SIRT3 SNPs validation in 640 individuals, functional analyses and new insights into SIRT3 stability

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Abstract. Sirtuins are critical players within multiple cellular pathways such as stress response, apoptosis and energy metabolism. They are associated with metabolic and degenerative diseases, the pathogenesis of cancer and are key elements in the regulation of cellular life span. From within the 7 known human sirtuins, SIRT3 recently stepped out of the shadow of SIRT1 showing strong effects on stress response, apoptosis, cell cycle and energy metabolism, mimicking effects of caloric restriction. We have identified two non-synonymous human SIRT3 SNPs and evaluated their impact on SIRT3 activity and stability. We assessed their influence on cellular energy metabolism in relation to SIRT1 and identified SIRT3 to increase cellular respiration by 80% when compared to SIRT1, which increased cellular respiration by only 30%.

Introduction

The sirtuins represent a family of enzymes that contain phylogenetically highly conserved catalytic domains that catalyze NAD^+-dependent protein deacetylation and/or ADP-ribosylation. In numerous model organisms they have been described to be important regulators of longevity and elements that are essential in the regulation of transcriptional silencing, apoptosis and cellular stress response. Also, they are known to be essential in the regulation of cellular metabolism, the pathogenesis of cancer and degenerative diseases and thus, they have recently gained significant attention as molecular targets in the development of novel epigenetic therapeutics (1-4). To date, seven human sirtuins have been reported out of which SIRT1 has been the most intensively studied (2-4).

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More recently, SIRT3, the major mitochondrial deacetylase came to the fore, presenting itself with a major impact on energy metabolism, stress response and with distinct effects on apoptosis and cell cycle regulation (Fig. 1B, C, E and F) (5-12). In analogy to known SIRT1 effects within the cell nucleus, SIRT3 interacts with mitochondrial substrate isoforms of (cytosolic or nuclear) SIRT1 substrates such as Acetyl CoA-synthetase and FOXO3a (Fig. 1B and D) (11,13). Also, a recent proteomic analysis identified 20% of mitochondrial proteins to be acetylated; including many metabolic enzymes and longevity regulators, all of which represent putative substrates for SIRT3, since SIRT3 knockout was shown to go along with global mitochondrial hyperacetylation (5,14). SIRT3 is a mitochondrial protein, which is being activated in the mitochondrial matrix through cleavage to the 28 kDa isoform by the matrix processing peptidase (MPP) (Fig. 1A) (15). Interestingly, SIRT3 activates the nuclear transcription of mitochondria related genes such as UCP1 (uncoupling protein 1), PGC-1α (peroxisome proliferator-activated receptor γ) and COX (cytochrome oxidase) IV and V, which activate CREB (7). Similar to the class I and II HDACs, the sirtuins are differentially expressed in different types of cancer and we have been able to show that SIRT1 and SIRT3 are overexpressed in acute myeloid leukaemia (unpublished data) (16,17). SIRT3 overexpression increases cell respiration, reduces the mitochondrial membrane potential and the level of reactive oxygen species, thus mimicking the effects of calorie restriction on cell metabolism and senescence (7).

The more recent literature on SIRT3 gives inconsistent information on the existence and impact of SIRT3 SNPs, e.g., on the relevance of SIRT3 SNPs in conjunction with survival in the elderly (18). In order to further elucidate the relevance of putative SIRT3 SNPs, we focussed our analyses on coding, non-synonymous SNPs (nSNPs), since these predicted an alteration in the SIRT3 amino acid sequence. The identification and validation of SIRT3 nSNPs could pave the way for further associative studies linking sirtuin biology with metabolic disease, organ degeneration and cancer.

