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Research Article

Potential mechanisms linking SIRT activity and hypoxic 2-hydroxyglutarate generation: no role for direct enzyme (de)acetylation

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2-Hydroxyglutarate (2-HG) is a hypoxic metabolite with potentially important epigenetic signaling roles. The mechanisms underlying 2-HG generation are poorly understood, but evidence suggests a potential regulatory role for the sirtuin family of lysine deacetylases. Thus, we hypothesized that the acetylation status of the major 2-HG-generating enzymes [lactate dehydrogenase (LDH), isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH)] may govern their 2-HG-generating activity. *In vitro* acetylation of these enzymes, with confirmation by western blotting, mass spectrometry, reversibility by recombinant sirtuins and an assay for global lysine occupancy, yielded no effect on 2-HG-generating activity. In addition, while elevated 2-HG in hypoxia is associated with the activation of lysine deacetylases, we found that mice lacking mitochondrial SIRT3 exhibited hyperacetylation and elevated 2-HG. These data suggest that there is no direct link between enzyme acetylation and 2-HG production. Furthermore, our observed effects of *in vitro* acetylation on the canonical activities of IDH, MDH and LDH appeared to contrast with previous findings wherein acetyl-mimetic lysine mutations resulted in the inhibition of these enzymes. Overall, these data suggest that a causal relationship should not be assumed between acetylation of metabolic enzymes and their activities, canonical or otherwise.

Introduction

2-Hydroxyglutarate (2-HG) is a non-canonical metabolite with potential signaling roles that is synthesized from the Krebs cycle metabolite α-ketoglutarate (α-KG). Among the enzymes thought to convert α-KG into 2-HG are cancer-linked forms of mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDH2) [1], as well as malate dehydrogenase type 2 (MDH2) [2] and lactate dehydrogenase (LDH) [3,4]. The mechanisms, which trigger a neomorphic 2-HG synthetic activity, are not fully elucidated.

It has been reported that a specific IDH2 mutation ($R_{172}K$) found in glioma cells facilitates 2-HG production [1], leading to the early characterization of 2-HG as an oncometabolite. In addition, hypoxia [3–5] and acidic pH [6–8] trigger 2-HG production by MDH2 and LDH. Acidic pH is thought to protonate α-KG, enhancing its access to the substrate-binding pocket of these enzymes [7]. However, it is unclear whether post-translational modification of these enzymes can also regulate 2-HG production.

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Lysine acetylation, regulated by the sirtuin family of NAD^+-dependent protein deacylases (SIRTs) [9], is emerging as an important regulatory mechanism for numerous metabolic processes [10–13]. We already demonstrated that an elevation in 2-HG levels observed in cardiac ischemic preconditioning (IPC) was associated with lysine deacetylation [14,15], and we and others showed that IPC-induced deacetylation occurs on LDH-A, IDH2 and MDH2 [15–17]. Intriguingly, both lysine deacetylation and the elevation in 2-HG seen in IPC were inhibited by the SIRT1 inhibitor splitomicin (Sp) [14,15]. As such, we hypothesized here that the 2-HG-generating capacity of these dehydrogenases may be regulated by lysine acetylation status.

Methods

Reagents

All chemicals and reagents were obtained from Sigma (St Louis, MO) unless otherwise stated. LDH was from bovine heart (L3916, Sigma), whereas MDH2 (M2634, Sigma) and $NADP^+$-dependent mitochondrial IDH2 (I2002, Sigma) were from porcine heart. HEK293 cells were from ATCC (Manassas, VA).

In vitro protein acetylation, quantitation and western blotting

Protein acetylation was performed similar to that performed in [18]. LDH, IDH2, or MDH2 or LDH (2 mg/ml) was incubated overnight at 37°C in 50 mM Tris–HCl (pH 8) plus 1.5 mM acetyl coenzyme A, followed by neutralization of pH prior to further analysis. For MDH2, acetylated protein was further subjected to deacetylation by incubation with recombinant human SIRT3 (ab125810, AbCam, Cambridge, MA).

