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Where in the cell is SIRT3?:

Functional localization of an NAD⁺-dependent protein deacetylase

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Abstract

Sirtuins are NAD⁺-dependent enzymes that have been implicated in a wide range of cellular processes, including pathways that affect diabetes, cancer, lifespan, and Parkinson's disease. To understand their cellular function in these age-related diseases, identification of sirtuin targets and their subcellular localization is paramount. Sirtuin 3 (SIRT3) a human homologue of Sir2, has been genetically linked to lifespan in the elderly. However, the function and localization of this enzyme has been keenly debated. A number of reports have indicated that SIRT3, upon proteolytic cleavage in the mitochondria, is an active protein deacetylase against a number of mitochondrial targets. In stark contrast, some reports have suggested that full length SIRT3 exhibits nuclear localization and histone deacetylase activity. Recently, a report comparing SIRT3^{-/-} and SIRT3^{+/+} mice have provided compelling evidence that endogenous SIRT3 is mitochondrial and appears to be responsible for the majority of protein deacetylation in this organelle. In the current issue of *Biochemical Journal*, Cooper *et al.* present additional results that address the mitochondrial and nuclear localization of SIRT3. Utilizing fluorescence microscopy and cellular fractionation studies, Cooper *et al.* determine that SIRT3 localizes to the mitochondria and is absent in the nucleus. Thus, this study provides additional evidence to establish SIRT3 as a proteolytically modified, mitochondrial deacetylase.

Keywords

SIRT3; Nucleus; Mitochondria; Deacetylation; NAD⁺

Class III histone deacetylases or Sirtuins are an evolutionarily conserved family of enzymes that utilizes NAD⁺ as a substrate to deacetylate the ε-amino group of lysine from acetylated proteins, yielding two additional products nicotinamide and O-acetyl-ADP-ribose[1]. In yeast, Sir2 (silent information regulator 2) transcriptionally silences the mating-type loci, telomeric DNA regions, and the ribosomal RNA locus by maintaining histones in the hypoacetylated state[2]. Strikingly, gene dosage of Sir2 in yeast, flies, and worms appears to mediate lifespan extension similar to the effects of caloric restriction, while the lack of Sir2 opposes this effect[2]. Additionally, sirtuins have been implicated in a number of cellular processes including cell survival, cell cycle regulation and genomic stability, fatty acid synthesis, and glucose and insulin homeostasis[3].

The seven members of the mammalian Sirtuin (SIRT1-7) family have various substrates and are compartmentalized to distinct cellular locations. The most studied sirtuin, SIRT1, is both nuclear and cytosolic and has been demonstrated to deacetylate Foxo1, p53, NF-κβ, Ku70, PGC-1α, and AceCS1[3]. SIRT2 deacetylates α-tubulin in the cytoplasm, while SIRT6 and

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SIRT7 mediate DNA repair and rRNA transcription, respectively, in the nucleus[3]. In the mitochondria, SIRT4 ADP-ribosylates and inhibits glutamate dehydrogenase, while the functional activities of SIRT5 remains unclear[3].

The localization and the cellular role of SIRT3 has been the subject of some controversy. Initial studies had suggested SIRT3 resides in the mitochondria and functions as an NAD⁺ dependant deacetylase[4,5]. Remarkably, a recent report suggested that upwards of one-fifth of detected mitochondria proteins are acetylated[6]. Given that SIRT3 is the only reported mitochondrial deacetylase with robust activity, this suggests that SIRT3 is likely the main mitochondrial deacetylase and may in part, control energy flux via post-translational modification of metabolic proteins[7]. However, several contradictory reports have suggested that SIRT3 resides in the nucleus and exerts epigenetic control by deacetylating histone substrates[8,9].

One source of debate arises from inconsistent reports of post-translational processing of the human and mouse SIRT3 homologs. Initially, human SIRT3 (hSIRT3) was reportedly expressed as a 44 kD protein and targeted to the mitochondria via an n-terminal localization sequence[4]. Upon entry into the mitochondrial matrix, hSIRT3 is processed at the N-terminus by a mitochondrial matrix peptidase into the activated 29 kD enzyme[4]. These conclusions are corroborated by the fact that truncations, as well as mutations in the N-terminal mitochondrial localization sequence, prevents the protein from proper mitochondrial localization, as well as efficient proteolytic cleavage[4]. In contrast, murine SIRT3 (mSIRT3) was suggested to lack the N-terminal mitochondrial localization sequence, and instead, is translated beginning at the conserved deacetylase domain [9–11]. Curiously, the same cDNA-encoded mSIRT3 has been shown to be associated with the mitochondrial inner membrane[11]. These studies raise the question of whether human and mice utilize different mechanisms to translocate SIRT3 into the mitochondria. A genomic observation may afford a resolution to this discrepancy. Cooper *et al.* present sequence analysis that indicates the initiator methionine of mSIRT3 exists further upstream in the mSIRT3 genomic region, resulting in a protein sequence that encodes a conserved mitochondrial targeting signal similar to hSIRT3. However, experimental evidence for the functionality of this differentially processed version of mouse SIRT3 has yet to be revealed.