In our study we validated two out of five putative non-synonymous coding SIRT3 SNPs (R80W and V208I) and evaluated their impact on SIRT3 enzymatic activity and protein stability in relation to wtSIRT3 and wtSIRT1. Interestingly,

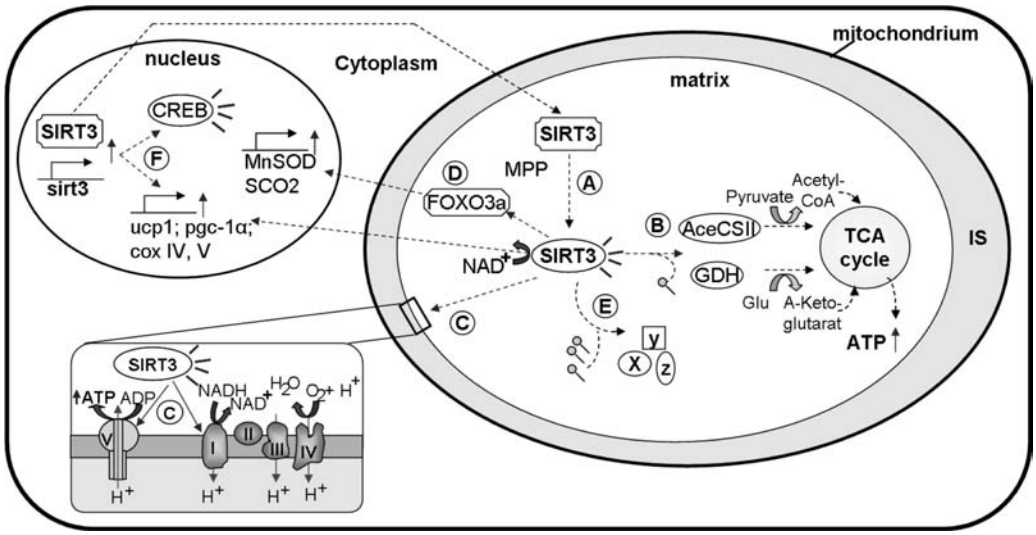

Figure 1. Overview on known SIRT3 interaction partners in energy metabolism. IS, intermembrane space. (A) SIRT3 processing via MPP; (B) SIRT3 deacetylates: AceCSII, GDH, Ku-70; (C) subunits of complex I and V; (D) FOXO3a; (E) 20% of mitochondrial proteins are acetylated, presenting putative SIRT3 substrates; (F) SIRT3 induces CREB and expression of ucp1, pgc-1α, complex IV and V.

Table I. Putative SIRT3 nSNPs.

dbSNP rs no.	AB assay no.	Sequence [Vic/Fam] (sense/anti sense = s/as)	Exon	Codon-position	AS-exchange
rs28365927	C_25753927_10	CTCC[A/G]GAAT (as)	1	1	R80W
rs11246020	C_25754220_10	GTGA[C/T]GTTG (as)	3	1	V208I
rs1734492	C_7503249_10	AGCC[C/G]GAGA (as)	3	1	R214G
rs1734491	C_7503273_10	GCAG[G/T]TGGC (as)	4	2	T255N
rs3020901	SIRT3_0901-0901	ACGC[C/T]GTGA (as)	6	1	G369S
rs7927733	SIRT3_5927-5927	TTTTC[A/C]GGTG (s)	Intron 5	---	Splice site

despite the growing number of SIRT3 publications and its manifold cellular functions, SIRT3 protein stability has not been addressed so far and is one of the topics in the study presented herein.

Materials and methods

Genomic DNA isolation. Genomic DNA from 640 healthy Caucasian donors was extracted from 5 ml peripheral blood by salting out with subsequent quantification on a Nanodrop ND-1000 Spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany).

In silico analyses. *In silico* analyses were performed on the dbSNP (NIH) (19), HapMap (20), and ABI (Applied Biosystems) databases (21). Analyses were focused on coding, non-synonymous SIRT3 (AF083108) SNPs. Previously validated SNPs from the literature were also included.

Allelic discrimination. Allele specific SNP-assays were performed by TaqMan-PCR on an ABI Prism 7700 Sequence Detection System (ABI, Darmstadt, Germany) in accordance with the instructions provided by the manufacturer (Table I).

