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SHORT COMMUNICATION

Adipocyte protein modification by Krebs cycle intermediates and fumarate ester-derived succination

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Abstract Protein *succination*, the non-enzymatic modification of cysteine residues by fumarate, is distinguishable from *succinylation*, an enzymatic reaction forming an amide bond between lysine residues and succinyl-CoA. Treatment of adipocytes with 30 mM glucose significantly increases protein succination with only a small change in succinylation. Protein succination may be significantly increased intracellularly after treatment with fumaric acid esters, however, the ester must be removed by saponification to permit 2SC-antibody detection of the fumarate adduct.

Keywords Succination · *S*-(2-succino)cysteine · Succinylation · Acetylation · Fumarate · Ester · Glutathione

Abbreviations

2SC	*S*-(2-succino)cysteine
MMF	Monomethylfumarate
DMF	Dimethylfumarate
GSH	Glutathione
Keap1	Kelch-like ECH-associated protein 1

Introduction

Our laboratory described a novel chemical modification of proteins, *S*-(2-succino)cysteine (2SC), formed by a Michael addition reaction that generates a thioether bond between fumarate and the cysteine sulfhydryl group; a non-enzymatic process termed *succination* of protein (Alderson et al. 2006). Subsequent studies indicated that 2SC is increased on adipocyte proteins during type 2 diabetes and is associated with glucotoxicity-driven mitochondrial stress (Nagai et al. 2007; Frizzell et al. 2011, 2012). The functional significance of succination in diabetes has been studied on several proteins including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Blatnik et al. 2008) and adiponectin (Frizzell et al. 2009). Succination appears to have an inhibitory function, e.g., inactivation of GAPDH in muscle of type 1 diabetic rats or blocking the polymerization and secretion of adiponectin from type 2 diabetic mouse adipose tissue (Blatnik et al. 2008; Frizzell et al. 2009). The increased succination of kelch-like ECH-associated protein 1 (Keap1) and mitochondrial Aconitase 2 (ACO2) has been described in renal cell carcinoma models derived from mutations in fumarate hydratase (Adam et al. 2011; Ternette et al. 2013). Modification of Keap1 by succination stabilizes Nrf2 and has been proposed to promote cancer survival through induction of the antioxidant response element (Adam et al. 2011; Ooi et al. 2011), while succination of ACO2 inhibits enzyme activity leading to disrupted mitochondrial metabolism (Ternette et al. 2013).

The term *succinylation* is similar to *succination,* and describes a different acyl modification of protein (Fig. 1a). Succinylation is proposed to occur via an enzymatic mechanism forming an amide bond between succinyl-CoA and a lysine residue, producing *N*-succinyl-lysine (Fig. 1a), although the conjugating enzyme has currently not been identified (Lin et al. 2012; Peng et al. 2011; Zhang et al. 2011). The presence of succinyllysine has been described on several proteins including; isocitrate dehydrogenase,

