Functional interplay between protein acylation and cellular metabolism in metabolic disorders
Pougovkina, O.A.

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Chapter 5

The role of aberrant protein succinylation in Succinyl-CoA Synthetase deficiency

Olga Pougovkina, Heleen te Brinke, Rob Ofman, Johannes N. Spelbrink, Ronald J.A. Wanders, and Vincent C.J. de Boer

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The role of aberrant protein succinylation in SCS deficiency

Introduction

Post translation modifications (PTMs) expand the functional diversity of the cellular proteome by modulating the function of proteins, albeit catalytic or not and thereby allow a rapid response to changes in the surrounding environment. Lysine acylation is a reversible PTM which involves covalent attachment of an acyl group to the positively charged ε-amino group of the lysine residue. The most well defined lysine acylation PTM is acetylation, the function and regulation of which has been thoroughly studied for decades. However, recent advances in proteomics have led to the identification of new acylation PTMs demonstrating that virtually all acyl-CoAs generated by intermediary metabolism can be used as donors for lysine acylation. These include among others, propionylation, butyrylation, malonylation, succinylation and glutarylation (Chen et al., 2007; Peng et al., 2011a; Tan et al., 2014; Zhang et al., 2011).

Lysine succinylation, along with malonylation and glutarylation changes the positive charge of lysine to negative and it is likely that this has a considerable impact on the function of the modified protein. These three types of lysine modification were shown to be regulated by the mitochondrial sirtuin SIRT5 (Du et al., 2011; Peng et al., 2011a; Tan et al., 2014). Succinylation was detected on many mitochondrial metabolic enzymes (Park et al., 2013; Rardin et al., 2013a) indicating that it may have a role in metabolic regulation. Elevated lysine succinylation was shown to decrease metabolic flux through mitochondrial β-oxidation and ketogenesis pathways in SIRT5 KO mice (Rardin et al., 2013a). Functional studies have demonstrated that SIRT5 regulates cellular respiration by desuccinylating pyruvate dehydrogenase complex (PDC) and succinate dehydrogenase (SDH), which represses their activity (Park et al., 2013).

In contrast to acetylation, it is unknown how other acylation modifications are catalyzed. Likewise, an enzyme with succinyl-transferase activity has not been identified. However, there are indications that acylation modifications can also occur non-enzymatically due to accumulation of specific acyl-CoAs during certain metabolic states of the cell. In another study we have shown that accumulation of butyryl-CoA, malonyl-CoA and propionyl-CoA caused by a specific enzyme deficiency in inborn errors of metabolism results in increased butyrylation, malonylation and propionylation, respectively (Pougovkina et al., 2014). The mechanism of non-enzymatic acylation is especially relevant in mitochondria, which due to its metabolic activity generates various acyl-CoAs through intermediary metabolism. In addition, the mitochondrial CoA pool is significantly higher than in other cellular compartments, which will inevitably lead to higher mitochondrial acyl-CoA levels. Succinyl-CoA takes part in several mitochondrial metabolic pathways: as an intermediate in the TCA cycle, as the product of catabolism of odd-chain fatty acids and some branched-chain amino acids, and it is also part of porphyrin biosynthesis (Berg et al., 2002).

Succinyl-CoA synthetase (SCS) deficiency is a severe inborn error of metabolism that results in defective flux through the TCA cycle. SCS is a mitochondrial enzyme in the TCA cycle that catalyzes the reversible conversion of succinyl-CoA and ADP or GDP to succinate and ATP or GTP (Johnson et al., 1998). SCS is a heterodimer
composed of α- and β-subunits. The α-subunit consists of SCS-G1 and is constant in all of the tissues. The β-subunit can be either SCS-A2 or SCS-G2 and determines the substrate specificity of the enzyme making it either ADP-forming or GDP-forming, respectively (Fig. 1A) (Johnson et al., 1998; Lambeth et al., 2004). The SCS-A2 subunit predominates in catabolic tissues such as brain and skeletal muscle, and the SCS-G2 subunit is mainly expressed in anabolic tissues, such as the liver and kidney (Lambeth et al., 2004). Until now, only patients with mutations in the genes encoding SCS-G1 and SCS-A2 have been reported (Carrozzo et al., 2007; Elpeleg et al., 2005; Ostergaard et al., 2007b; Randolph et al., 2011; Rivera et al., 2010; Rouzier et al., 2010; Sakamoto et al., 2011; Van Hove et al., 2010). SCS-G1 patients generally present with a more severe phenotype including marked acidosis with lactic aciduria, multi-organ failure and death within the first days of life (Rivera et al., 2010). Most patients with mutations in SCS-A2 have a milder phenotype being mostly normal at birth and developing the first symptoms after 3-6 months of life. SCS-A2 deficiency presents with hypotonia and muscle weakness, Leigh-like syndrome with hearing impairment, dystonia and short lifespan (Carrozzo et al., 2007; Elpeleg et al., 2005; Ostergaard et al., 2007b).