Cell culture, transfections. Hek293T and HeLa (DSMZ, Braunschweig) and H-JR-Mito-HeLa cells (Marinpharm, Luckenwalde), were cultured in the presence of 1% non-essential amino acids (Biochrom AG, Berlin, Germany). Cells were transiently transfected with either Nanofectin (PAA, Pasching, Austria) or Nucleofection for Amaxa-electroporation (Amaxa/Lonza, Köln, Germany) in accordance with manufacturers' instructions. Transfections of different vector constructs were performed with adjusted molar amounts.

Single protein monitoring through multiparametric biosensing. The impact of single overexpressed sirtuin proteins (wtSIRT3, $SIRT3_{R80W}$, $SIRT3_{V208I}$, wtSIRT1) on cellular metabolism was monitored subsequent to transient transfection of equimolar amounts of eukaryotic expression constructs into HeLa cells and real-time *in vitro* monitoring of glycolysis, respiration and cell adhesion with the Bionas biosensor chip system (Bionas, Rostock, Germany) (22).

Histone isolation. Histone proteins were extracted from 293T cells as previously published after incubation with 25 nM Trichostatin A (TSA) (Sigma Aldrich, Deisenhofen, Germany) and 5 mM nicotinamide (NAM) (Sigma Aldrich) (23).

Immunoprecipitation and SIRT3 activity test via histone deacetylation assay. Flag tagged wtSIRT3, $SIRT3_{R80W}$, $SIRT3_{V208I}$ were immunoprecipitated and used for *in vitro* deacetylation assays as previously described (11).

Protein stability analyses. Twenty-four hours after transient transfection of wtSIRT3, $SIRT3_{R80W}$ or $SIRT3_{V208I}$ HeLa cells were incubated with cycloheximide (Sigma Aldrich) in order to block protein synthesis for up to 48 h. Protein samples were prepared at different time points and analyzed by Western blotting.

Western blotting. Western blots were performed according to standard protocols on nitrocellulose membranes (Biorad, Hercules, CA) and visualized by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK).

Plasmids and antibodies. Eukaryotic expression constructs that have been used in this study: hSIRT3-Flag (GenBank AF083108, pcDNA3.1) (kindly provided by E. Verdin, The Gladstone Institute, San Francisco, CA) and hSIRT1-Flag (GenBank AF083106, pCMVtag4a; Gibco Invitrogen, Carlsbad, CA). Site-directed mutagenesis (QuikChange Mutagenesis Kit, Stratagene, Cedar Creek, TX) was carried out to generate the $hSIRT3_{H248Y}$-FLAG, $hSIRT3_{R80W}$, $hSIRT3_{V208I}$ constructs. SIRT3 wt, SNP and inactive mutants were further cloned into the pEGFP-C1 vector (Clontech Laboratories, Saint-Germain-en-Laye, France) via EcoRI restriction sites. All construct sequences were verified by direct DNA sequencing. Antibodies that were used for immunoblotting: anti-Flag M2, anti-tubulin (all from Sigma-Aldrich), anti-SIRT3 (Imgenex, San Diego, CA), anti-c-Myc (own production), anti-H4 (ab31827; Abcam, Cambridge, UK) and antiH4-Lys16 (Upstate Biotechnology, Hamburg), anti-rabbit (Dianova, Hamburg), anti-mouse (Dianova).

Statistical analysis. SNPs were analyzed for concordance with the Hardy-Weinberg equilibrium with the online calculator of Michael H. Court (http://www.tufts.edu/~mcourt01).

Results and Discussion

Mitochondrial dysfunction is linked to a number of metabolic diseases, degenerative disorders and cancer. Based on the multifaceted mitochondrial functions of SIRT3, SIRT3 nSNPs could be of pathophysiological significance in this context.

Identification and validation of putative SIRT3 nSNPs. *In silico* analyses of three SNP databases [dbSNP (19), HapMap (20), and ABI (21)] for putative SIRT3 nSNPs (AF083108) allowed the identification of six candidate SNPs (Table I, Fig. 2), of which one SNP was located within a putative splice site (19).

