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► **To cite this version:**

Helen M. Cooper, Johannes N. Spelbrink. The human Sirt3 protein deacetylase is exclusively mitochondrial. *Biochemical Journal*, 2008, 411 (2), pp.279-285. 10.1042/BJ20071624 . hal-00478931

HAL Id: hal-00478931

<https://hal.science/hal-00478931>

Submitted on 30 Apr 2010

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The human Sirt3 protein deacetylase is exclusively mitochondrial.

Helen M. Cooper and Johannes N. Spelbrink*

Institute of Medical Technology and Tampere University Hospital, Tampere, Finland.

*To whom correspondence should be addressed:

Tel: +358 3 35518598; Fax: +358 3 35517710; Email: hans.spelbrink@uta.fi

Short title: **The human Sirt3 protein deacetylase is exclusively mitochondrial.**

Keywords: **Sirtuin, deacetylase, mitochondrial, Leptomycin B, import, immunofluorescence**

Abbreviations: HsSirt3, human Sirt3, HAT, histone acetyl transferase, HDAC, histone deacetylase, AceCS2, mitochondrial acetyl CoA synthetase (2), HEK293, human embryonal kidney 293, U2OS, U2 osteosarcoma, CRM1, chromosome region maintenance 1, LMB, leptomycin B, NP40, nonidet-P40, POLG2, polymerase gamma accessory subunit, P2HA, HA tagged POLG2, siRNA, small interfering RNA, M (as in M143), methionine, mtCOXII, cytochrome *c* oxidase subunit II, cDNA, complementary DNA, EST, expressed sequence tag

Abstract

It has recently been suggested that perhaps as many as 20% of all mitochondrial proteins are regulated through lysine acetylation while Sirt3 has been implicated as an important mitochondrial protein deacetylase. It is therefore of crucial importance that the mitochondrial localization of potential protein deacetylases is unambiguously established. While mouse Sirt3 was recently shown to be mitochondrial, human Sirt3 (HsSirt3) was reported to be both nuclear and mitochondrial and to relocate from the nucleus to the mitochondrion upon cellular stress. We show here using various HsSirt3 expression constructs and a combination of immunofluorescence and careful subcellular fractionation that in contrast to the earlier report human Sirt3 is exclusively mitochondrial. We discuss possible experimental explanations for these discrepancies. In addition, we suggest on the basis of the analysis of public genome databases that the full-length mouse Sirt3 protein is a 37 kDa mitochondrial precursor protein contrary to the previously suggested 29 kDa protein.

Introduction

An important aspect of the regulation of chromatin remodelling and consequently gene-expression involves post-translational modifications of histones, abundant nuclear DNA-binding proteins with regulatory and packaging functions [1, 2]. These modifications include phosphorylation, methylation and acetylation. Acetylation of lysine residues involves histone acetyl transferases (HATs) such as P300/CBP, while deacetylation involves histone deacetylases, or HDACs. HDACs can be divided in three distinct families based on their domain architecture, and originally based on similarity with yeast HDACs [3]. Class I and II HDACs share similar catalytic domains, whereas the Class III 'Sir' or Sirtuin family of deacetylases are catalytically distinct and depend on NAD⁺ as a cofactor. Although the names suggests HDACs are specific for histones, this is not the case. Indeed, many proteins have been identified as targets of HATs and HDACs, many of them transcription factors, but also for example the cytoplasmic protein tubulin, a major structural component of the microtubular network [4]. In addition, in recent years acetylation/deacetylation has been show to play an important role in protein stability [5].

The deacetylase Sirt3 as well as Sirt4 and 5 were recently identified as mitochondrial members of the Sir family of deacetylases [6-8], which suggested the possibility of acetylated mitochondrial protein substrates. A recent proteomics approach to identify lysine-acetylated proteins indeed has suggested that as many as 20% of all mitochondrial proteins are regulated by lysine acetylation [9]. The first bona fide human Sirt3 (HsSirt3) substrate to be identified was the mitochondrial acetyl CoA synthetase or AceCS2 [10, 11] and further work has now shown that Sirt3 in mouse is a global

mitochondrial deacetylase involved in the regulation of steady-state acetylation levels of many proteins [12]. Whereas Sirt3 is a deacetylase, Sirt4 has been shown to be an ADP-ribosylase functioning in the regulation of Insulin secretion [13, 14]. The identification of Sirt3 and Sirt4 substrates and the involvement of Sirt1 in e.g. metabolic syndrome has suggested roles of several Sirt proteins in the regulation of whole organism metabolism in response to food availability [15-18]. Finally, both Sirt3 and 4 but not Sirt 5 have been implicated in nutrient sensing via mitochondrial NAD⁺ levels [19].

In order to understand the consequences of mitochondrial protein acetylation and deacetylation it is of crucial importance to unambiguously establish the mitochondrial localization of HDACs. The human Sirt3 has an additional 142 amino acids at its N-terminus. Whereas the mouse protein is exclusively mitochondrial [12], it was recently suggested that the N-terminal extension of human Sirt3 mediates an initial localization to the nucleus [20] and mitochondrial relocation occurs upon stress, induced by its own overexpression, by UV damage or etoposide. A second family II HDAC, HDCA7 has been shown to be localized in the inner mitochondrial membrane and nucleus and relocate to the cytoplasm upon apoptosis inducing stress [21]. In this paper we re-address the issue of HsSirt3 nuclear localization by using similar approaches [20] and show contrary to the original publication that human Sirt3 is exclusively mitochondrial similar to the mouse protein.