Interestingly, both patients with SCS-G1 and SCS-A2 deficiencies present with mtDNA depletion syndrome (MDS) (Carrozzo et al., 2007; Elpeleg et al., 2005; Ostergaard et al., 2007a; Ostergaard et al., 2007b; Rivera et al., 2010), which links the TCA cycle to mtDNA homeostasis. Consequently these patients were reported to have lower activity of individual OXPHOS complexes in muscle mitochondria, with exception of complex II that is completely encoded by the nuclear genome (Elpeleg et al., 2005; Ostergaard et al., 2007a). Manifestation of MDS is most prominent in the muscle tissue of the patients and some studies were not able to detect this phenotype in the primary fibroblasts of the patients (Elpeleg et al., 2005; Ostergaard et al., 2007a). However, in some cases reduced OXPHOS activity and mtDNA depletion were also detected in patient fibroblasts (Carrozzo et al., 2007; Rivera et al., 2010). Currently, the mechanism causing MDS in SCS deficiency is unknown. Because nucleoside diphosphate kinase (NDK), an enzyme essential for mtDNA replication (Bradshaw and Samuels, 2005), was shown to interact with SCS-A2 in prokaryotes and eukaryotes (Kadrmis et al., 1991; Kavanaugh-Black et al., 1994; Kowluru et al., 2002), there are speculations that the absence of SCS may lead to reduced activity of NDK and thus mtDNA depletion.

In this study we demonstrate that the level of succinylation is increased in SCS deficient patient fibroblasts. Furthermore, we report that SCS-deficient cells have a partial deficiency in the OXPHOS system and are more glycolytic. To investigate whether hypersuccinylation in these cells is linked to OXPHOS deficiency we have reduced the level of succinylation by overexpression of SIRT5. Finally, we have analyzed the effect of succinylation on the activity of NDK.
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Figure 1. Lysine succinylation is elevated in SCS deficient cells. (A) Schematic representation of the TCA cycle and the reversible conversion of succinyl-CoA to succinate catalyzed by SCS. SCS deficiency is caused by mutations in subunit G1 or A2 of SCS which results in accumulation of succinyl-CoA. (B) Lysine succinylation levels in cell lysates of fibroblasts from SCS-A2 and SCS-G1 deficient patients are increased as compared to control fibroblasts analyzed using α-succinyllysine western blot. Actin was used as loading control and SCS-A2 and SCS-G1 western blots confirm the absence of the respective protein. 33 µg protein was loaded per lane. (C) Immunofluorescence imaging of SCS-G1 deficient fibroblasts transfected with pDsRed2-Mito and stained with α-succinyllysine antibody and DAPI. The overlay of the images shows co-localization of protein succinylation with mitochondria. (D) Knockdown of SCS-A2 and SCS-G1 in HEK293 cells. Mitochondria enriched fractions were obtained by differential digitonin treatment of cells and mitochondrial lysine succinylation was assessed with α-succinyllysine antibody. Mitochondrial VDAC was used as loading control and SCS-A2/SCS-G1 western blots confirm efficient knockdown. 15 µg protein was loaded per lane.
Chapter 5

Results

*SCS deficiency leads to increased mitochondrial protein succinylation*

Due to the metabolic block of succinyl-CoA conversion to succinate, we expected succinyl-CoA to accumulate in SCS deficient cells (Fig. 1A). In an in vitro experiment we have observed that succinyl-CoA reacts with mouse mitochondrial protein lysate in a time dependent manner (Fig. S1A). As this suggests that accumulation of succinyl-CoA could lead to increased succinylation, we hypothesized that SCS deficient cells have elevated protein succinylation. We therefore analyzed protein succinylation in fibroblasts of two SCS deficient patients, one with SCS-G1 and the other with SCS-A2 deficiency. To assess succinylation levels we performed α-succinyllysine western blot analysis and quantified succinyllysine with LC-MS. As expected, protein succinylation in SCS deficient patient cells was substantially increased compared to control cells (Fig. 1B and S1B). As shown previously, the absence of SCS-G1 leads to complete depletion of SCS-A2 (Fig. 1B). SCS-A2 deficient fibroblasts on the other hand, still contain SCS-G1 (Fig. 1B), which could explain the difference in the severity of clinical presentation between SCS-G1 and SCS-A2 deficient patients (Rouzier et al., 2010). Nevertheless, succinylation levels as evaluated by western blot analysis and through succinyllysine quantification by LC-MS are comparable in SCS-G1 and SCS-A2 deficient cells (Fig. 1B and S1B).