$SIRT3_{R80W}$ is localized at the N-terminal end, which is being processed after mitochondrial import; $SIRT3_{V208I}$, $SIRT3_{R214G}$ and $SIRT3_{T255N}$ are part of the conserved active deacetylase domain and $SIRT3_{G369S}$ is located within the SIRT3 C-terminus.

Validation of putative SIRT3 nSNPs from in silico data. Putative SIRT3 nSNPs (Table I) were analyzed for allelic frequencies in 640 DNA samples from healthy donors by quantitative Taqman PCR SNP analysis and were then compared to database data (if available). While our analyses confirmed two nSNPs, $SIRT3_{R80W}$ and $SIRT3_{V208I}$, the other four candidate SNPs were identified to be either false positives or at least not present in Caucasians (Fig. 3) since they did not reveal any allelic variations.

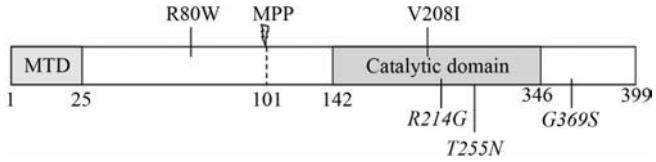

Figure 2. Identified and validated SIRT3 nSNPs localised to functional SIRT3 domains. SNPs identified as false positive in the Caucasian population are presented in italic letters. MTD, mitochondrial target sequence; MPP, matrix processing peptidase.

Genotype frequency analyses for SNP $SIRT3_{R80W}$ identified 72% to be homozygous (C/C), 23.5% heterozygous (C/T) and 4.5% homozygous (T/T) for the wt and variant alleles respectively. At the beginning of our analyses the databases (dbSNP Build ID: build 126) gave only information about $SIRT3_{R80W}$ frequencies in people of African origin (80.6% G/G, 19.4% A/G). In fact, for Caucasians, data on $SIRT3_{R80W}$ are given in the HapMap database with the following frequencies: 71.7% wt, 26.5% heterozygous and 1.8% homozygous for variant allele, which is close to the data that have been generated in our laboratory.

For $SIRT3_{V208I}$ we revealed a genotype frequency of 60% homozygous (G/G), 35% heterozygous (G/A) and 6% homozygous (A/A) for the wt and variant alleles, compared to 72, 23 and 5% in the databases, respectively (20) (Fig. 3). However, the data that have been generated in our laboratory are statistically more reliable, since our sample size of 640 significantly exceeds the HapMap sample size of 113.

Our results on non-validated SIRT3 nSNPs are in accordance with HapMap data, revealing 100% homozygous wt genotypes for false positive SNPs (19,20). Genotype frequencies for $SIRT3_{R80W}$ (1.866) and $SIRT3_{V208I}$ (0.087) were both in accordance with the Hardy-Weinberg equilibrium (p=0.172 and 0.766, respectively).

As a result of our study we found databases to give an incorrect picture for SIRT3 SNP allelic frequencies, since these are based on unvalidated information. The recent literature on the topic therefore offers not only insufficient but also quite inconsistent data on SIRT3 SNPs and their potential functional impact.

Rose *et al* postulated an impact of SIRT3 SNP_{G477T} on survival in the elderly (24). This SNP did not lead to a change in the amino acid level (Ser159Ser) and it was shown by subsequent analyses by the authors themselves, that the putative impact of this SNP was due to a linkage disequilibrium with a VNTR (variable number of tandem repeats) allele specific enhancer located in intron 5 of SIRT3 (25). Since we had focussed our analyses on nSNPs and a recently reported meta-analysis on 2,461 samples by Lescai *et al* was not able to confirm the effect of this SNP or the VNTR allele specific enhancer, they were not considered in the context of this study (18). In addition, in that study no positive association