Materials and Methods

Reagents and antibodies

All common reagents used in this study were of analytical grade. Leptomycin B was purchased from Sigma (St. Louis, USA), Optiprep™ (Iodixanol) from Axis-Shield (Oslo, Norway). Antibodies used in this study were as follows: HDAC7 (sc-11421) and Sirt3 (sc-49744) from Santa Cruz (Santa Cruz, USA); anti c-myc 9E10 monoclonal from Roche Molecular Biochemicals (Mannheim, Germany); anti-FLAG M2 monoclonal from Sigma; anti-HA monoclonal HA.11 from Covance (Richmond, USA); anti Sirt3 from Abcam (40006) (Cambridge, UK); anti mtCOX II, Alexa Fluor® 488 or 568 secondary antibodies from Invitrogen (Carlsbad, USA). HDAC1 and nucleoporin antibodies were from Santa Cruz (Santa Cruz, USA) and Zymed (San Francisco, USA), respectively (kind gifts from Keijo Viiri, Institute of Medical Technology Tampere). HsSirt3 siRNAs and non-targeting siRNAs were from Dharmacon as follows:

	Catalog no	Sense sequence of siRNA duplex
HsSirt3 #1	D-004827-01	CAA CGU CAC UCA CUA CUU UUU
HsSirt3 #2	D-004827-02	GAA CUC CCA UUC UUC UUU CUU
HsSirt3 #4	D-004827-04	GGA GUG GCC UGU ACA GCA AUU
Non-targeting	D-00121-01-05	UAG CGA CUA ACA CAU CAA

Cloning of expression constructs

Full length HsSirt3 was originally obtained by RT-PCR using highly purified HeLa mRNA, and cloned in pcDNA3.1(-) /Myc-His A (Invitrogen) using Not I and BamHI.

Truncated (Δ 1-142) HsSirt3-Myc-His and HsSirt3-FLAG were obtained by PCR amplification from the original full-length construct and cloned either in pcDNA3.1(-)

/Myc-His A or pcDNA5/FRT/TO (for FLAG) (Invitrogen). The FLAG epitope was introduced at the C-terminus by PCR and followed immediately by a stop-codon (all primer sequences can be obtained upon request). All constructs were fully verified by sequencing.

Stable inducible cell-lines, transient transfections and siRNA transfections

Stable inducible cell lines expressing HsSirt3-FLAG and POLG2-HA were generated and maintained as previously described [22]. To induce expression the indicated amount of doxycycline (Sigma) was added to the growth medium from an ethanol stock, and cells were processed for further analyses following the required induction. U2OS and HeLa cells were maintained in DMEM medium (Cambrex Bioscience) with 2 mM L-glutamine (Cambrex Bioscience) and 10% FCS (Euroclone). For immunofluorescence cells were transfected using TransIT[®]-LT1 transfection reagent (Mirus, Madison, USA) according to the manufacturer's instructions and 1 µg plasmid per 6-well plate well. Leptomycin B was added as required at 5 ng/ml 2-3 hours prior to processing for immunofluorescence or Western blot analysis. For the RNAi, cells were transfected with a total of 420 pmol of siRNAs/10 cm plate, either a combination of 3 HsSirt3 specific siRNAs or a single non-targeting siRNA, using Lipofectamine 2000 reagent at 30 µl/420 pmol of RNA according to the manufacturers' instructions. Cells were grown for 48hrs after transfection.

Immunofluorescence

For immunofluorescent detection cells were grown on coverslips in 6 well plates.

Following transfection for 1-2 days and Leptomycin B treatment cells were fixed using

either 3.3% paraformaldehyde (PFA) in cell culture medium for 25 min or in methanol for 5 min at -20°C [23]. This was followed by three washes in PBS and lysis for 10 min with 0.5% Triton X100 in PBS/10% fetal calf serum (FCS) after PFA fixation. No lysis step was performed after methanol fixation. Primary and secondary antibodies were incubated at recommended concentrations in PBS/10%FCS for 1 hr-o/n. Mitotracker[®] Red CMXRos treatment was done prior to fixation essentially as described [24]. Slides were mounted using ProLong[®] Gold antifade with DAPI (Invitrogen). Image acquisition using confocal microscopy was carried out as described [25], using an Andor iXon DV885 EMCCD camera and the Andor iQ software (Andor, Belfast N. Ireland). Images were further processed using Photoshop CS2.