Because succinyl-CoA is generated by mitochondrial metabolism, we expected most of the succinylation proteins resulting from the SCS deficiency to be mitochondrial. To investigate this, we have examined the subcellular localization of succinylation in SCS-G1 deficient fibroblasts using immunofluorescence. For this we transfected SCS-G1 deficient fibroblasts with pDsRed2-Mito to label the mitochondria, fixed the cells and stained them with α-succinyllysine antibody to label the succinylated proteins. Merging of mitochondrial (red) and succinylation (green) signals showed a substantial overlap indicating that most of the succinylation is indeed mitochondrial (Fig. 1C). Staining of SCS-A2 deficient cells with α-succinyllysine

![Figure 2](image-url)

Figure 2. SCS deficient cells have impaired mitochondrial respiration. (A) Oxygen consumption rate (OCR) in control fibroblasts, SCS-A2 deficient and SCS-G1 deficient fibroblasts analyzed using XF-96 Seahorse analyzer. Oligomycin (ATP synthase inhibitor), FCCP (uncoupler) and Rotenone with Antimycin A (AA) (complex I and III inhibitors) were injected during the run to assess mitochondrial respiration. (B) Extracellular acidification rate (ECAR) in control fibroblasts, SCS-A2 deficient and SCS-G1 deficient fibroblasts (G1) analyzed using XF-96 Seahorse analyzer. (C) Graphical representation of correlation between oxidative and glycolytic metabolism in control and SCS deficient fibroblasts. The data was derived from the measurements in 2A and 2C.
also gave a strong mitochondrial signal, which was considerably lower in the case of control cells (Fig. 1SC).

To test whether inhibition of SCS would lead to increased protein succinylation in another cell model, we used shRNA to knockdown SCS-G1 and SCS-A2 in HEK293 cells. Considering mitochondrial localization of succinylation in SCS deficient cells, we directly analyzed protein succinylation in enriched mitochondrial fractions of HEK293 cells with SCS knockdowns. There was a considerable increase in mitochondrial succinylation in the cells handled with shSCS-A2 and shSCSG1 as compared to the control (Fig. 1D). This further supports our observation that decreased mitochondrial flux of succinyl-CoA due to SCS deficiency leads to increased mitochondrial protein succinylation.

**SCS deficient cells present with defective OXPHOS**

Next, we analyzed cellular respiration in SCS-G1 and SCS-A2 deficient cells to evaluate the previously reported OXPHOS deficiency in SCS deficient patient cells (Carrozzo et al., 2007; Rivera et al., 2010). We assessed mitochondrial respiration in both patients cell lines by measuring the oxygen consumption rate (OCR) using the Seahorse analyzer. The assay was performed in DMEM containing 25 mM glucose and 2 mM L-glutamate as substrates. To get a complete overview of mitochondrial function we measured the OCR at basal state without any compounds injected, the ATP turnover by injecting ATP synthase inhibitor oligomycin and maximum respiratory capacity by injecting the uncoupler FCCP , which reflects the maximum achievable OXPHOS activity under these assay conditions. The achieved respiratory profile clearly indicated mitochondrial dysfunction in SCS deficient cells. We detected decreased basal respiration and maximum respiration in both patient cell lines as compared to control (Fig. 2A).

In the same assay run we measured the extracellular acidification rate (ECAR) to assess the rate of glycolysis in these cells. As shown in Figure 2B, SCS deficient cells were more glycolytic than the control. The decreased mitochondrial respiration and increased rate of glycolysis point to a shift from oxidative to glycolytic metabolism (Fig. 2C), which usually accompanies mitochondrial dysfunction.

Overall these results demonstrate that these patient fibroblasts present with defective OXPHOS that was reported to be most severe in muscle cells (Carrozzo et al., 2007; Elpeleg et al., 2005; Ostergaard et al., 2007a; Rivera et al., 2010). Most importantly, these cells also have increased mitochondrial succinylation and we therefore directed our further research to investigate the role of succinylation in mitochondrial dysfunction observed in this disorder.

**SIRT5 overexpression reduces succinylation and improves OXPHOS in SCS deficient cells**

Because mitochondrial succinylation is regulated by SIRT5 (Park et al., 2013; Rardin et al., 2013a), we overexpressed SIRT5 in SCS deficient cells to directly analyze the effect of succinylation on mitochondrial function (Fig. 3A). To determine whether SIRT5 overexpression reduced protein succinylation, we quantified succinyllysine levels by LC-MS. Indeed, SIRT5 overexpression significantly reduced
protein succinylation in SCS deficient fibroblasts (Fig. 3B). Next, we assessed how SIRT5 overexpression affected mitochondrial function in SCS deficient cells. For this we evaluated the maximum rate of respiration and the glycolytic rate in these cells. For both SCS-A2 and SCS-G1 deficient cells, SIRT5 overexpression increased the maximum respiratory capacity (Fig. 3C and 3SA). SIRT5 overexpression had no effect on mitochondrial respiration of control fibroblasts (Fig. 3C and 3SA). The glycolytic rate, on the other hand, decreased in SCS deficient cells as the result of SIRT5 expression (Fig. 3E and 3SB), indicating metabolic switch in these cells from glycolytic to oxidative metabolism. This is likely to be the direct result of the increased OXPHOS capacity in these cells.