To monitor protein acetylation, naïve and acetylated proteins were resolved on 12% SDS–PAGE gels, transferred to nitrocellulose and probed with pan anti-acetyl-lysine (K-Ac) or anti-SIRT3 antibodies (Cell Signaling, Danvers, MA) at 1:1000 dilution, followed by HRP-linked secondary antibody (GE Biosciences, Pittsburgh, PA) at 1:2500 and enhanced chemiluminescent detection (Pierce, Rockford, IL). In addition for MDH2, acetylation at the previously published K_{239} site [17,19] was determined by mass spectrometry in the University of Rochester Proteomics and Mass Spectrometry Core Facility. In brief, proteins were reduced and alkylated (DTT, IAA), trypsinized (Pierce) desalted (C18 Tips, Pierce), dried and suspended in 0.1% TFA. Peptides were separated by C18 HPLC (Easy nLC-1000, Thermo Fisher), with a custom column and an elution gradient of 0.1% formic acid in water → 0.1% formic acid in acetonitrile over 30 min. LC effluent was diverted to a Q Exactive Plus mass spectrometer (Thermo Fisher), operated in a data-dependent mode, with a full MS1 scan (400–1400 m/z, resolution 70 000 at m/z 200, AGC target of 10^6, maximum injection time 50 ms), followed by eight data-dependent MS2 scans (resolution 35 000, AGC target of 10^5, maximum injection time 120 ms). Isolation width was 1.5 m/z, offset 0.3 m/z and normalized collision energy 27. Raw data were searched using Mascot (Matrix Science) within the Proteome Discoverer software (Thermo Fisher), using the SwissProt *Sus scrofa* database with <2 missed cleavages, MS1 tolerance 10 ppm and MS2 tolerance 25 μm. Carbamidomethyl was set as a fixed modification, with methionine oxidation, lysine acetylation and N-term acetylation as variable modifications. Percolator was used as the FDR calculator, and filtering peptides were with q-value >0.01. Protein acetylation was also monitored using the trinitrobenzene sulfonic acid (TNBS) assay for free lysine ε-amino groups [20], following removal of excess acetyl-CoA from proteins by size-exclusion chromatography (Bio-Rad Micro Bio-Spin™ P-6).

Enzyme activities and LC–MS/MS metabolite assays

Activities of LDH, IDH2 or MDH2 were measured spectrophotometrically at 340 nm, representing either the consumption of NADH (for LDH and MDH) or the generation of NADPH (for IDH) [6]. In addition, aliquots from spectrophotometric cuvettes were withdrawn for the quantitation of selected reaction substrates or products by LC–MS/MS, as recently reported [6]. Note that the LC–MS/MS method employed was not capable of discerning L vs. D enantiomers of 2-HG. As such, we assume herein that 2-HG from LDH or MDH2 is L-2-HG, while that from IDH2 is D-2-HG [1,3,4].

Animals and primary cells

Male littermate wild-type (WT) and *Sirt3*$^{-/-}$ mice on a C57BL/6J background were used at 2 months of age. All mice were maintained in a pathogen-free vivarium with a 12 h light/dark cycle and food and water available *ad libitum*. All procedures conformed to the National Institutes of Health Guide for the Care and Use of

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Laboratory Animals and were approved by the AAALAC-accredited University of Rochester Committee on Animal Resources.

For cellular hypoxia experiments, HEK293 cells were placed in a hypoxic chamber (pO_2 <0.1%) for 20 h as previously described [6]. Primary adult mouse cardiomyocytes were isolated as previously described [21]. Cytosolic pH was measured by fluorescent microscopy using the pH-sensitive probe BCECF as previously described [6]. To model ischemia, cardiomyocytes were plated on laminin-coated 25 mm glass cover slips (25 000 cells per slide) in 35 mm dishes, followed by the placement of a second cover slip to exclude oxygen and limit extracellular volume. We previously reported on a similar method using cartridges within the Seahorse XF apparatus to achieve similar ends [21,22]. Cells in the center of the cover slip were imaged after 10 min.

Mitochondria were isolated from WT or *Sirt3*$^{-/-}$ hearts as previously described [23]. The activity of mitochondrial L-2-HG dehydrogenase was measured spectrophotometrically and by LC–MS/MS as previously described [6].

Statistics

Significance between groups was determined by ANOVA, assuming a normal distribution, followed by *post hoc* Student's *t*-test. Differences were considered statistically significant at $P < 0.05$, and graphs show means ± standard errors.

Data sharing

The full original data set for this work is available on the FigShare website (https://doi.org/10.6084/m9.figshare.5170531).

