Broad Spectrum Lysine Acylation Profiling to Investigate Regulation of SIRT5 using Four Different Acyl-Lysine PTM Antibodies

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INTRODUCTION

Acylation refers to a form of post-translational protein modification that is mediated through the attachment of functional groups to lysine residues via acyl linkages.

In recent years such acylation-mediated modifications namely acetylation, succinylation, formylation, butyrylation, propionylation, malonylation, glutarylation and crotonylation have been identified and validated by proteomic analyses. Accumulating evidence links this group of post-translational modifications (PTMs) with the metabolic and signaling pathways of the cell. As a member of the sirtuin family of NAD$^+$-dependent histone deacetylases, Sirt5 has been shown to remove acetyl, succinyl, malonyl and glutaryl groups from modified lysine residues indicating the broad spectrum of substrates regulated by Sirt5, especially in the context of metabolic pathways.

To better understand the regulatory landscape of Sirt5 and to identify Sirt5-associated pathways, we conducted a large-scale lysine acylation profiling study using PTM antibodies targeting acetyl, succinyl, malonyl and glutaryl lysine modifications in the livers of wild type and Sirt5 knock-out mice. Quantitative analysis (LFQ) of identified lysine acylation sites was performed and showed upregulation of lysine succinylation and malonylation in the livers of Sirt5 knock-out mice. Interestingly, although Sirt5 was initially identified as a lysine deacetylase, Sirt5 knock-out mice did not show as many upregulated lysine acetylation sites as those corresponding to succinylation or malonylation.

Summary: Collectively, the proteins identified in the current study covered several major metabolic pathways, including the TCA cycle and fatty acid degradation, which are heavily regulated by Sirt5. Quantitative changes of lysine acylation sites were mapped onto protein interaction networks in order to better understand the cross-talk between different types of acylation.

METHODS

Preparation of Liver Peptides of Wild Type and Sirt5 Knock-out Mice

The livers of wild type and Sirt5 knock-out mice were harvested as described before (1). A total of 40 mg of soluble protein from each liver was reduced, alkylated and digested by trypsin. Digested peptides were desalted over SEP PAK C18 columns and lyophilized.

Immunoaffinity Purification (IAP) of Lysine Acylated Peptides

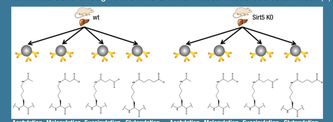
IAP of lysine acylated peptides from mouse liver tryptic peptides was performed using the PTMScan® protocol as previously described (2). Briefly, 200 µg of each lysine acylation antibody (acetylation, succinylation, malonylation and glutarylation) was conjugated to Protein A beads (Roche) overnight at 4˚C and then washed extensively with PBS. A total of 10 mg of mouse liver tryptic peptides was dissolved in 1.4 ml of IAP buffer and mixed with PTM Ab beads and incubated for 2 hours at 4˚C. The beads were washed twice with 1 ml of IAP buffer and three times with 1 ml of HPLC grade water. Peptides were eluted from beads with 0.15% TFA. Eluted peptides were desalted over tips packed with Empore C18 and eluted with 40% acetonitrile in 0.1% TFA. Eluted peptides were dried under vacuum.

LC-MS/MS Analysis and Database Searching

Enriched acylated peptides were separated on a 100 µm X 15 cm reversed-phase column and eluted using a 120-min linear gradient of 5%–30% acetonitrile in 0.125% formic acid delivered at 300 nl/min using an Easy nLC (Thermo Fisher). Tandem mass spectra were collected in a data-dependent manner with an Orbitrap Elite® mass spectrometer. All MS2 spectra were searched using SEQUEST® (v. 28 (rev. 12), 1998–2007) against the NCBI mouse database including forward and reverse sequences. Static carbamidomethylation of cysteine (+57.0215) was required, and appropriate lysine acylation [Ac-K (+42.0106), Suc-K (+100.0160), Mal-K (+86.0004), Glu-K (+114.0317) and methionine oxidation (+15.9949) were allowed with a maximum of four modifications per peptide. Peptide spectral matches were filtered to a 1% false discovery rate using the linear discriminant analysis module of Core. Replicate injections were run for each enrichment.

Peptide Quantification and KEGG Pathway Analysis

Filtered pepXML files containing only acyl lysine peptides and raw data files were processed by Skyline v2.6 (3). Quantitative data was evaluated and clustered in Spotfire® DecisionSite v 9.1.2. KEGG pathway analysis of identified proteins with Ac-K was done through the DAVID Bioinformatics Resources version 6.7 (4).



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(1) Tan M., and Zhao Y. (2014) *Cell Metabolism* 19, 605–617. **(2)** Guo A., and Comb MJ. (2014) *Mol. Cell. Proteomics* 13, 372–387. **(3)** Schwartz D., and Gygi, SP. (2005) *Nat. Biotechnol.* 23, 1391–1398. **(4)** Schilling B., and Gibson, BW. (2012) *Mol. Cell. Proteomics* 11: 202–214. **(5)** Huang DW., and Lempicki RA. (2009) *Nat. Protoc.* 4: 44–57.

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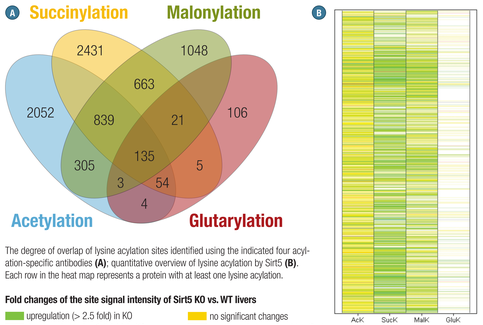
Table 1: Summary of the identified lysine acylation sites and associated proteins.