To further investigate the changes of mitochondrial function in SCS deficient cells overexpressing SIRT5, we measured mitochondrial respiration in glucose deficient medium containing galactose and L-glutamine as substrates. The absence of glucose makes the cells more dependent on OXPHOS and we therefore expected the effect of desuccinylation by SIRT5 to be more pronounced. Also in this experimental set-up we observed direct evidence of mitochondrial dysfunction in SCS deficient cells. Using the results of the respiratory measurements from Seahorse we have derived three parameters to evaluate mitochondrial function: maximum respiratory capacity.
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Figure 4. SIRT5 overexpression leads to improved mitochondrial function in SCS deficient cells. In (A) - (D) the figures are derived from data obtained using XF-96 Seahorse Analyzer. The assay was performed in glucose deficient medium. (A) Maximum respiratory capacity, spare respiratory capacity and ATP turnover rate measured in control and SCS deficient fibroblasts. (B) Comparison of maximum respiratory rate between control and SCS deficient fibroblasts expressing empty vector or SIRT5-flag. Mean ± SD, n = 8 and *** indicates p < 0.001. (C) Comparison of spare respiratory capacity between control and SCS deficient fibroblasts expressing empty vector or SIRT5-flag. Mean ± SD, n = 8 and *** indicates p < 0.001. (D) Comparison of ATP turnover rate between control and SCS deficient cells expressing empty vector or SIRT5-flag. Mean ± SD, n = 8 and *** indicates p < 0.001. (E) Western blot analysis with α-flag to confirm SIRT5-flag overexpression in HEK Flp-In cells. (F) ATP turnover rate derived from OCR measurements before and after the injection of oligomycin. Mean ± SD, n = 15, ** indicates p < 0.01 and *** indicates p < 0.001.
capacity, spare respiratory capacity (SRC) and ATP turnover rate. SRC is the difference between the basal respiration and maximum respiration, which is a measure of the ability of the OXPHOS system to respond to an increase in the energy demand and is also an evaluation of mitochondrial function. ATP turnover rate was estimated from the decrease in respiration induced by inhibiting ATP synthase with oligomycin. All of the three parameters were significantly lower in SCS deficient cells as compared to control values (Fig. 4A). Interestingly, SIRT5 overexpression significantly increased all of these parameters in SCS deficient cells (Fig. 4B, C and D). This links increased protein succinylation in SCS deficient cells to OXPHOS deficiency observed in these cells. SIRT5 overexpression has significantly decreased the maximum respiratory rate and spare respiratory capacity in the control (Fig. 4B and C). This goes in line with a previously published study demonstrating that SIRT5 represses cellular respiration through desuccinylation of its targets PDC and SDH (Park et al., 2013).

Because SIRT5 overexpression in SCS deficient cells caused a decrease in the rate of ATP turnover (Fig. 4D), we investigated the effect of an increased level of succinylation on ATP production in HEK Flp-In cells overexpressing SIRT5-flag. To induce an increase in succinylation, we exposed these cells to 50 mM succinate for three days. We induced SIRT5-flag expression in these cells by adding doxycycline to the medium (Fig. 4E). We then assessed cellular respiration in these cells and determined the rate of ATP turnover which was indeed significantly lower in cells exposed to succinate (Fig. 4F). Inducing SIRT5 expression in these cells gave a significant rise in the ATP turnover rate in the cells exposed to succinate (Fig. 4F).

Succinylation represses the activity of nucleoside diphosphate kinase

Since it was previously suggested that mtDNA depletion observed in patients with SCS deficiency is the result of a lowered activity of NDK (Elpeleg et al., 2005), we analyzed whether NDK is a substrate for lysine succinylation and whether succinylation can impact its enzymatic activity. Our observation that accumulation of succinyl-CoA in cells resulted in protein hypersuccinylation and that so far no enzyme with lysine succinyltransferase has been identified, might suggest that succinyl-CoA could react with proteins non-enzymatically. Indeed, in vitro incubations of NDK with increasing concentrations of succinyl-CoA (Fig. S5A) or succinic anhydride (Fig. S5B) resulted in an increase in the extent of lysine succinylation of NDK.

To investigate the effect of succinylation on NDK activity we overexpressed flag-tagged human mitochondrial NDK in control fibroblasts and immunopurified the protein using α-flag immunoprecipitation. Cellular fractionation of cells after transfection showed that the transfected NDK was indeed localized in the mitochondria (Fig. 5A) and we were able to efficiently isolate NDK using flag-immunoprecipitation (Fig. 5B). Succinylation of purified NDK with succinic anhydride (Fig. 5C) resulted in a significant decrease of its enzymatic activity. Whereas the Km for the substrate TDP of succinylated human mitochondrial NDK (0.46 ± 0.04 mM) was not different from the non-succinylated enzyme (0.44 ± 0.11 mM), the Vmax of succinylated NDK was 37% lower than the Vmax of non-succinylated NDK (Fig. 5D).
Desuccinylation of NDK by SIRT5 rescues NDK activity

Next, we analyzed whether SIRT5 would desuccinylate NDK and thereby rescue the decrease in its activity caused by succinylation. Indeed, incubating succinylated purified NDK with SIRT5 and NAD$^+$ resulted in loss of its lysine succinylation (Fig. 5E).