Results

We recently demonstrated a role for SIRT1 in the elevation of 2-HG observed in cardiac ischemic preconditioning (IPC) [14]. In addition, 2-HG is elevated in cells exposed to hypoxia [3,4]. However, since these are distinct experimental systems (intermittent whole organ ischemia vs. prolonged cellular hypoxia), it is not clear if SIRT1 is also required for the latter phenomenon. To investigate this, we measured 2-HG levels in HEK293 cells exposed to hypoxia with or without the SIRT1 inhibitor Sp. As expected, hypoxia markedly increased 2-HG levels (Figure 1A), and notably, this effect was blunted by Sp, suggesting a similar role for SIRT1 in both hypoxic situations.

LDH, IDH2 and MDH2 are enzymes capable of generating 2-HG [1,3,4,6,7] and are also thought to be targets for SIRT-mediated deacetylation [15–17,19,24,25]. Thus, we performed an *in vitro* analysis of the relationship between acetylation and 2-HG generation using purified enzymes (LDH, IDH2 and MDH2). Lysine 5 within LDH-A was identified as an acetylation site [26], with acetyl-mimetic mutation of this residue (K_5Q) resulting in enzyme inhibition. In contrast with these findings from mutational studies, when we acetylated purified LDH *in vitro* (Figure 1B), we observed no effect on its canonical activity (pyruvate + NADH → lactate + NAD^+) (Figure 1C). Furthermore, 2-HG generation from α-KG by LDH was not affected by acetylation (Figure 1D). Since Sp is thought to inhibit cytosolic SIRT1 [27–31], and LDH is cytosolic, we also tested whether Sp treatment of HEK293 cells had any effect on cytosolic LDH activity, but found no effect (control: 952 ± 23 vs. Sp: 987 ± 29 nmol/min/mg protein, means ± SEM, $N = 6$, $P = 0.36$). Taken together, these data suggest that acetylation does not affect the enzymatic properties of LDH, and that SIRT1 may regulate hypoxic cellular 2-HG via other mechanisms (see below).

Next, we examined the effects of acetylation on IDH2. Purified $NADP^+$-dependent mitochondrial IDH2 was acetylated *in vitro* (Figure 2A), and its canonical reaction (i.e. $NADP^+$ + isocitrate → α-KG + NADPH) was monitored both spectrophotometrically and by LC–MS/MS [6]. Acetylated IDH2 (Ac-IDH2) exhibited significantly higher activity than the naive enzyme, measured as either NADPH or α-KG generation (Figure 2B). This result contrasts with a previous study [32], wherein an acetyl-mimetic IDH2 mutation ($K_{413}Q$) demonstrated lower activity.

In terms of 2-HG generation, Figure 2C shows that both naive and Ac-IDH2 could use α-KG plus NADPH as substrates, but neither enzyme generated substantial corresponding amounts of 2-HG. Instead, IDH2 is known to operate in reverse, using α-KG in a reductive carboxylation reaction to produce isocitrate [6,33]. Taken together, these data suggest that acetylation boosted the canonical activity of IDH2 (i.e. isocitrate → α-KG), with no effect on its non-canonical activities (2-HG generation or reductive carboxylation). The fact

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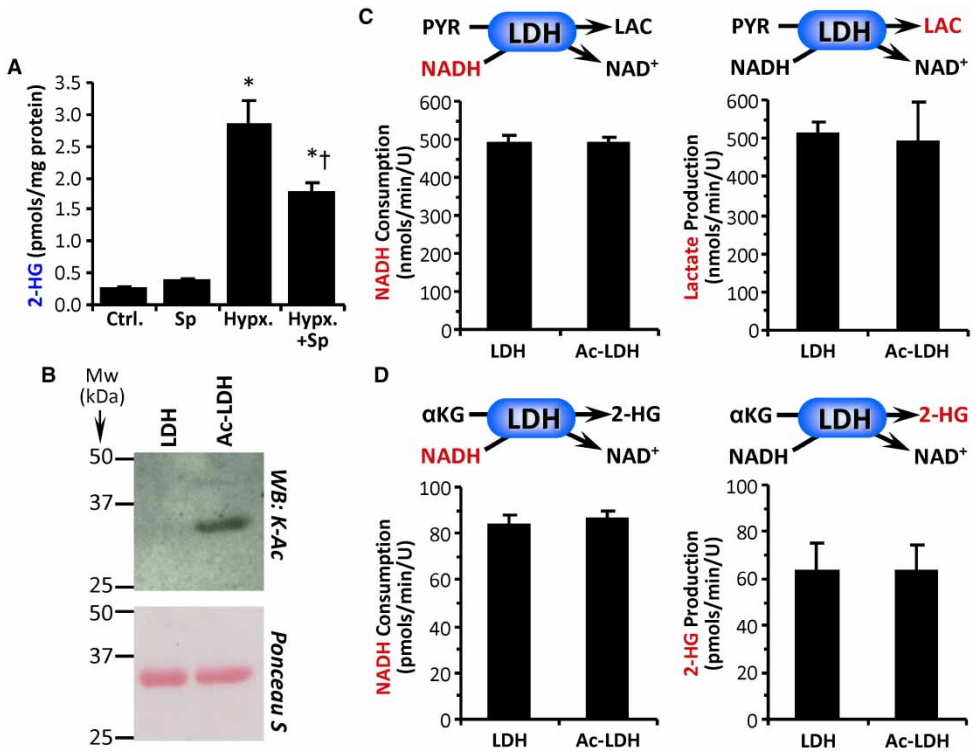