	Acetylation	Malonylation	Succinylation	Glutarylation
Site	4,953	3,036	5,713	329
Protein	2,927	2,168	2,259	338

Figure 1

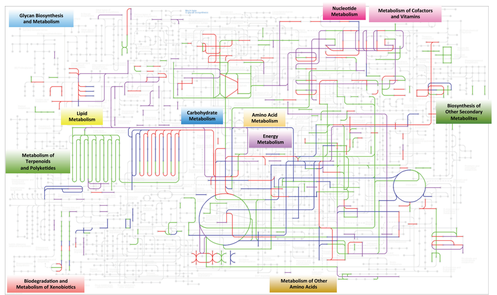


The degree of overlap of lysine acylation sites identified using the indicated four acyl-ation-specific antibodies **(A)**; quantitative overview of lysine acylation by Sirt5 **(B)**. Each row in the heat map represents a protein with at least one lysine acylation.

Fold changes of the site signal intensity of Sirt5 KO vs. WT livers

upregulation (> 2.5 fold) in KO
downregulation (< -2.5 fold) in KO
both upregulation and downregulation in KO
no significant changes
no site acylations were identified

Figure 2: The involvement of lysine acylation in metabolic pathways.

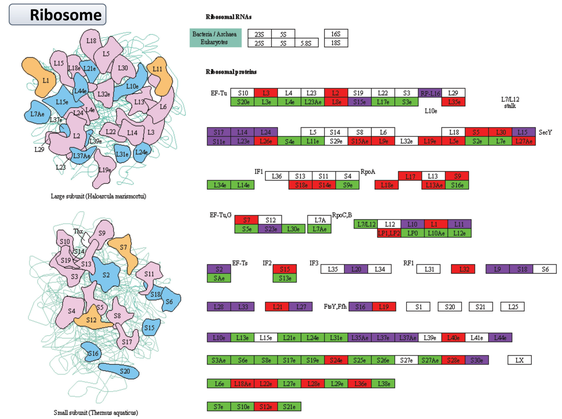


The number of acylation types identified on the protein

1 2 3 4

Each line connecting two metabolites represents an enzyme.

Figure 3



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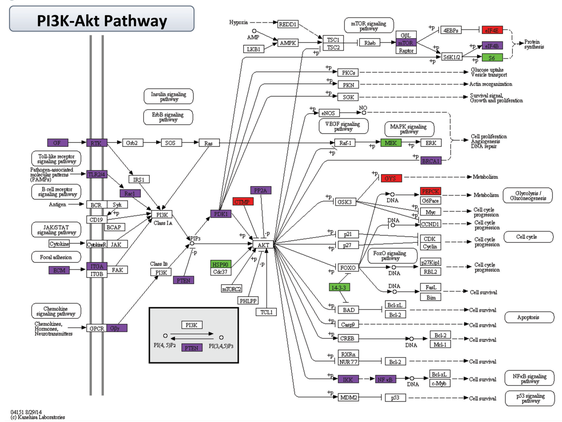
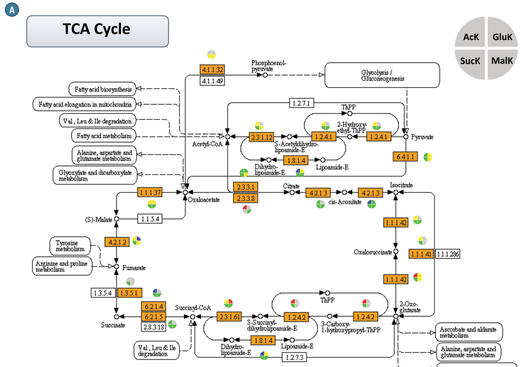


Identification of lysine acylation in ribosomal subunits **(A)**; involvement of lysine acylation in the PI3K-Akt pathway **(B)**.

The number of acylation types identified on the protein

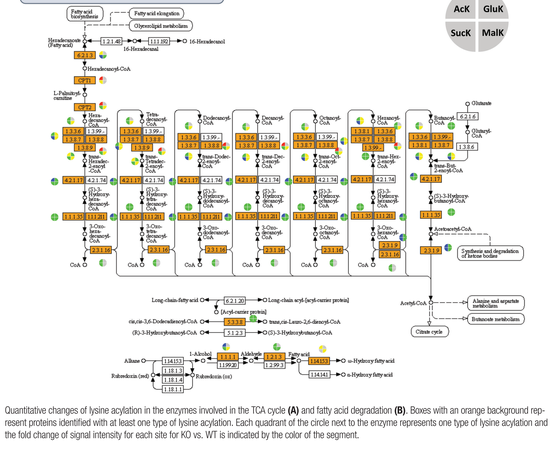
1 2 3 4

Figure 4



Changes in acylation levels in KO liver

upregulation (> 2.5 fold) in KO
downregulation (< -2.5 fold) in KO
both upregulation and downregulation in KO
no significant changes
no site acylations were identified



Quantitative changes of lysine acylation in the enzymes involved in the TCA cycle **(A)** and fatty acid degradation **(B)**. Boxes with an orange background represent proteins identified with at least one type of lysine acylation. Each quadrant of the circle next to the enzyme represents one type of lysine acylation and the fold change of signal intensity for each site for KO vs. WT is indicated by the color of the segment.

upregulation (> 2.5 fold) in KO
downregulation (< -2.5 fold) in KO
both upregulation and downregulation in KO
no significant changes
no site acylations were identified