We then tested whether SIRT5-mediated desuccinylation would rescue the decrease in NDK activity caused by succinylation. Succinylating NDK decreased its activity by 25%. Interestingly, desuccinylation by SIRT5 resulted in only a 13% lower activity than non-succinylated NDK, indicating that SIRT5 partially rescued the aberrant effect of succinylation.

To confirm our observation in a more physiological context, we co-expressed NDK and SIRT5 in SCS-G1 deficient fibroblasts, and measured NDK activity. Because SCS-G1 deficient cells have no SCS at all, this set-up enabled us to analyze the effect of succinylation on NDK activity and not of its physical interaction with SCS. Co-expression of SIRT5 resulted in a decrease level of NDK succinylation (Fig. 5H). Furthermore, we observed a significant increase in NDK activity in cells co-transfected with SIRT5 as compared to the control co-transfection with an empty vector (Fig. 5I). These results suggest that SIRT5 does indeed regulate NDK succinylation levels in mitochondria thereby preserving its enzymatic activity.

SIRT5 desuccinylates mitochondrial transcription factor A in SCS-G1 fibroblasts

Next, we set out to investigate whether SIRT5 controls lysine succinylation of other mitochondrial proteins involved in mtDNA maintenance and replication. For this we analyzed succinylation levels of mitochondrial transcription factor A (TFAM), which is essential for maintenance of the mitochondrial genome (Larsson et al., 1998) in SCS-G1 deficient fibroblasts and control fibroblasts. TFAM protein levels were substantially decreased in the SCS-G1 deficient cells as compared to the controls (Fig. 5J), whereas the amount of TFAM that came down upon succinyllysine immunoprecipitation was higher than in control cells. Thus, TFAM was hypersuccinylated in SCS-G1 deficient cells. To determine whether SIRT5 was able to desuccinylate endogenous TFAM we expressed SIRT5 in SCS-G1 deficient fibroblasts and assessed TFAM succinylation. Indeed, SIRT5 overexpression reduced succinylation levels of endogenous TFAM in SCS-G1 deficient cells (Fig. 5K).

Discussion

In this study we analyzed the functional impact of hypersuccinylation in SCS deficient cells. We demonstrated that defective metabolic flux through the TCA cycle caused by SCS deficiency results in increased lysine succinylation in patients fibroblasts and that this succinylation is primarily mitochondrial. Furthermore, we linked hypersuccinylation in these cells to defective OXPHOS, as decreasing succinylation through SIRT5 overexpression increased OXPHOS activity and made the cells less glycolytic. Finally, we investigated the effect of succinylation on enzymatic activity of NDK, an enzyme essential for mtDNA replication, and found that NDK was less active when succinylated. Consequently, NDK activity could be partially rescued by desuccinylation with SIRT5.

SCS deficient patients present with multiple respiratory chain deficiencies,
Figure 5. SIRT5 desuccinylates and partially rescues the activity of nucleoside diphosphate kinase (NDK). (A) Western blot of cytosolic and mitochondrial fraction in fibroblasts overexpressing human mitochondrial flag tagged nucleoside diphosphate kinase (NDK-flag). (B) Purification of NDK-flag using flag immunoprecipitation (IP) analyzed by α-NDK western blot. (C) Purified mitochondrial NDK was incubated with (+) or without (-) 6 mM succinic anhydride (SA) for 15 minutes at room temperature in 20mM ammonium bicarbonate buffer. Succinylation of NDK was assessed with α-succinyllysine western blot analysis. (D) Enzyme kinetics of succinylated and non-succinylated mitochondrial NDK studied by using different concentrations of TDP (0-1.6 mM) as substrate. Error bars represents
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primarily in muscle tissue, which is caused by mtDNA depletion (Carrozzo et al., 2007; Elpeleg et al., 2005; Ostergaard et al., 2007a; Rivera et al., 2010). In line with these results, our analysis of mitochondrial respiration in SCS deficient patient fibroblasts has confirmed that OXPHOS activity is indeed decreased whereas glycolysis is increased. Overexpressing SIRT5 reduced succinylation levels in patient cells and increased OXPHOS activity. In accordance with this, patient fibroblasts overexpressing SIRT5 also became less glycolytic thereby clearly making a shift to oxidative metabolism. We interpret these results to suggest that there is a link between hypersuccinylation and defective OXPHOS in these cells.

In principle, succinylation can contribute to molecular pathogenesis of OXPHOS deficiency in two ways: by directly suppressing activity of OXPHOS complexes or by suppressing activity of mtDNA maintenance proteins and thereby inducing mtDNA depletion. So far, there is evidence that succinylation is involved in metabolic regulation, as proteomic studies have detected succinylation on many metabolic enzymes (Park et al., 2013; Rardin et al., 2013a). Furthermore, site specific succinylation was shown to activate mitochondrial respiration through induction of PDC and SDH as desuccinylation of these complexes by SIRT5 suppressed their activity (Park et al., 2013). Consistently, we observed a decrease in maximum respiratory capacity and spare respiratory capacity in control cells overexpressing SIRT5. In contrast to this, SIRT5 overexpression induced an increase in mitochondrial respiration in SCS deficient cells. This possibly distinguishes physiological succinylation that has a regulatory role and pathological succinylation which is a consequence of a metabolic disorder. In the case of the latter, SIRT5 appears to play a ‘detoxifying’ role by removing aberrant succinylation.