Figure 1. Cellular hypoxia, LDH acetylation and canonical vs. 2-HG-generating activity.

(**A**) HEK293 cells were exposed to hypoxia with or without the SIRT1 inhibitor Sp, followed by the measurement of 2-HG levels. Data are means ± SEM, $n = 5$. *$P < 0.05$ vs. control (Ctrl.). †$P < 0.05$ vs. hypoxia alone (Hypx.). (**B**) Purified LDH was acetylated *in vitro* with acetyl-CoA (see Methods), followed by western blot probe with K-Ac antibody. Ponceau S-stained membrane below indicates protein loading. Images representative of at least four independent experiments. (**C**) Canonical activity of naive and acetylated LDH (Ac-LDH) with pyruvate and NADH as substrates was measured spectrophotometrically as NADH consumption (left panel) or by LC–MS/MS as lactate production (right panel). (**D**) Non-canonical activity of LDH with α-KG and NADH as substrates was measured spectrophotometrically as NADH consumption (left panel) or by LC–MS/MS as 2-HG production (right panel). All enzyme rate data are means ± SEM, $n = 4$–7. Reactions monitored are shown above each graph, with the metabolite measured shown in red font.

that acetylation did affect IDH2 canonical activity suggests that the lack of an effect on non-canonical activities (negative result) was not simply due to confounding experimental issues, or an insufficient degree of acetylation (see below).

The mitochondrial enzyme MDH2 can also produce 2-HG [3,4,6,7], and notably lysine 239 of MDH2 is differentially acetylated in mice lacking the mitochondrial deacetylase SIRT3 [17]. Furthermore, an acetyl-mimetic $K_{239}Q$ mutant protein exhibits lower MDH2 activity [17], suggesting that acetylation at this site is inhibitory. We acetylated purified MDH2 *in vitro* [18] and verified this by western blot. As shown in Figure 2A (and in agreement with results from others [18]), this acetylation event was also reversed by incubation with human recombinant SIRT3 + NAD^+. In addition, mass spectrometric analysis of naïve vs. acetylated MDH2 (Ac-MDH2; Figure 3B–D) revealed a difference of 42 mass units in the y^{1+} ion for the peptide-containing K_{239} (IQEAGTEVVK underlined in Figure 2C), confirming acetylation at this site. Furthermore, a TNBS assay for free lysine ε-amino groups [20] revealed that the *in vitro* acetylation method resulted in occupancy of 74.4 ± 2.7% of lysines within MDH2.

Surprisingly, as shown in Figure 3E, the canonical MDH2 enzymatic reaction (i.e. OAA + NADH → malate + NAD^+) was no different between naïve vs. Ac-MDH2 (Note that the normal forward reaction, malate → OAA, is energetically unfavorable, so the enzyme is routinely assayed in the reverse direction.). Furthermore, with α-KG as a substrate, the enzymatic reaction to generate 2-HG was also unaffected by acetylation status