Subcellular fractionation and Western blot analysis

Cells were isolated by centrifugation and washed once with ice cold PBS. For hypotonic lysis the cell pellet was resuspended by gentle pipetting in 2-3 volumes of ice-cold homogenization buffer (4 mM Tris-HCl, pH7.8, 2.5 mM NaCl, 0.5 mM MgCl₂, 0.1 mM PMSF), kept on ice for 6 min, then homogenized in a glass homogenizer with 20-25 strokes of a tight-fitting pestle. Disruption of the cells was monitored by microscopy. One-ninth volume of 2M sucrose, 10 mM Tris-HCl, 1 mM EDTA pH 7.8 was added following lysis and nuclei and cell debris were pelleted by centrifugation at 1,200xg max for 3 min at 4 °C. Mitochondria from the post-nuclear supernatants were recovered by centrifugation at 12,000xg max for 3 min at 4 °C. Mitochondrial pellets were washed once with 1 ml of ice-cold sucrose wash solution (0.25 M Sucrose, 25 mM KCl, 5 mM MgCl₂, 20 mM Tricine-KOH, pH 7.8) and the mitochondrial pellet lysed for 15 min on

ice in 50 mM TrisHCl pH7.5, 300 mM NaCl, 1 mM EDTA, 1% Triton X100. An equal volume of 2x Laemmli sample buffer was added and the sample was denatured at 95°C for 5 min prior to SDS-PAGE. Nuclear pellets following hypotonic lysis were either extracted first by 0.5% NP40 in 50 mM TrisHCl pH7.5, 150 mM NaCl, again pelleted and extracted with 20 mM Tris-HCl at pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1 mM PMSF, 0.5 mM DTT [26] for 20 min and centrifuged at 20,000 xg for 30 min. Alternatively, the low-speed pellet obtained from hypotonic lysis was further purified on a 25%/30%/35% Optiprep gradient to obtain purer nuclear fractions free of whole cell and mitochondrial contaminations according to the manufacturers protocol, and then extracted by high salt as above. Western blot analysis by Enhanced Chemiluminescence was done essentially as described [27]. Western blot analysis used Prestained Broad-Range markers from Biorad. Peroxidase-coupled secondary anti-mouse or rabbit was obtained from Vector Laboratories. In some instances the Supersignal[®] West Femto Maximum kit (Pierce) was used for detection according to the manufacturer's protocol. Detection and quantification used a Biorad Chemi Doc XRS system.

Results and Discussion

As others we observe a clear mitochondrial localization of HsSirt3 upon transient or stable overexpression in various human cell lines, including HeLa, U2OS, and HEK293 FlpIn[™] TREx[™] a stable inducible cell line engineered to express HsSirt3-FLAG. It has been suggested that overexpression per se is enough to induce HsSirt3 translocation from

the nucleus to the mitochondria [20] but that preventing nuclear export by inhibition of CRM1 by leptomycin B would retain the protein in the nucleus. We chose to similarly test leptomycin B (LMB) in combination with immunofluorescence in U2OS cells following transient transfection of various HsSirt3 expression plasmids because these cells spread well and have a large surface area of their cytoplasm. In addition, an advantage of using transient transfection is that a large variation can be observed in expression levels with cells expressing the recombinant protein at high levels but also cells in which the protein is expressed at levels just above the detection limit. Thus cells not treated with LMB showed a clear mitochondrial localization of HsSirt3 irrespective of the level of expression or the epitope tag, being either the FLAG or myc epitope (Fig1, panels A1-3, and not shown). Treatment of these cells with LMB did not show any evidence of a nuclear fluorescent signal suggestive of nuclear retention of HsSirt3 (Fig1, panels B1 and B2). We used a commercial antibody against HDAC7 in co-staining to demonstrate the efficacy of the LMB treatment showing a clear redistribution of HDAC7 from mostly cytoplasmic to mostly nuclear, as previously observed [28]. Similar results were observed using HeLa cells although results were sometimes more difficult to interpret due to clustering of mitochondria in the nuclear periphery (Fig1, panel C). Unfortunately commercial antibodies against HsSirt3 worked very poorly in immunofluorescence unless the protein was overexpressed, suggesting that endogenous HsSirt3 expression levels were relatively low in the cells examined. Finally, we transfected cells to express an HsSirt3 variant that lacked the first 142 amino acids previously suggested to be important for initial nuclear localization where it was suggested to be processed prior to mitochondrial re-localization [20]. Our hypothesis was

that if indeed the full-length protein is processed in the nucleus to yield a protein that is mitochondrial-import competent, deletion of the first 142 amino-acids should result in a protein that is mainly mitochondrial. In addition, it has been suggested that mouse Sirt3 starts at the equivalent of human methionine 143 [29, 30], but is nevertheless a mitochondrial protein [12] (see also below). The results (Fig 1D) show however that the human variant lacking amino acids 1-142 is present in the cytoplasm and at high expression levels also in the nucleus. LMB treatment of these cells did not alter this distribution (not shown). Co-staining with Mitotracker® CMXRos did not show any evidence of mitochondrial targeting of truncated HsSirt3 (not shown).

