, SIRT2, is an NAD+ dependent tubulin deacetylase, Mol. Cell. 11 (2003) 437–444 http://www.ncbi.nlm.nih.gov/pubmed/12620211 , Accessed date: 26 December 2013.

[94] H.-L. Cheng, R. Mostoslavsky, S. Saito, J.P. Manis, Y. Gu, P. Patel, R. Bronson, E. Appella, F.W. Alt, K.F. Chia, Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice, Proc. Natl. Acad. Sci. U. S. A. 100 (2003), https://doi.org/10.1073/pnas.1934713100 10794-9.

[95] M. Tanno, J. Sakamoto, T. Miura, K. Shimamoto, Y. Horio, Nucleocytoplasmic shuttling of the NAD+ dependent histone deacetylase SIR2T1, J. Biol. Chem. 282 (2007) 6823–6832, https://doi.org/10.1074/jbc.M609554200.

[96] Q. Jin, T. Yan, X. Ge, C. Sun, X. Shi, Q. Zhai, Cytoplasm-localized SIRT1 enhances apoptosis, J. Cell. Physiol. 213 (2007) 88–97, https://doi.org/10.1002/jcp.21091.