Mitochondrial localization of SIRT3 has been demonstrated in three separate fluorescent microscopy studies utilizing a C-terminal GFP fusion expression construct [4,5,12]. In addition, cellular fractionation experiments in which nuclear, cytosolic, and mitochondrial components are separated by differential centrifugation and probed for SIRT3 with C-terminal specific antibodies demonstrate that SIRT3 is enriched in the mitochondria[4]. Cooper *et al.* provide additional support for the lack of nuclear SIRT3 by utilizing stringent nuclear fractionation techniques. While these studies utilize SIRT3 overexpression, several additional lines of evidence support endogenous SIRT3 mitochondrial residence. Endogenous SIRT3 has been detected in the mitochondria of HEK293 cells, and decreased protein levels were observed by siRNA knockdown[13]. Additionally, endogenous SIRT3 was clearly distinguishable from myc-tagged SIRT3 as a faster migrating band in western blotting experiments of fractionated mitochondria[4]. Collectively, this evidence supports SIRT3 mitochondrial localization regardless of the expression level in cells. A study comparing mSIRT3^{+/+} and mSIRT3^{-/-} mice has provided the strongest evidence of endogenous mSIRT3 mitochondrial localization; careful isolation and subcellular fractionation of mSIRT3^{+/+} liver lysates demonstrated that SIRT3 is exclusively localized to the mitochondria and absent from the nucleus and cytoplasm[7]. Furthermore, SIRT3^{-/-} mice demonstrate global hypocetylation of mitochondrial proteins, and adding recombinant SIRT3 to mitochondrial extracts from SIRT3^{-/-} animals reduces protein acetylation. These

combined studies strongly support the conclusion that SIRT3 localizes to the mitochondria and acts as the predominant NAD⁺ dependant deacetylase.

In dramatic contrast, two studies have suggested that full length SIRT3 is localized to the nucleus. One study proposes SIRT3 is localized to the nucleus upon co-expression of SIRT5[9]. Until the mechanism of how SIRT5 influences the biological activities of SIRT3 are demonstrated, the caveats of forced SIRT3 and SIRT5 co-overexpression remain. A separate study reported that SIRT3 localizes to the nucleus and translocates to the mitochondria only upon cellular stress, such as overexpression or an oxidative challenge[8,9]. Although an intriguing possibility, the antibody employed in these studies raises questions as to whether all the endogenous SIRT3 was detected. Because the antibody was raised against the N-terminal domain of SIRT3, the same domain that is proteolytically clipped upon mitochondrial translocation, the use of this antibody in immunofluorescent studies would make it improbable for detection of mitochondrial SIRT3. Seemingly contradictory, the same study reported that an antibody raised to the C-terminal domain of SIRT3 clearly identifies endogenous SIRT3 in the mitochondria fraction of 293F cells[8]. By measuring SIRT3 levels with antibodies to portions of SIRT3 that are not proteolytically processed, Cooper *et al.* demonstrated internally consistent data that discerns endogenous and overexpressed SIRT3.

Lastly, SIRT3 has been identified as a direct NAD⁺ dependant deacetylase for the substrates AceCS2 and GDH[7,14,15]. *In vitro* assays demonstrate that full length SIRT3 lacked measurable NAD⁺ dependant deacetylation, but upon cleavage of the mitochondrial localization sequence, gains deacetylase activity towards histone peptide[4,5]. When amino acids 1–119 are removed, recombinantly expressed SIRT3 displays high catalytic activity against histone H4 peptide as well as acetylated AceCS2 *in vitro* [14]. This contrasts with a separate study where unprocessed full length SIRT3 could exchange nicotinamide in the presence of acetylated histone peptide and NAD⁺[8], an assay that has been used to demonstrate catalytic activity of sirtuins. Although nicotinamide exchange indicates that SIRT3 is mechanistically competent for NAD-cleavage, the caveat of this assay is that it does not measure the full deacetylation reaction. Therefore, it is problematic to conclude that full length SIRT3 is a functioning protein deacetylase.

In this issue of *Biochemical Journal*, Cooper *et al.* re-examine the localization of hSIRT3 via fluorescence localization and subcellular fractionation experiments, as well as provide insight to the genomic nature of mSIRT3. In doing so, additional compelling evidence supports the conclusion that endogenous SIRT3 resides in the mitochondria as a highly active protein deacetylase. Alternative cellular localization of SIRT3 cannot be excluded and further investigations will be necessary to provide convincing evidence that SIRT3 resides elsewhere under “stress”. Among the critical questions yet to be answered, it is not yet clear how SIRT3 expression activates the nuclear transcription of mitochondria-related genes such as UCP1, PGC-1 α , and COX IV and V[11]. As one possibility, SIRT3 may deacetylate proteins involved in metabolic pathways or signaling cascades that emanate from the mitochondria and signal to the nucleus. These signals could exist as deacetylated proteins or metabolites, such as O-AADPr or nicotinamide.

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