To further corroborate the immunofluorescence experiments we relied on Western blot analysis and careful subcellular fractionation. Although the use of a hypotonic lysis method to obtain mitochondrial fractions from cultured cells is common practice, it is not appropriate to use the resulting nuclear pellet without further purification, as was done in the earlier demonstration of nuclear localization of HsSirt3 [20]. The problem is that the nuclear fraction resulting from hypotonic lysis typically contains 20-30 % unbroken cells and has a considerable mitochondrial contamination that is not always clear in a subsequent high salt extraction because many mitochondrial contaminants, especially membrane proteins, will be in the unused pellet following centrifugation of the high salt extract. To illustrate this problem we repeated essentially the same procedure as previously published [20], and furthermore tried to remove the nuclear mitochondrial contamination from the nuclear pellet by an additional NP40 detergent extraction which is a detergent commonly used to prepare crude nuclear fractions from whole cells. The

results in Figure 2A show that it is essentially impossible to avoid a mitochondrial contamination of a crude nuclear fractionation, even following an additional NP40 step. Apart from cells overexpressing HsSirt3-FLAG we used a cell line engineered to express an HA tagged version of the accessory subunit of mitochondrial DNA polymerase gamma POLG2 (P2HA), which is a well established mitochondrial protein (see e.g. [31]). Although with immunofluorescence this protein is clearly mitochondrial, the use of the crude isolation method shows also this protein to be present in all fraction including the high-salt 'nuclear' extract.

To obtain a clean nuclear fraction we subjected the low-speed nuclear pellet fractions of hypotonic lysates to a further purification step using an iodixanol gradient fractionation. Because nuclei are of considerably higher density than mitochondria and whole cells, this procedure yields a very pure nuclear fraction essentially free from mitochondrial contaminants. The resulting fraction was again subjected to a high salt extraction and the various fractions were subjected to Western blot analysis. Using inducible HEK293 FlpIn™ TREx™ cells this furthermore allowed us to use very short induction times similar to the immunofluorescence approach previously used [20] which suggested that short induction showed apparent nuclear HsSirt3 localization. Both the tagged overexpressed HsSirt3 as well as the endogenous protein were detected with commercial Sirt3 antibodies. The results (Fig. 2B) unambiguously demonstrate that both HsSirt3-FLAG and endogenous HsSirt3 are mitochondrially localized with no evidence of protein residing in the nucleus. Similar results were also obtained with regular HEK293 cells not engineered to overexpress any protein (not shown). Enrichment of typical nuclear proteins in the iodixanol nuclear fraction was demonstrated by reprobing

membranes for HDAC1 (Fig. 2B) and e.g. nucleoporin (not shown) showing that the high salt extraction had worked. Neither the short induction nor LMB treatment (not shown) had any effect on the above results. Strong overexpression for a longer period (2-3 days) or for 1-1,5 hours showed the presence of the 44 kDa precursor HsSirt3-FLAG protein. This was detected with the FLAG antibody but more importantly also with one of the commercial HsSirt3 antibodies showing this antibody is capable of detecting also the endogenous precursor protein. The precursor form of HsSirt3-FLAG was clearly detectable in the mitochondrial lysates but was not observed on any occasion in clean nuclear extracts. A potential precursor form of the endogenous protein was not observed on any occasion in the nuclear fraction. In fact, as most inducible systems, the cell culture system used in this study is slightly leaky resulting in steady-state HsSirt3-FLAG in non-induced cells that is comparable with endogenous HsSirt3 steady-state levels (see below). Non-induced cells that continuously express HsSirt3-FLAG at very low levels did not show the HsSirt3-FLAG precursor form in their mitochondrial (or nuclear) extracts, but only upon a short 1-1,5 hour induction the precursor form became visible in the mitochondrial lysates but not in the nuclear lysates and without a visible increase in the processed form. These data show that the precursor is generally shortlived (not visible in the 'leaky' non-induced cells but visible after short induction) and mitochondrially targeted where it is further processed to yield the mature protein as previously suggested [6].

To demonstrate that the band tentatively assigned to the endogenous HsSirt3 is indeed HsSirt3, we treated cells with HsSirt3 siRNA. Figure 2C shows that in cells overexpressing HsSirt3-FLAG, siRNA treatment for 48 hours had the desired effect of

knocking down HsSirt3-FLAG expression, albeit modestly. Furthermore, the same treatment in regular HEK293 cells increased acetylation of AceCS2 as detected by an anti-acetyl lysine antibody, as previously described [10] (not shown). Treatment of siRNA and subcellular fractionation followed by Western blot analysis shows that indeed the assigned endogenous mitochondrial HsSirt3 band is HsSirt3.

Combined the above results unequivocally demonstrate the mitochondrial localization of HsSirt3 and suggest that processing of the full length protein very likely takes place in the mitochondrion. There are various explanations for the discrepancy of our results with the previous results [20]. First, as we have shown here, the subcellular fractionation previously used to obtain a nuclear fraction was flawed in that any crude nuclear fraction derived from hypotonic lysis will still contain both whole cells and mitochondria. Furthermore the use of an antibody marker to detect a mitochondrial membrane protein is not appropriate to exclude contamination from the nuclear high-salt extract. Second, the previous paper heavily relied on an antibody that was specific for the N-terminus of HsSirt3 but the specificity of that antibody for HsSirt3 was not adequately demonstrated and various appropriate immunofluorescence controls were lacking. For example, an HsSirt3 siRNA experiment to demonstrate a reduction or complete loss of the nuclear fluorescent signal assumed to be from the precursor form of HsSirt3 was not shown. Finally, some of the images that appear to suggest nuclear retention of signal upon LMB treatment could be explained by clustering of a mitochondrial signal around and on top of the nucleus.