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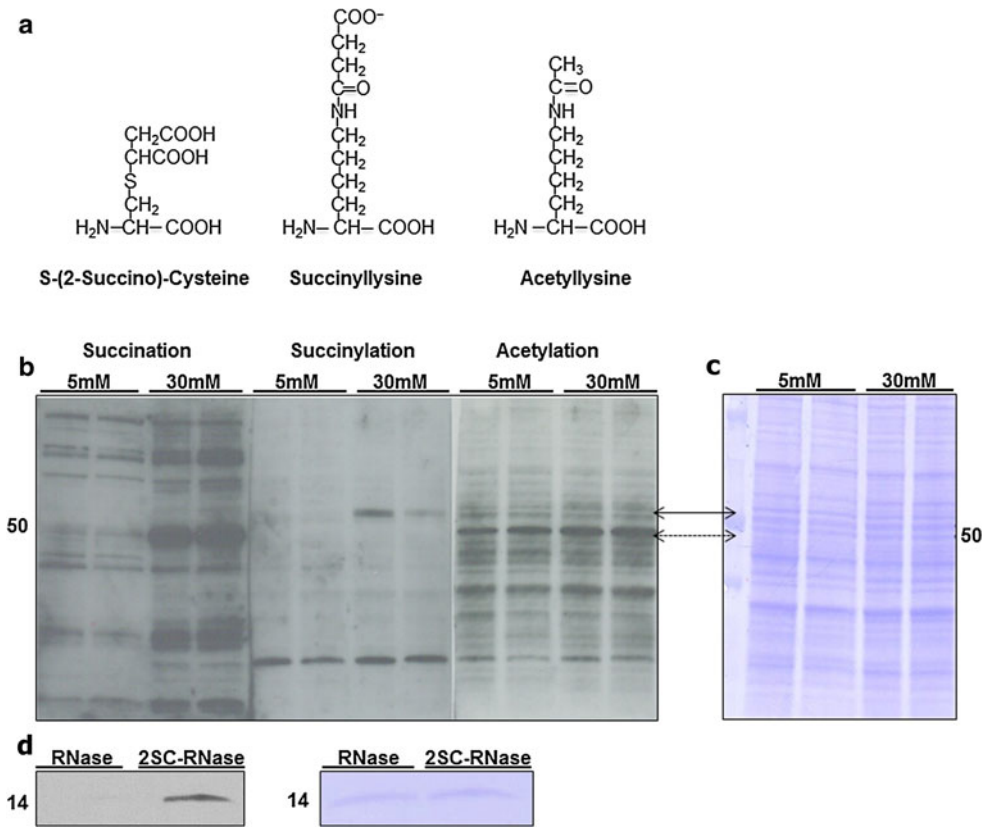

Fig. 1 Succination versus succinylation and acetylation in 3T3-L1 adipocytes. **a** Structures of *S*-(2-succino)-cysteine, succinyllysine, and acetyllysine. **b** Adipocytes were matured in 5 or 30 mM glucose for 8 days. A western blot was performed on 30 µg of protein using antibodies specific for succinated proteins (*lanes 1–4*) succinylated proteins (*lanes 5–8*) and acetylated proteins (*lanes 9–12*). Two representative lanes are shown for each treatment. A molecular weight marker is shown on the *far left*. **c** A representative Coomassie stain of the blots in Fig. 1b depicts equal loading of protein in each lane. The *solid arrow* indicates the protein band with significant increase in succinylation and *dashed arrow* indicates protein with significant increase in succination. **d** A western blot was performed on 0.5 µg of RNase and 2SC-RNase (positive control prepared after incubation of RNase in 500 mM fumarate for 18 h). A Coomassie stain of the immunoblot indicates equal protein loading of RNase and 2SC-RNase

serine hydroxymethyltransferase, GAPDH and histone proteins (Du et al. 2011; Xie et al. 2012; Zhang et al. 2011). Succinylation of specific histone sites is proposed to result in gene inactivation and obstruction in the association of negatively charged DNA with the histones (Xie et al. 2012). Recently, succinylation has been shown to be reversible through interaction of *N*-succinyl-lysine with the desuccinylase sirtuin-5 (Du et al. 2011; Newman et al. 2012; Zhou et al. 2012), prior to this only sirtuins 1-3 were known as deacetylases. In contrast to both succinylation and acetylation, succination is believed to be irreversible due to the stability of the thioether bond (Alderson et al. 2006; Frizzell et al. 2009).

In addition, we have studied the relationship between succination and modification of proteins by fumarate esters. Fumarate esters rapidly enter the cell where they react quickly with available thiols, including intracellular glutathione (Ghoreschi et al. 2011; Schmidt and Dringen 2010; Thiessen et al. 2010). Both monomethylfumarate (MMF) and dimethylfumarate (DMF) are used clinically for treatment of psoriasis and neurological diseases (Ghoreschi et al. 2011; Gold et al. 2012; Papadopoulou et al. 2010). Fumarate ester treatment has been shown to induce beneficial changes in the inflammatory cell profile, and recently DMF has been shown to modify Keap1 resulting in an Nrf2-mediated antioxidant response in neurons (Linker et al. 2011). We have examined the reactivity of MMF, DMF and fumarate within adipocytes in vitro. The purpose of this article is to compare the levels of protein succination, succinylation and acetylation in high glucose (30 mM)-treated adipocytes and to describe a procedure to detect protein succination by fumarate esters.