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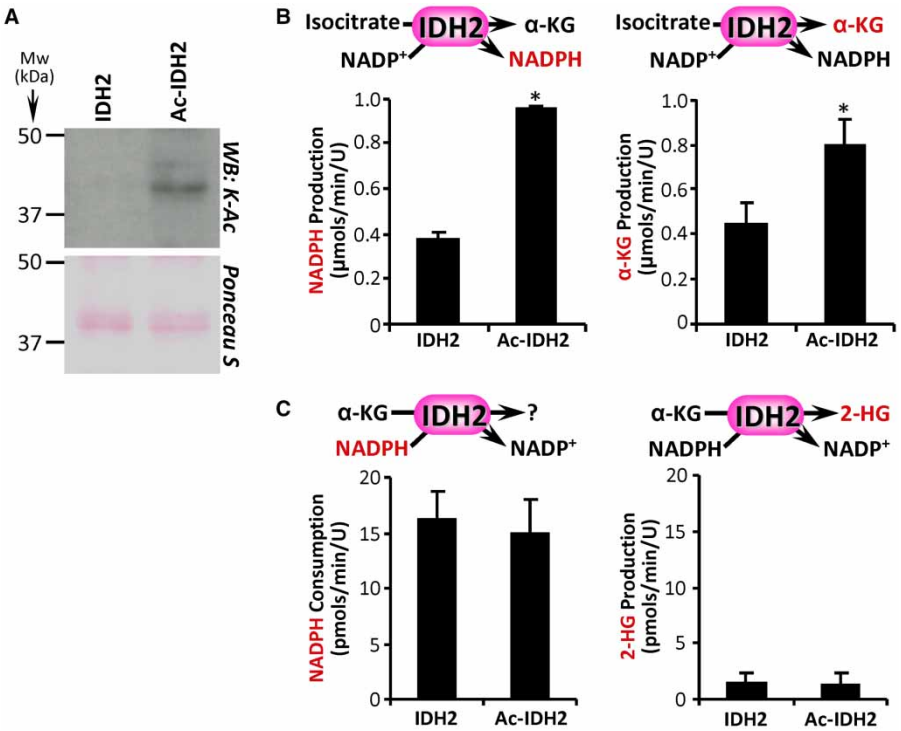

Figure 2. IDH2 acetylation and canonical vs. 2-HG-generating activity.

(**A**) Purified IDH2 was acetylated *in vitro* with acetyl-CoA (see Methods), followed by western blot probe with K-Ac antibody. Ponceau S-stained membrane below indicates protein loading. Images representative of at least four independent experiments. (**B**) Canonical activity of naive and Ac-IDH2 with isocitrate and $NADP^+$ as substrates was measured spectrophotometrically as NADPH production (left panel) or by LC–MS/MS as α-KG production (right panel). (**C**) Non-canonical reductive activity of IDH2 with α-KG and NADPH as substrates was measured spectrophotometrically as NADPH consumption (left panel) or by LC–MS/MS as 2-HG production (right panel). All enzyme rate data are means ± SEM, $n = 4$. $^*P < 0.05$ between naive and Ac-IDH2. Reactions monitored are shown above each graph, with the metabolite measured shown in red font.

(Figure 3F). Overall, these data suggest that neither canonical nor alternative activities of MDH2 were affected by acetylation.

To further investigate the possible role of lysine acetylation as a regulator of 2-HG generation, we examined mice with deletion of *Sirt3* (Figure 4A), a mitochondrial SIRT known to deacetylate LDH, IDH2 and MDH2 [15–17,19,24,25]. As shown in Figure 4B,C, $Sirt3^{-/-}$ hearts exhibited the expected hyperacetylation of cardiac mitochondrial proteins (vs. WT), but surprisingly this was accompanied by a 3.5-fold elevation in 2-HG. Such a result contrasts with the situation in both IPC [14] and cellular anoxia (Figure 1A), wherein deacetylation is associated with elevated 2-HG. Taken together, these data support a disconnect between acetylation and 2-HG generation.

Nevertheless, these data raise an intriguing question as to the mechanism of 2-HG elevation in $Sirt3^{-/-}$ mice. One possibility may be inhibition of the mitochondrial 2-HG catabolic enzyme L-2-HG dehydrogenase (L-2-HGDH) [34]. However, as shown in Figure 4D, we observed no difference in L-2-HGDH activity between $Sirt3^{-/-}$ and WT cardiac mitochondria.

Another potentially important regulator of 2-HG generation is acidic pH [6,7]. However, we did not observe any significant difference in pH between cardiomyocytes isolated from WT or $Sirt3^{-/-}$ hearts (Figure 4E,F). Notably (and in agreement with previous studies), $Sirt3^{-/-}$ hearts exhibited elevated NADH levels (Figure 4C), a phenomenon that has been attributed to the inhibition of mitochondrial respiratory complex I (NADH ubiquinone oxidoreductase) [23,35]. It is thought that excessive NADH accumulation (also known as *reductive stress*) is a key driver of the α-KG → 2-HG reaction [36], and together, these findings suggest that a probable cause of elevated 2-HG in $Sirt3^{-/-}$ hearts is an increased drive from NADH.