One of the premises to suggest that human Sirt3 behaves differently has been the absence of an N-terminal extension in the predicted mouse Sirt3 protein [20, 29, 30], i.e.

the mouse protein has been predicted to start at the methionine equivalent to human M143. Since the human and mouse protein are very highly conserved from M143 onward and we have not seen any evidence of mitochondrial localization of a truncated human variant starting at M143 (Fig 1D) we questioned the original mouse protein prediction and its localization [29, 30]. In addition both the human and mouse proteins when starting at M143 have a very poor mitochondrial targeting prediction. For these reasons we searched the mouse genome initially using the mouse cDNA sequence coding for the 29 kDa Sirt3. This analysis using the 'ab initio RNA' database yielded several predicted mRNAs from the Celera mouse genome (sequence provided in suppl Figure 1) that when translated would give a larger protein more compatible with the full length human protein. Using this prediction we looked for confirmation in the EST databases by using the 5' end of the cDNA upto and including the equivalent of the human M143 codon. This confirmed that the predicted mRNAs that would code for a larger mouse Sirt3 protein do exist *in vivo* (as also indicated in Suppl Figure 1). It should be noted here that the larger protein variant was originally also predicted by Yang et al. [29] but was discarded on the basis of it being rare and on a poor alignment with the predicted full-length human protein. For reasons unknown, this alignment did not take into consideration the possibility of large gaps. In stead it was proposed that the major Sirt3 mRNA in mouse would be created by an 8 nucleotide extension to the mRNA derived from the 5' end of exon 2. ESTs that agree with this can indeed be found in abundance, but curiously the intron-exon boundary resulting from the 8 nucleotide extension of exon 2 of mouse Sirt3 is less a consensus boundary than the one without this extension. Since the Sirt3 genomic region is gene dense we suggest that the abundant ESTs with the 8

nucleotide extension result from (pre)-mRNA of the gene adjacent to mouse Sirt3, coding for a 26S proteasomal subunit and transcribed in the opposite orientation.

When we searched for protein homologues using our prediction for the mouse full length protein the best hits were with various predicted mammalian Sirt3 proteins such as those from horse, pig, and cow. Sequence alignment of these proteins showed an excellent alignment (Fig 3), with all proteins having a predicted N-terminal stretch of 75-77 amino-acids prior to the equivalent of human M143. In addition, all proteins had an excellent prediction for mitochondrial localization. Of the various proteins in the alignment the mouse protein is the only one to have a second methionine 14 amino acids downstream of the first methionine that we assign here as M1. Further experiments are required to test whether M1 or the downstream methionine (M15) is preferentially used.

In conclusion, based on experimental data we show here the unambiguous and exclusive localization of human Sirt3 in mitochondria. In addition, based on the reanalysis of the mouse Sirt3 gene and transcripts we suggest that mouse Sirt3 has an N-terminal mitochondrial targeting sequence similar to that predicted for other mammals, but shorter than the human protein. This is supported by the observation that a truncated human protein that starts at the previously predicted mouse N-terminus is not mitochondrially targeted despite a high degree of similarity with the mouse protein from this residue onwards. Nevertheless, the endogenous mouse protein has been demonstrated unequivocally to be mitochondrial [12]. Our results greatly simplify various ambiguous published results relating to the function of the Sirt3 protein and should help in clarifying its potential roles in metabolism. First, based on our data there is no support for a nuclear function of human Sirt3. Second, the effects of mouse Sirt3 overexpression on for

example PGC1 α in brown adipocytes [30] is likely an artefact caused by the overexpression of a truncated mouse Sirt3 that is targeted not to mitochondria but to the cytoplasm. Although Shi et al. did show apparent co-localization with the mitochondrial dye Mitotracker® this appears to be an experimental artefact, possibly caused by severe overexpression, since the Mitotracker® staining does not actually show a typical mitochondrial network as normally seen with mouse 3T3 cells that were used in that study. Finally, as an additional cautionary note, the HDAC7 antibody used in this study was identical to the antibody used by Bakin et al [21], who suggested a mitochondrial localization of HDAC7. Our immunofluorescence results in U2OS cells (Fig 1, panels B1-2) and similar results obtained using C4-2 cells (not shown) showed nuclear/cytoplasmic localization of HDAC7 and thus also seem to contradict this previous report. In addition, when we expressed in various cell lines an HDAC7-FLAG variant of essentially the same sequence as reported [21] we see the same nuclear/cytoplasmic pattern as observed with the endogenous protein and the same LMB responsiveness. Nevertheless, since in our opinion it is difficult to fully exclude a mitochondrial localization of at least a minor fraction of HDAC7 we are hesitant to fully exclude a mitochondrial role for this protein.

Acknowledgements:

We like to acknowledge Outi Kurronen and Merja Jokela for their skilful technical assistance, Howy Jacobs, Anu Wartiovaara-Suomalainen and Brendan Battersby for many discussions, Sjoerd Wanrooij for his help in creating the inducible HsSirt3 and POLG2 cell lines and Fenne Koning for the initial characterization of the HsSirt3 siRNAs used in this study. This research was financially supported by the Academy of Finland (Grants 110689, 103213 and CoE funding), the Sigfrid Juselius Foundation, Tampere University Hospital Medical Research Fund and the University of Tampere.