Materials and methods

Cell culture

3T3-L1 adipocytes were differentiated and matured as previously described (Nagai et al. 2007). Adipocytes

matured in 5 mM glucose for 8 days were treated with 100 μM MMF, DMF or (sodium) fumarate for 24 h or with 500 μM MMF, DMF or fumarate for 48 h. Cellular protein was collected by lysing the cells in radioimmunoprecipitation assay buffer (RIPA) [50 mM Tris/HCl, 150 mM NaCl, 1 mM ethylenediaminetetra acetic acid (EDTA), 1 % Triton X-100, 0.1 % sodium dodecyl sulfate (SDS) and 0.5 % sodium deoxycholate, pH 7.4, 2 mM diethylenetriaminepenta acetic acid (DTPA) with the addition of 2 mM sodium orthovanadate, 2 mM sodium fluoride and a protease inhibitor cocktail (P8340, Sigma-Aldrich)] as described (Nagai et al. 2007).

Protein saponification

200 μg protein from cell lysates incubated with 80 % dimethyl sulfoxide (DMSO), 1 or 0.2 mM potassium hydroxide (KOH), and 85 mM EDTA at room temperature for 30 or 35 min, with vortexing at 5 min intervals. The pH was adjusted to 7, and the protein was precipitated with 90 % acetone before being resuspended in 100 μL RIPA buffer. The pH was again adjusted to 7 prior to protein quantification.

Western immunoblotting

Protein quantification and western blotting were performed as previously described (Nagai et al. 2007). Anti-acetyl lysine antibody was from Cell Signaling (Cell Signaling, Beverly, MA). The polyclonal anti-2SC antibody was prepared as described previously (Nagai et al. 2007). Anti-succinyl lysine antibody was obtained from Dr. Zhongyi Cheng, PTM Biolabs Inc., Chicago, IL.

Analysis of glutathione concentration

Glutathione (GSH) was measured using Arbor Assay Glutathione Fluorescent Detection Kit. Adipocytes treated with 100 μM fumarate and MMF for 24 h and 100 μM DMF for 20 min were used for determining GSH concentrations. Data were analyzed in Sigmaplot 11, using a one way ANOVA to compare cellular GSH concentrations between treatments.

Results

Succination versus succinylation and acetylation

Adipocytes cultured in 30 versus 5 mM glucose had a significant increase in succinated proteins as detected by anti-2SC antibody (Fig. 1b, lanes 1–4), confirming the increased succination previously observed in the diabetic adipocyte (Nagai et al. 2007; Frizzell et al. 2009). However, both succinylation and acetylation show limited increases between adipocytes matured in 30 versus 5 mM glucose (Fig. 1b, lanes 5–8 and 9–12). Both succinylation and acetylation only appeared to increase on one protein ~60 kDa in 30 mM adipocytes (Fig. 1b, c, solid arrow), a slightly higher molecular weight than the prominent 2SC protein at ~50 kDa in 30 mM adipocytes, known to be tubulin (Fig. 1b, c, dashed arrow). Positive staining of 2SC-RNase and not unmodified RNase in Fig. 1d confirms specificity of antibody staining.

Fumarate versus fumarate esters

Initial treatment of adipocytes after 48 h in 500 μM fumarate and MMF did not increase protein succination as expected, while 500 μM DMF treatment showed slight increases in succination (Fig. 2a). Although fumarate added exogenously to the growth medium does not appear to enter the cell readily or increase 2SC, it was expected that MMF and DMF would enter the cell and result in increased 2SC-modified proteins. In its absence, we speculated that the ester may not have been completely removed intracellularly, preventing recognition by the anti-2SC antibody. To eliminate the ester and allow detection of the fumarate adduct the protein was saponified by base hydrolysis (Fig. 2b). Analysis of adipocytes treated with 500 μM MMF and DMF then resulted in the detection of multiple succinated proteins (Fig. 2b), although individual protein bands were not resolved due to intense staining post-saponification when compared to control 5 mM adipocytes (Fig. 2b). To detect specific bands the adipocytes were cultured in medium containing 100 μM MMF, DMF or fumarate for 24 h and again saponified but in a lower KOH concentration (0.2 mM) for 35 min. Milder saponification treatment allowed the detection of specific bands equivalent to the proteins that are increasingly succinated in cells treated with 30 mM glucose, suggesting identical protein targets (Fig. 2c, lanes 3, 4 and 5–8). Intracellular glutathione (GSH) levels were unaffected after fumarate and MMF treatment whereas DMF treatment significantly decreased GSH in adipocytes (Fig. 2d).