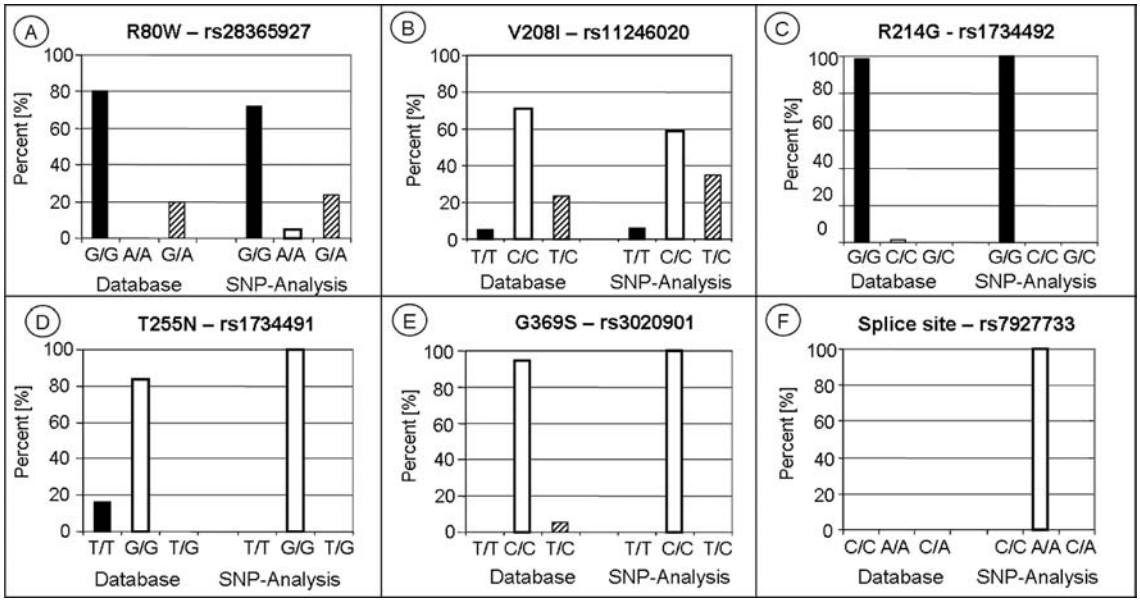

Figure 3. SIRT3 SNPs - distribution of allelic frequencies in 640 healthy donors.

was reported except for one SIRT3 SNP (rs939915), which we had, however, identified to be an SNP of the neighbouring gene psmd13, but unfortunately not a SIRT3 SNP.

Functional analyses of validated SIRT3 nSNPs

Impact of SIRT3 SNPs on protein activity. Both validated SIRT3 nSNPs, $SIRT3_{R80W}$ and $SIRT3_{V208I}$ go along with changes in the SIRT3 amino acid sequence and may affect SIRT3 protein levels and SIRT3 functional activity. Based on the SIRT3 histone deacetylase activity on histone $H4_{K16}$ (26), SIRT3 deacetylation activity was assessed for wtSIRT3 and its SNP mutants on hyperacetylated H4 histone proteins in order to further assess their impact on SIRT3 activity (Fig. 4A). Western blot analyses showed reduced signals of acetylated $H4_{K16}$ without significant differences for wtSIRT3 and the SIRT3 nSNPs. Incubation with the sirtuin specific inhibitor NAM as well as the SIRT3 inactive mutant completely abrogated both, wt and nSNPs SIRT3 deacetylase activity.

Impact of SIRT3 SNPs on protein stability. For SIRT3 stability analyses HeLa cells were transfected with SIRT3-flag vectors (wt, R80W, V208I). Twenty-four hours post transient transfection cells were incubated with the protein inhibitor CHX and analyzed for up to 72 h of CHX treatment by Western blotting (Fig. 4B). To visualize the effect of CHX we checked for c-Myc protein levels since c-Myc has a short half-life (<30 min) (27). C-Myc signals were strongly reduced and absent after less than 2 h of CHX protein inhibition, while stable signals during treatment with the CHX solvent DMSO indicated that the solvent had no influence. In contrast to the rapid reduction of signals for c-Myc, the signals for the mitochondrial, mature SIRT3 (maSIRT3) remained constantly elevated with a first decrease after 34 h of CHX treatment.