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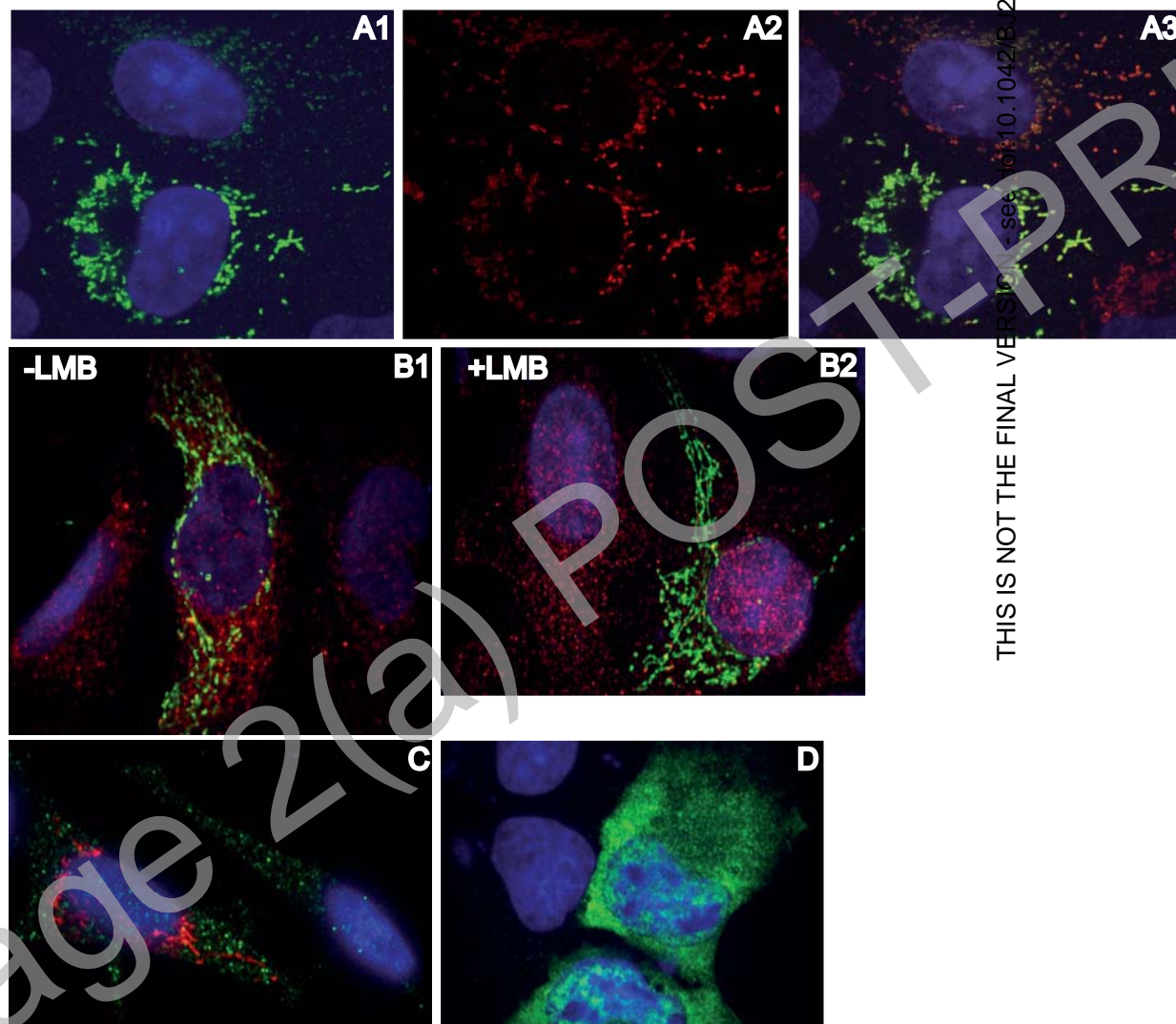
Legends to Figures:

Figure 1: Overexpressed HsSirt3 is mitochondrial irrespective of expression level or presence of Leptomycin B. HsSirt3-FLAG was expressed in human U2OS cells following transient transfection (panels A1-A3). 2 Days following transfection cells were stained with Mitotracker[®] Red (A2) and processed for immunofluorescence using an anti-FLAG antibody (A1). A merge image is shown in panel A3. The results show that irrespective of expression levels HsSirt3-FLAG is mitochondrially localized. (Panels B1-B2) U2OS cells were transfected as in panels A and treated (B2) 2-3 hrs with 5 ng/ml Leptomycin B (LMB). HsSirt3- FLAG is shown as green fluorescence while endogenous HDAC7, which was used to show that LMB is an effective inhibitor of nuclear export in these cells, is shown as red fluorescence. Results show that LMB had no discernable effect on HsSirt3-FLAG localization, while it showed a clear increase in the HDAC7 nuclear signal compared to non-LMB treated cells. Similar results were observed for HeLa cells treated with LMB (panel C and not shown, HsSirt3-FLAG shown as red fluorescence and HDAC7 as green fluorescence) although the LMB effect on HDAC7 in this case was not as clear, but reproducible. Of particular notice in this image is the clustering of mitochondria in the nuclear periphery in LMB treated HeLa cells, giving the false impression that some of the red fluorescence is nuclear. (Panel D) Human Sirt3 Δ 1-142 with a myc tag was transiently transfected in U2OS cells. Immunofluorescence showed that this protein is located in the cytoplasm and at high expression levels, as shown here, also in the nucleus.