Discussion

Protein succination, succinylation, and acetylation are distinguished by amino acid specificity, nature of the chemical bond and reaction mechanism. Although succination increases significantly in adipocytes matured in 30 versus 5 mM glucose (Nagai et al. 2007, Fig. 1b), succinylation and acetylation only appear to increase on a few proteins, suggesting that the increase in succination is

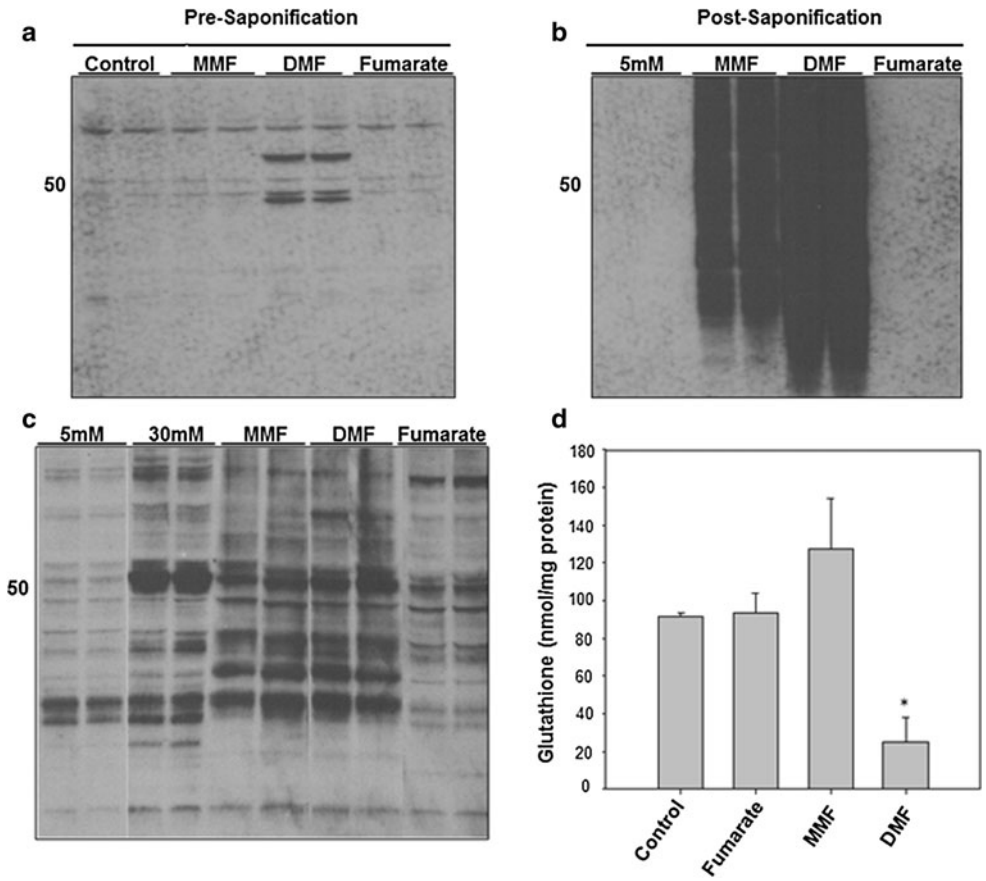

Fig. 2 Detection of protein succination in adipocytes after fumarate ester treatment. **a** Adipocytes were matured in 5 mM glucose and treated with 500 μM MMF, DMF or fumarate for 48 h. Control adipocytes remained in 5 mM glucose for the 48 h treatment. A western blot was performed on 30 μg of protein and anti-2SC antibody was used to detect succinated protein. **b** Adipocytes were matured in 5 mM glucose and treated with 500 μM MMF, DMF or fumarate for 48 h before saponification to eliminate the ester group from the alkyl fumarate-treated cells (1 mM KOH, 30 min). The succinated proteins post-saponification were detected by western blotting 30 μg of protein using the anti-2SC antibody (protein concentration determined based on final volume of sample). **c** Adipocytes matured in 5 or 30 mM glucose were treated with 100 μM MMF or DMF for 24 h or 5 mM fumarate for 8 days. Control adipocytes remained in 5 mM glucose for 48 h. To improve detection of protein after fumarate ester treatment the saponification procedure was optimized (0.2 mM KOH, 35 min). The merged image shows two representative lanes for each treatment. **d** Reduced glutathione concentration (GSH) in adipocytes matured in 5 mM glucose and treated with 100 μM fumarate or MMF for 24 h and 100 μM DMF for 20 min. $n = 3$ for each treatment. $^{*}p < 0.05$