It has been suggested previously that mtDNA depletion in SCS deficiency is caused by decreased activity of NDK (Elpeleg et al., 2005; Ostergaard et al., 2007a), however no specific mechanism has been proposed. We observed that hypersuccinylation of NDK reduced its activity in vitro and desuccinylation of NDK by SIRT5 partially rescued its enzymatic activity. Overexpressing SIRT5 in SCS-G1 deficient cells decreased succinylation of NDK and significantly increased its activity. Because NDK activity is rate-limiting in mtDNA replication in post mitotic tissues
(Bradshaw and Samuels, 2005) even modest changes in its activity can significantly influence mtDNA amounts. Moreover, we found that another mtDNA maintenance protein, TFAM, is hypersuccinylated in SCS-G1 deficient cells and overexpression of SIRT5 in these cells decreased TFAM succinylation. This together with the fact that one succinylation site has been previously identified on TFAM in SIRT5 KO cells (Park et al., 2013) suggests that SIRT5 could be regulating TFAM succinylation. Furthermore, it is possible that SIRT5 regulates succinylation of other mtDNA proteins, as for example one succinylation site was detected on deoxyguanosine kinase (DGUOK) (Park et al., 2013) which is an enzyme involved in the regulation of mitochondrial nucleotide pools. Importantly, DGUOK deficiency is associated with MDS (Eriksson and Wang, 2008; Mandel et al., 2001).

SCS-G1 and SCS-A2 deficient patients vary in clinical presentation however MDS was reported for both deficiencies. The mechanism of mtDNA depletion in these patients is obscure as SCS is not directly related to mtDNA maintenance. Our results establish a connection between mitochondrial dysfunction in this disorder and increased succinylation. In addition, we provide evidence that hypersuccinylation of NDK could lead to its decreased activity in SCS deficient cells. Further proteomic and functional studies are necessary to fully elucidate the role of succinylation in the pathophysiology of SCS deficiency. Advances in this area are promising because aberrant protein succinylation can be counteracted with SIRT5 and therefore activators of SIRT5 could be used as therapy in SCS deficient patients.

Materials and methods

SDS –PAGE and Western blotting

Cells were harvested by trypsinization, resuspended in lysis buffer (PBS, 0.1% triton and deacylase inhibitors: 1 µM trichostatin A and 10 mM nicotinamide) and sonicated at 40J/W/s on ice. Samples were ran on NuPAGE 4-12% gradient gels (Invitrogen), followed by transfer of proteins to nitrocellulose. The following antibodies were used: α-succinyllysine antibody (#401 PTM Biolabs), NDK (#18348-1-ap Proteintech), TFAM (kind gift of Prof. Rudolf Wiesner, University of Cologne), SUCLG1 (#H00008802 Abnova), SUCLA2 (#sc-68912 Santa Cruz) and HA (#16918 Abcam). Antibodies for β-actin (#A5441 monoclonal, Sigma-Aldrich) and VDAC (#14734 Abcam) were used as loading controls. IR-dye based secondary antibodies (LI-COR) were used to detect antibody signals using the Odyssey scanner (LI-COR).

Cell culture conditions

SCS deficient fibroblasts, control fibroblasts and HEK293 cells were routinely cultured in DMEM containing 25 mM HEPES, 2% Pen/Strep and 10% FBS. To induce SIRT5 overexpression in HEK Flp-In cells 10 ng/ml doxycycline was added to the medium. For succinate exposure the cells were incubated in DMEM containing 25 mM succinate. For Seahorse respiratory analysis with HEK 293 cells, the cells were exposed to succinate with and without doxycycline for three days.

Succinyllysine analysis in fibroblasts
Cell pellets were resuspended in 50 mM NH$_4$CO$_3$ buffer containing deacetylase inhibitors (1 µM Trichostatin A and 10 mM nicotinamide) followed by sonication at 40J/W/S. To digest the protein into amino acids, samples were incubated with pronase (53702, Calbiochem), at a protein to pronase ratio of 10:1, in 50 mM NH$_4$CO$_3$ for 4 hours at 37°C. The reaction was stopped with 5 volumes of acetonitrile, 10 µl 2.5 mM D4-labeled L-lysine internal standard (DLM-2640 Cambridge Isotopes Laboratories) and 10 µl 10 µM D8-labelled acetyllysine internal standard (D-6690 CDN Isotopes). The samples were briefly vortexed and centrifuged at 14000 rpm 4°C for 10 minutes followed by solvent evaporation at 40°C under a gentle stream of nitrogen. Samples were then taken up in 0.01% heptafluorobutyric acid and analyzed with LC-MS/MS.