Figure 2: The precursor and processed form of overexpressed HsSirt3 as well as endogenous HsSirt3 are exclusively mitochondrial. (A) 293 FlpIn-TREx cells were induced for 2 days with 5 ng/ml DC to overexpress either HsSirt3-FLAG (S3FL), POLG2-HA (P2HA) or nothing and a crude mitochondrial fraction was prepared, the low speed 'nuclear' pellet obtained after hypotonic lysis was first extracted with a small volume of NP-40 lysis buffer (NP40 extract) and the resulting pellet of this NP-40 extract was subjected to high-salt extraction (nuclear fraction). The results illustrate the problem of obtaining a clean nuclear extract using this crude isolation procedure as even the well established mitochondrial POLG2 protein is found in all fractions: any conclusions based on this method regarding potential nuclear localization of proteins are unwarranted. (B) Purification of mitochondrial fractions by hypotonic lysis and subsequent nuclear fractionation using a iodixonal step-gradient. Indicated cell lines were non-induced (-) or induced (+) with 5 ng/ml DC for the indicated time in hours. Equivalent amounts of the starting material for the mitochondrial and nuclear fraction were loaded in each lane on a single gel. Membranes were probed with various antibodies starting with the Santa Cruz HsSirt3 polyclonal antibody. Indicated are mature HsSirt3-FLAG and endogenous HsSirt3 (based on the experiment shown panel C) and based on their mobility which was just below a 30 kDa marker band. The asterisk indicates the HsSirt3-FLAG precursor protein that was observed at variable levels in mitochondrial fractions and with various induction levels and times. A short 1,5 hour induction (as shown here) also showed this precursor in the mitochondrial fraction but not in the nuclear fraction even upon long exposure of the membranes (not shown). These results are in clear contradiction with published results [20] and do not show any evidence of nuclear HsSirt3-FLAG or of

endogenous HsSirt3 in cells that weakly or not at all overexpress HsSirt3FLAG (lanes P2HA). (C) HsSirt3 siRNA (HsS3) or non-targeting siRNA (nt) had the desired effect of reducing both the overexpressed HsSirt3-FLAG protein and the tentatively assigned endogenous HsSirt3 band both in HsSirt3-FLAG and in P2HA expressing 293FlpIn-TREx cells. The knockdown effect was confirmed by reprobing the membrane for a mitochondrial marker protein mtCOX II. Observed knockdown after 48 hours typically was 30-40%. In similar experiments, the same siRNA oligo's resulted in increased acetylation of overexpressed AceCS2-FLAG compared to nt treated controls, as previously shown by others [10] (not shown).

Figure 3: The predicted mouse Sirt3 is highly similar to the predicted proteins from cow, pig and horse. The predicted mouse Sirt3 protein based on the predicted cDNA sequence in Supplementary Figure 1 was Blastp searched against the non-redundant NCBI protein database (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). A multiple sequence alignment (using the ClustalW server at <http://www.ebi.ac.uk/>) is shown with the best matching proteins predicted for horse (accession XP_001489390), pig (NP_001103527), and cow (XP_879073). The arrow indicates the methionine equivalent to human Sirt3 methionine 143. This is also the residue originally predicted to be the start of the mouse protein (for details see [20, 29, 30] and the main text). A multiple sequence alignment including the human and chicken proteins is shown in Supplementary Figure 2.



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Fig. 1, Cooper and Spelbrink