related to the lack of enzymatic removal of 2SC, whereas both succinyllysine and acetyllysine are enzymatically reversible, preventing their accumulation (Du et al. 2011; Newman et al. 2012; Zhou et al. 2012). As a consequence protein succination may have a more prominent role in altering intracellular function in the diabetic adipocyte versus other Krebs cycle derived modifications.

We additionally investigated an alternative method to increase succination; treatment with fumaric acid esters. Initially anti-2SC antibody was unable to detect succination in adipocytes treated with MMF or DMF due to retention of the ester group. A previous study examining the effects of MMF in astrocytes identified Keap1 as a target protein with an adduct mass of 130 Da (Linker et al. 2011), implying that the ester had not been removed as the mass of fumarate adduct alone would be expected to be 116 Da. The difference in molecular weights suggested that fumarate esters react with proteins but the ester must be removed to detect succination. We have demonstrated a saponification procedure which successfully removes the ester to permit detection of MMF and DMF adducts by the anti-2SC antibody. However, a slight loss of total protein concentration occurs during hydrolysis, therefore the use of fumarate esters may not be ideal as a method to increase succination in vitro.

In previous studies we demonstrated that fumarate reacts slowly with thiols with a $pK_a \sim 9$ e.g., *N*-acetylcysteine (Alderson et al. 2006), whereas fumarate esters have been reported to react rapidly and decrease GSH concentrations in neuronal cells (Ghoreschi et al. 2011; Schmidt and Dringen 2010; Thiessen et al. 2010). The decrease in GSH observed in adipocytes treated with DMF, coupled with the

large increase in succinated proteins confirms that fumarate esters react quickly with both protein thiols and GSH. Protein succination may be an important and unexplored mechanism of action of fumaric acid esters.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Adam J, Hatipoglu E, O'Flaherty L, Ternette N, Sahgal N, Lockstone H, Baban D, Nye E, Stamp GW, Wolhuter K, Stevens M, Fischer R, Carmeliet P, Maxwell PH, Pugh CW, Frizzell N, Soga T, Kessler BM, El-Bahrawy M, Ratcliffe PJ, Pollard PJ (2011) Renal cyst formation in Fh1-deficient mice is independent of the Hif/Phd pathway: roles for fumarate in KEAP1 succination and Nrf2 signaling. Cancer Cell 20:524–537

Alderson NL, Wang Y, Blatnik M, Frizzell N, Walla MD, Lyons TJ, Alt N, Carson JA, Nagai R, Thorpe SR, Baynes JW (2006) *S*-(2-Succinyl)cysteine: a novel chemical modification of tissue proteins by a Krebs cycle intermediate. Arch Biochem Biophys 450:1–8

Blatnik M, Frizzell N, Thorpe SR, Baynes JW (2008) Inactivation of glyceraldehyde-3-phosphate dehydrogenase by fumarate in diabetes formation of *S*-(2-Succinyl)cysteine, a novel chemical modification of protein and possible biomarker of mitochondrial stress. Diabetes 57:41–49

Du J, Zhou Y, Su X, Yu JJ, Khan S, Jiang H, Kim J, Woo J, Kim JH, Choi BH, He B, Chen W, Zhang S, Cerione RA, Auwerx J, Hao Q, Lin H (2011) Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase. Science 334:806–809

Frizzell N, Rajesh M, Jepson MJ, Nagai R, Carson JA, Thorpe SR, Baynes JW (2009) Succination of thiol groups in adipose tissue proteins in diabetes succination inhibits polymerization and secretion of adiponectin. J Biol Chem 284:25772–25781

Frizzell N, Lima M, Baynes JW (2011) Succination of proteins in diabetes. Free Radical Res 45:101–109