Subcellular fractionation

Enriched mitochondrial fractions were obtained using digitonin fractionation. Cells were trypsinized and resuspended in 0.5 ml of cell buffer (150 mM KCl, 25 mM Tris, 2 mM EDTA, and 12.5 mM KPi at pH 7.4). A volume of 1 ml cell suspension (1.5 mg protein/ml) was incubated 200 µg digitonin for 5 minutes at 37°C. The samples were then centrifuged at 500 x g at 4°C for 5 minutes to obtain pellets containing mitochondria but free of cytosol. The pellet was resuspended in the required buffer for subsequent experiments.

Immunofluorescence colocalization studies

SCS deficient and control fibroblasts were plated on coverslips in 6-well plates. On the next day the cells were transiently transfected with pDsRed2-Mito. Two day after the transfection the cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton. To visualize protein succinylation the cells were stained with α-succinyllysine antibody (1:500) and Alexa Fluor 488 dye. Leica TCS SP8 X Confocal Microscope was used for imaging.

Seahorse mitochondrial respiratory flux analysis

On the day prior to the respiratory analysis, SCS deficient and control fibroblasts were plated at 30,000 cells/well in Seahorse 96 well culture plates followed by overnight incubation in regular DMEM. 30 minutes before the run, the medium was replaced with DMEM (Sigma-Aldrich #5030) supplemented with 5 mM glucose and 2mM L-glutamine. The DMEM did not contain sodium bicarbonate, pen/strep/fungizone and serum. To analyze mitochondrial respiration, oxygen consumption rate (OCR) was measured following injections of oligomycin (1.5 µM final concentration), FCCP (1 µM), antimycin (2.5 µM) and rotenone (1.25 µM). To measure respiratory flux in glucose deficient medium, on the day prior to the respiratory analysis the cells were plated in glucose-free DMEM containing 5 mM galactose and 2 mM L-glutamine. Also the medium used during the run (DMEM Sigma-Aldrich #5030) did not contain glucose, but was supplemented with 5 mM galactose and 2 mM L-glutamine.

Nucleoside diphosphate kinase (NDK) activity measurement

NDK activity was analyzed using a coupled enzyme assay according to (Bergmeyer
et al., 1986) on a Cobas-Fara centrifugal analyzer. In brief, ADP production by the phosphotransfer reaction of NDK from thymidine 5’-diphosphate (TDP) and ATP was coupled with pyruvate kinase (PK) and lactate dehydrogenase (LDH) to NADH oxidation. The reaction mixture consisted of 50 mM Tris pH 7.4, 75 mM KCl, 5 mM MgCl₂, 0.3 mM NADH, 1 mM ATP, 1 mM phosphoenolpyruvate, 2U/ml PK and 2U/ml LDH. The reaction was started with TDP (0-1.6 mM final concentration).

**Succinylation of NDK with succinic anhydride and succinyl-CoA**

NDK was succinylated using succinic anhydride (Sigma) in 0.1 M NaHCO₃ pH 8.3 at room temperature for 15 min or with succinyl-CoA at 37°C in 50 mM KPi buffer pH 8.0 for 30 minutes. Succinyl-CoA stock solutions were prepared and stored in formic acid 20 mM pH 3.5.

**Expression and purification of recombinant SIRT5-His**

SIRT5-His expression plasmid was obtained from Addgene (a gift of Cheryl Arrowsmith Addgene plasmid #25487) and was expressed in *E. coli* BL21DE3 strain. Transformed BL21DE3 cultures were grown in terrific broth medium containing ampicillin (100µg/ml) at 37°C until OD of 0.25 was reached after which the culture was allowed to cool down to 22°C. After one hour SIRT5 expression was induced with 500 mM IPTG and the cells were harvested 18 hours after the induction. Pellets of 3-4 mg were aliquotted, snap-frozen and stored at -80°C. For purification of SIRT5-His one pellet was resuspended in 2.5 ml lysis buffer (25 mM Hepes, 500 mM NaCl and 42.67 µg/mL lysozyme) and incubated on an overhead shaker for 30 minutes at 4°C. Cells were then sonicated at 8W for 15 seconds 10 times. Insoluble debris was removed by centrifugation for 15 minutes, 16000 rpm at 4°C. Imidazole at pH 8.0 was added to the supernatant to a final concentration of 30 mM. A Ni-NTA column was prepared using 0.4 ml HisLink Protein Purification Resin suspension. The column material was first equilibrated by washing five times with 1 ml wash buffer (25 mM Tris pH 8.0, 500 mM NaCl and 30 mM imidazole) and then resuspended in 2.5 ml of wash buffer. The suspension was then transferred into a disposable plastic column, and the column was allowed to run dry by gravity flow. After this, the supernatant from the *E. coli* lysate was loaded onto the column and allowed to pass through. The column was washed five times with 1 ml wash buffer. Next, SIRT5 was eluted with 5 column volumes (200 µl) of elution buffer (25 mM Tris pH 8.0, 100 mM NaCl, and 500 mM imidazole). For all of the desuccinylation experiments 10 µl of freshly eluted SIRT5 was used.