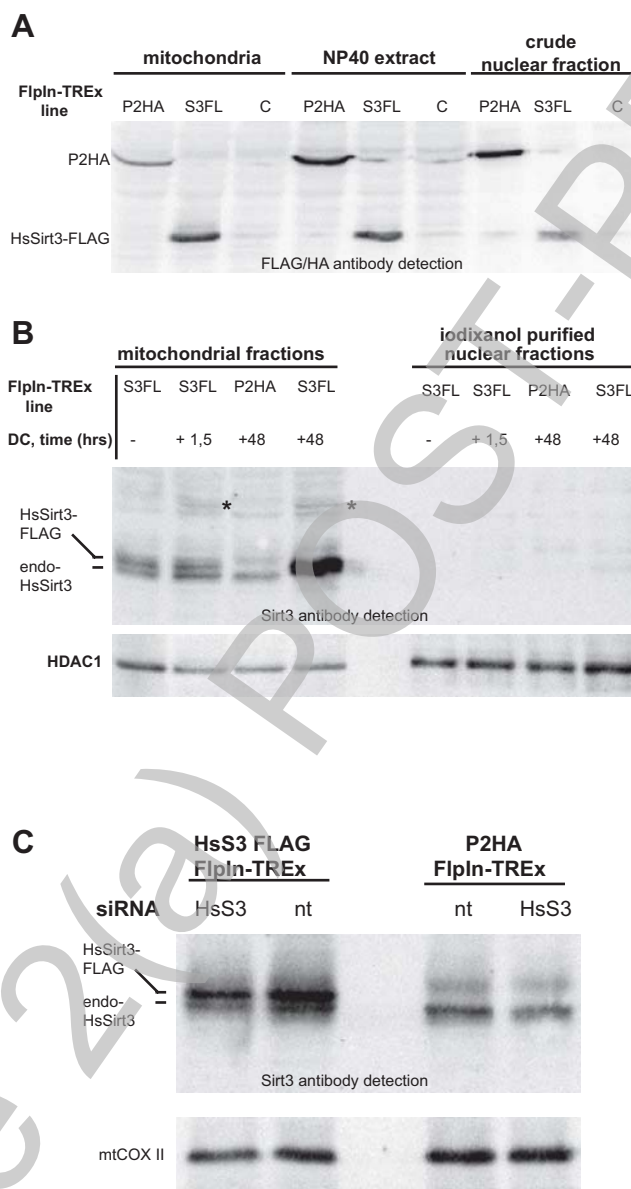


Fig. 2, Cooper and Spelbrink

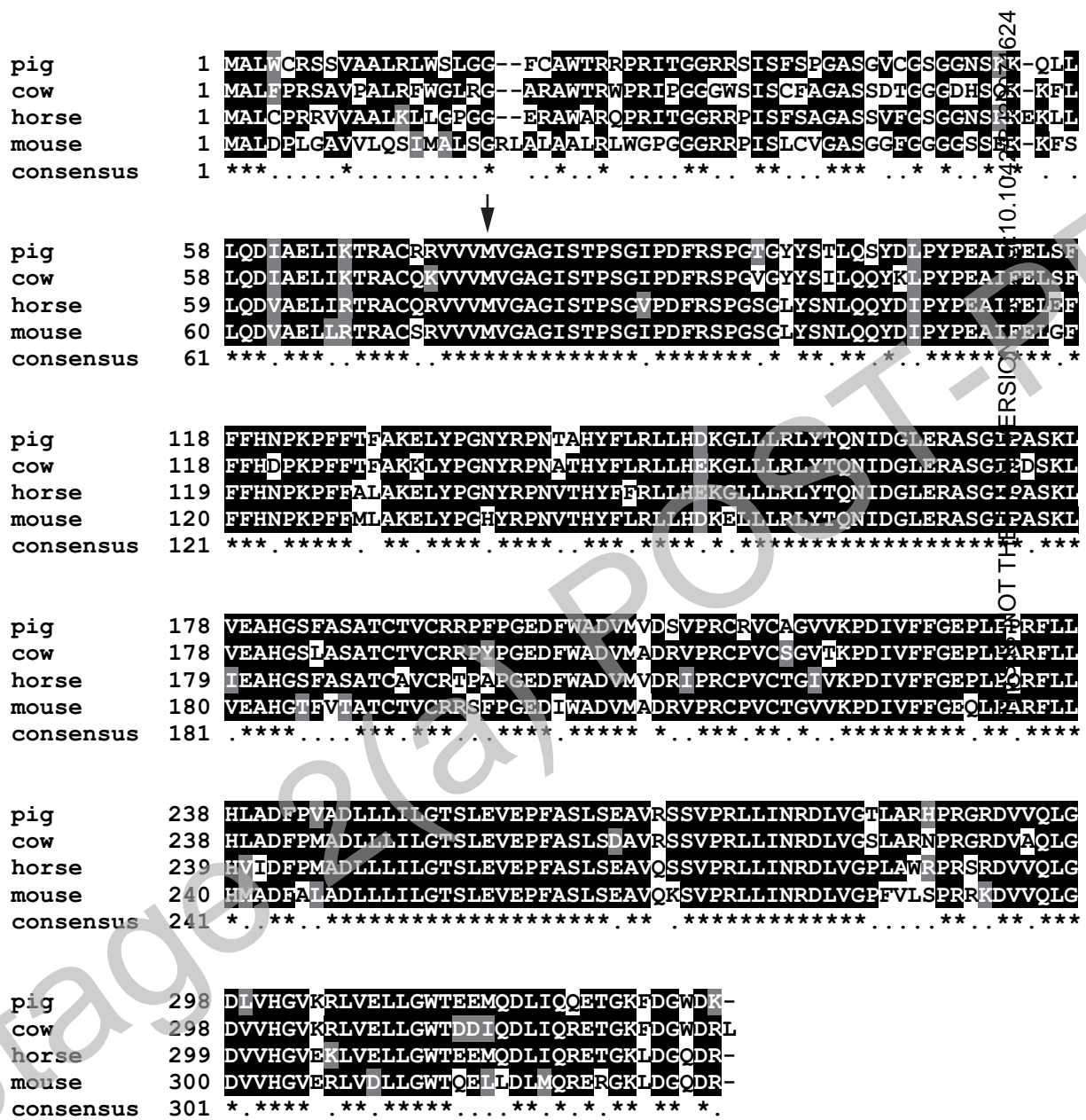


Fig 3. Cooper & Spelbrink