Our stability analyses, which again showed no difference between wt and SNP mutants, offer the first evidence for SIRT3 stability. While the mean half-life of mitochondrial proteins in mammalian cells is around 3.5 to 5 days, for some proteins the half-lives are as short as 20 to 80 min (28). The SIRT3 half-life has been determined to be around 48-60 h, which appears to be rather short as it is ranges below the mean half-life for most mitochondrial proteins. Also, it appears to be more stable than it was predicted *in silico* (Expasy, ProtParam software), which anticipated a half-life of around 4.4 h for mammalian SIRT3 (29).

Monitoring of SIRT3 bioactivity. We further analyzed the influence of wtSIRT3, its SNP mutants and wtSIRT1 on cellular metabolism (Fig. 5) subsequent to transient transfection on a biosensor chip system, which gives the opportunity to analyse the effect of a specific protein on cell metabolism in living cells through real-time monitoring of metabolically relevant parameters, such as oxygen consumption, acidification rate and cell adhesion allowing conclusions towards cellular energy metabolism, specially respiration and glycolysis.

These analyses show that SIRT3 overexpression led to a significant increase of cellular respiration by 40%, which was significantly stronger than the effect that was observed for an overexpression of SIRT1 (15%). While SIRT3 overexpression had a strong impact on respiration, it had no influence on cellular adhesion or glycolysis (data not shown). In concordance with our results that were obtained in reference to the potential impact of SIRT3 SNPs on SIRT3 activity and stability, the effects of SIRT3 nSNPs on cellular metabolism were without relevance.

Our study is the first direct comparative analysis of SIRT3 and SIRT1 on cellular metabolism. The stronger increase of cellular respiration for SIRT3 may be based on the fact that SIRT3 has both indirect (7,30) and direct activating effects on energy metabolism, especially respiration in contrast to SIRT1 (Fig. 1) (6). All these data help to explain the significant increase of respiration due to SIR3 overexpression and emphasize the important role of SIRT3 in cellular energy metabolism. Taking into account a transfection efficiency of 50-60% in our experiments the observed increase