**Immunoprecipitation (IP) of NDK-flag**

NDK flag transfected fibroblasts were trypsinized and lysed in 500 µl of IP buffer (50 mM Tris/HCl pH 7.4, 1% Triton, 150 mM NaCl, 0.5 mM EDTA, 10 mM NAM, 1 mM TSA and protease inhibitors (Roche)) by sonication at 40 J/W/S and the lysate was centrifuged for 2 minutes at 2000 rpm. Protein content was determined in the supernatant and the volume of all samples was adjusted to 600 µl, so that the amount of protein in the input was equal for each sample. Then, 100 µl was saved as input and 500 µl was used for the IP (300-500 µg protein). NDK-flag was purified
with 20 µl of α-flag M2 agarose beads (Sigma) per sample. After addition of the beads the samples were rotated on an overhead shaker for 2 hours at 4°C. After the incubation the beads were washed four times with 1 ml IP buffer. NDK-flag was eluted by incubating the beads three times in 25 µl of IP buffer containing 100 µg/ml of flag peptide for 2 minutes at RT. Eluted NDK-flag was collected by centrifuging the samples for 30 seconds at 2,000 rpm at 4°C and pooling together the elutes.

Desuccinylation of NDK with recombinant SIRT5

Desuccinylation assay with SIRT5 was performed as described in (Du et al., 2011). In brief, the assay was carried out in 20 mM Tris pH 7.5, 1 mM DTT and 1 mM NAD⁺ for 1 hour at 37°C. Assay buffer without NAD⁺ was used as negative control. NDK (35 µl) succinylated by succinic anhydride and freshly purified SIRT5-His (10 µl) were added to the assay. The final assay volume was 60 µl.

Immunoprecipitation (IP) of succinylated proteins from enriched mitochondrial fractions

Pellets containing enriched mitochondrial fraction from fibroblasts was resuspended in 500 µl of PBS 0.1% Triton, 10 mM NAM and protease inhibitors (Roche). The samples were sonicated three times at 40 J/W/s. Equal protein amount were used for each IP (200-400 µg). Succinylated proteins were purified from the mitochondrial lysate using α-succinyllysine beads (PTM Biolabs #402). The beads were washed three times with 0.5 ml PBS 0.1% Triton and resuspended in this buffer (1:1 ratio). A volume of 20 µl of bead suspension was added to each sample and incubated overnight on an overhead shaker at 4°C. Following the incubation, the supernatant was removed after centrifuging the samples at 1400 rpm for 30 seconds at 4°C. The beads were then washed four times with 0.5 ml PBS 0.1% Triton, 10 mM NAM and 1 mM TSA. Succinylated proteins were eluted by incubating the beads three times with 20 µl HCl/ 0.2M glycine pH 2.0 and then neutralized with NaOH.

Lysine succinylation of mouse liver mitochondrial lysates

On the day of the experiment, purified mitochondria were resuspended in 50 mM Tris/HCl pH 8.0, 150 mM NaCl and deacetylase inhibitors (1 µM trichostatin A and 10 mM nicotinamide) at a concentration of 2.5 µg protein/µl. The succinylation reactions were started by adding 1 mM of succinyl-CoA in a reaction volume of 50 µl. The samples were incubated for different time intervals from 0 to 60 minutes at 37°C. The reactions were stopped by adding NuPAGE sample buffer (Invitrogen) and heating the samples at 70°C for five minutes.
Chapter 5

References

The role of aberrant protein succinylation in SCS deficiency


Figure 1S. (A) Western blot analysis of succinyllysine in mouse mitochondrial lysate incubated with 1 mM of succinyl-CoA for different time intervals (0-60 minutes). 10 µg protein was loaded per lane. (B) Quantification of succinyllysine in control, SCS-A2 deficient and SCS-G1 deficient fibroblasts with LC-MS. (C) Immunofluorescence imaging of SCS-G1 deficient fibroblasts stained with α-succinyllysine antibody and DAPI. The overlay of the images shows co-localization of protein succinylation with mitochondria.
The role of aberrant protein succinylation in SCS deficiency

A

**mitochondrial respiration**

![Graph showing mitochondrial respiration](image)

**glycolytic rate**

![Graph showing glycolytic rate](image)

Figure 3S. (A) Oxygen consumption rate (OCR) in control fibroblasts, SCS-A2 deficient fibroblasts and SCS-G1 deficient cells expressing empty vector pcDNA3 or SIRT5-flag, measured using XF-96 Seahorse analyzer (* indicates p < 0.05). (B) Extracellular acidification rate (ECAR) measured using XF-96 Seahorse analyzer (* indicates p < 0.05).

A

succinyl-CoA (mM):

| 0.06 | 0.12 | 0.25 | 0.5 | succinylated NDK |

B

SA (mM):

| 0.6 | 0.75 | 1.5 | 3 | succinylated NDK |

Figure 5S. (A) Succinylation of purified NDK by incubations with different concentrations of succinyl-CoA (0.06-0.12 mM). Succinyl-CoA stock solutions were prepared and stored at pH 3.0 in formic acid buffer. Succinylation was performed at pH 8.0. (B) Succinylation of purified NDK by incubations with different concentrations of succinic anhydride (0-6 mM). Succinylation was performed at pH 8.0.