Frizzell N, Thomas SA, Carson JA, Baynes JW (2012) Mitochondrial stress causes increased succination of proteins in adipocytes in response to glucotoxicity. Biochem J 445:247–254

Ghoreschi K, Brück J, Kellerer C, Deng C, Peng H, Rothfuss O, Hussain RZ, Gocke AR, Respa A, Glocova I, Valtcheva N, Alexander E, Feil S, Feil R, Schulze-Osthoff K, Rupec RA, Lovett-Racke AE, Dringen R, Racke MK, Röcken M (2011) Fumarates improve psoriasis and multiple sclerosis by inducing type II dendritic cells. J Exp Med 208:2291–2303

Gold R, Linker RA, Stangel M (2012) Fumaric acid and its esters: an emerging treatment for multiple sclerosis with antioxidative mechanism of action. Clin Immunol 142:44–48

Lin H, Su X, He B (2012) Protein lysine acylation and cysteine succination by intermediates of energy metabolism. ACS Chem Biol 7:953–956

Linker RA, Lee DH, Ryan S, van Dam AM, Conrad R, Bista P, Zeng W, Hronowsky X, Buko A, Chollate S, Ellrichmann G, Brück W, Dawson K, Goelz S, Wiese S, Scannevin RH, Lukashev M, Gold R (2011) Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. Brain 134:678–692

Nagai R, Brock JW, Blatnik M, Baatz JE, Bethard J, Walla MD, Thorpe SR, Baynes JW, Frizzell N (2007) Succination of protein thiols during adipocyte maturation a biomarker of mitochondrial stress. J Biol Chem 282:34219–34228

Newman JC, He W, Verdin E (2012) Mitochondrial protein acylation and intermediary metabolism: regulation by sirtuins and implications for metabolic disease. J Biol Chem 287:42436–42443

Ooi A, Wong JC, Petillo D, Roossien D, Perrier-Trudova V, Whitten D, Min BW, Tan MH, Zhang Z, Yang XJ, Zhou M, Gardie B, Molinié V, Richard S, Tan PH, Teh BT, Furge KA (2011) An antioxidant response phenotype shared between hereditary and sporadic type 2 papillary renal cell carcinoma. Cancer Cell 20:511–523

Papadopoulou A, D'Souza M, Kappos L, Yaldizli O (2010) Dimethyl fumarate for multiple sclerosis. Expert Opin Investig Drugs 19:1603–1612

Peng C, Lu Z, Xie Z, Cheng Z, Chen Y, Tan M, Luo H, Zhang Y, He W, Yang K, Zwaans BM, Tishkoff D, Ho L, Lombard D, He TC, Dai J, Verdin E, Ye Y, Zhao Y (2011) The first identification of lysine malonylation substrates and its regulatory enzyme. Mol Cell Proteomics 10:M111.012658

Schmidt MM, Dringen R (2010) Fumaric acid diesters deprive cultured primary astrocytes rapidly of glutathione. Neurochem Int 57:460–467

Ternette N, Yang M, Laroyia M, Kitagawa M, O'Flaherty L, Wolhulter K, Igarashi K, Saito K, Kato K, Fischer R et al (2013) Inhibition of mitochondrial aconitase by succination in fumarate hydratase deficiency. Cell Rep 3:689–700

Thiessen A, Schmidt MM, Dringen R (2010) Fumaric acid dialkyl esters deprive cultured rat oligodendroglial cells of glutathione and upregulate the expression of heme oxygenase 1. Neurosci Lett 475:56–60

Xie Z, Dai J, Dai L, Tan M, Cheng Z, Wu Y, Boeke JD, Zhao Y (2012) Lysine succinylation and lysine malonylation in histones. Mol Cell Proteomics 11:100–107

Zhang Z, Tan M, Xie Z, Dai L, Chen Y, Zhao Y (2011) Identification of lysine succinylation as a new post-translational modification. Nat Chem Biol 7:58–63

Zhou Y, Zhang H, He B, Du J, Lin H, Cerione RA, Hao Q (2012) The bicyclic intermediate structure provides insights into the desuccinylation mechanism of human sirtuin 5 (SIRT5). J Biol Chem 287:28307–28314