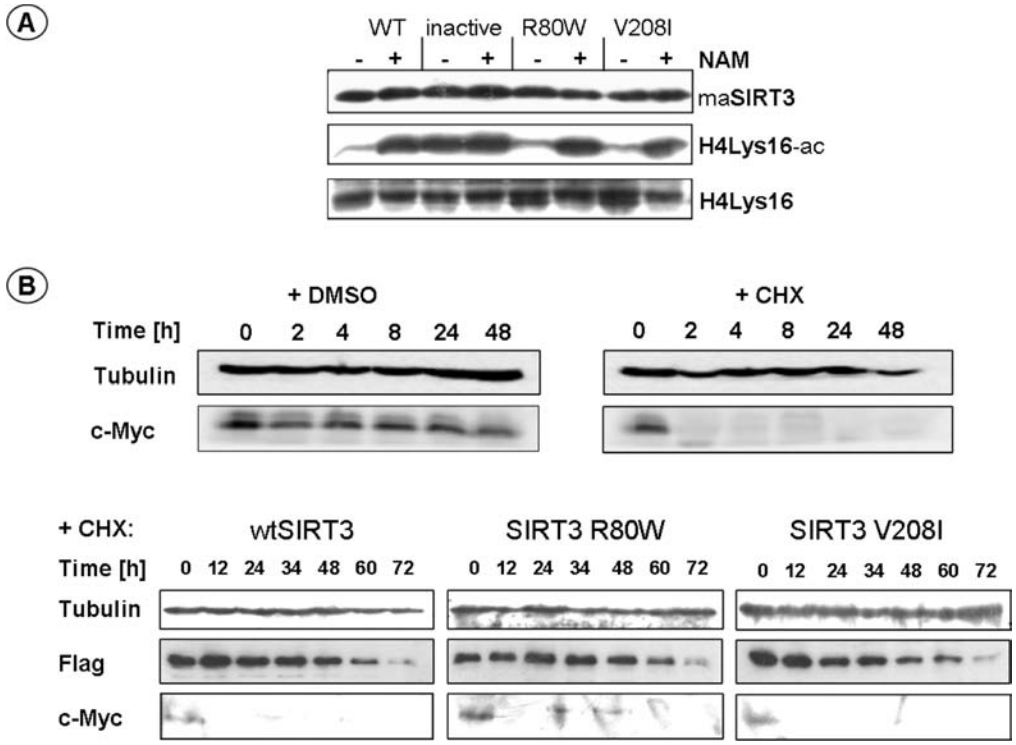

Figure 4. Influence of SIRT3 SNPs on SIRT3 activity and stability. (A) Histone deacetylation assay. Same amounts of purified hyperacetylated histones (H4) and wtSIRT3, $SIRT3_{H248Y}$ (inactive) $SIRT3_{R80W}$ and $SIRT3_{V208I}$ were incubated for 20 min in HDAC-assay-buffer (with NAD) and with or without NAM. (B) SIRT3 stability assay. SIRT3 (wt, R80W, V208I) transfected cells were incubated with CHX, DMSO and kinetically analyzed via Western blotting for tubulin, c-Myc and maSIRT3 signals.

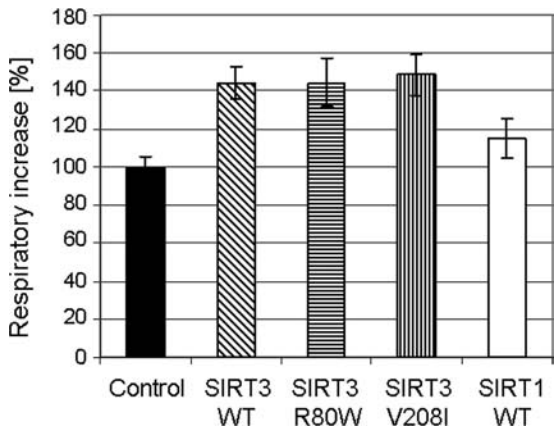

Figure 5. Influence of wtSIRT3 and functional impact of SIRT3 SNPs on cellular respiration in comparison to wtSIRT1. Control approaches were performed with empty vector, SIRT3 inactive mutant and without transfection, respectively.

of respiration of 40 and 15% did not reflect the full potential of SIRT3 and SIRT1, which may be up to around 80 and 30% respectively, assuming a transfection efficiency of 100%. This would be in concordance with preliminary *in vitro* data showing an increase of respiration of 80% in HIB1B cells overexpressing mouse SIRT3 (7).

Possible explanations for the lack of impact of SIRT3 nSNPs. Despite their localization within the conserved deacetylase domain of SIRT3 and the identification of a potential glycosylation site in position aa 207-210 (31), the SNP_{V208I} was not expected to have a strong influence on protein levels since the change in the amino acid was between two amino acids of the same biochemical group.

SNP_{R80W} results in a change from a positively charged, highly basic amino acid into an aromatic, neutral amino acid, which was anticipated to have functional impact. The lack of functional consequences of SNP_{R80W} may in part be explained by its N-terminal localisation, which is most essentially processed after mitochondrial import. The postulated role of the N-terminus in nuclear localisation and function of SIRT3 seems not be affected either (8).

Taken together, our analyses provide the first evidence that SIRT3 is a rather stable protein, with a half-life of more than 48 h and reveal that SIRT3 has only two nSNPs, which however seem to have no functional impact. Furthermore, we show that in living cells SIRT3 has strong activating effects on cellular respiration which are significantly higher than the effect of SIRT1. These results further prompts the emerging importance of SIRT3 in the context of cell survival, metabolism, degenerative disease and cancer and point out that the impressive functions of SIR2 in model organisms (like yeast, *Caenorhabditis elegans*, and *Drosophila* melanogaster) can only be sufficiently assessed in humans in combinational analyses that consider all or at least the most important sirtuin isoforms, under which SIRT1 and SIRT3 are the most important candidates.

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