Biochemical Journal (2017) **474** 2829–2839
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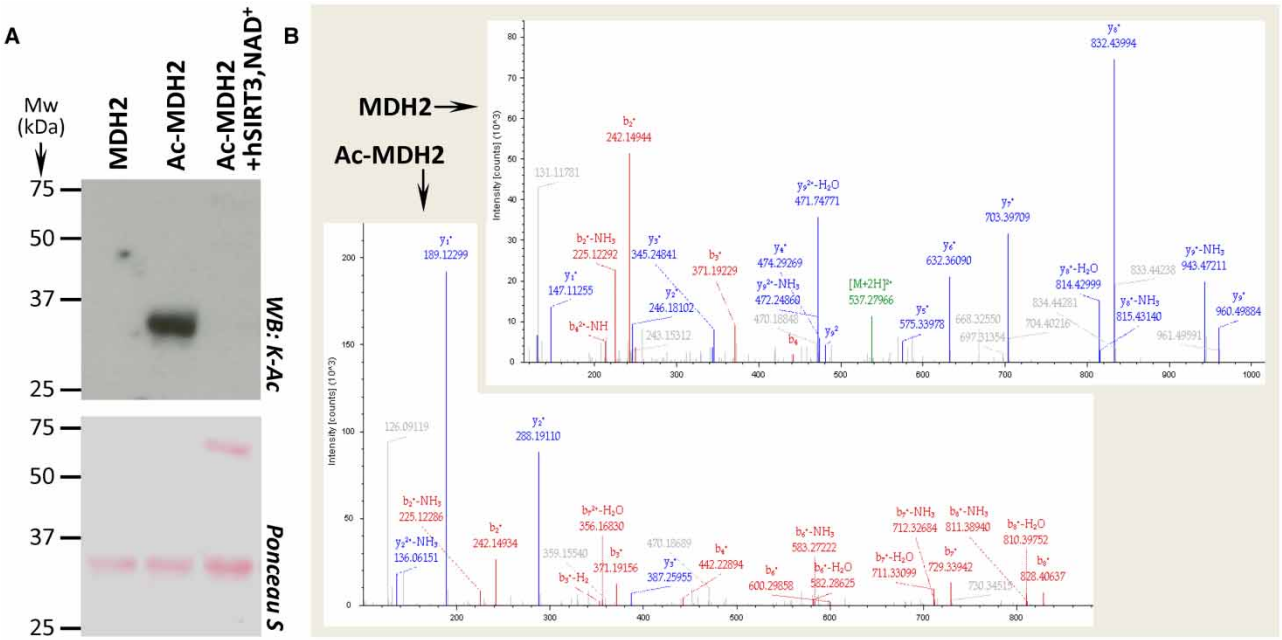

C

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1   MLSALARPAG AALRRSFSTS AQNNAKVAVL GASGGIGQPL
41  SLLLKNSPLV SRLTLYDIAH TPGVAADLSH IETRATVKGY
81  LGPEQLPDCL KGCDVVVIPA GVPRKPGMTR DDLFNTNATI
121 VATLTAACAQ HCPDAMICII SNPVNSTIPI TAEVFKKHGV
161 YNPNKIFGVT TLDIVRANAF VAELKGLDPA RVSVPVIGGH
200 AGKTIIPLIS QCTPKVDFPQ DQLSTHTGRI QEAGTEVVKA
241 KAGAGSATLS MAYAGARFVF SLVDAMNGKE GVVECSFVKS
281 QETDCPYFST PLLLGKKGIE KNLGIGKISP FEEKMIAEAI
321 PELKASIKKG EEFVKNMK
```

D

MDH2	Ac-MDH2	Seq'		MDH2	Ac-MDH2
114.091	114.091	b^{1+}	I		
242.150	242.150	b^{2+}	Q y^{9+}	960.499	1002.51
371.193	371.193	b^{3+}	E y^{8+}	832.441	874.452
442.230	442.230	b^{4+}	A y^{7+}	703.398	745.410
499.251	499.251	b^{5+}	G y^{6+}	632.361	674.372
600.399	600.299	b^{6+}	T y^{5+}	575.340	617.351
729.341	729.341	b^{7+}	E y^{4+}	474.292	516.303
828.410	828.410	b^{8+}	V y^{3+}	345.250	387.260
927.478	927.478	b^{9+}	V y^{2+}	246.181	288.192
			K y^{1+}	147.113	189.123

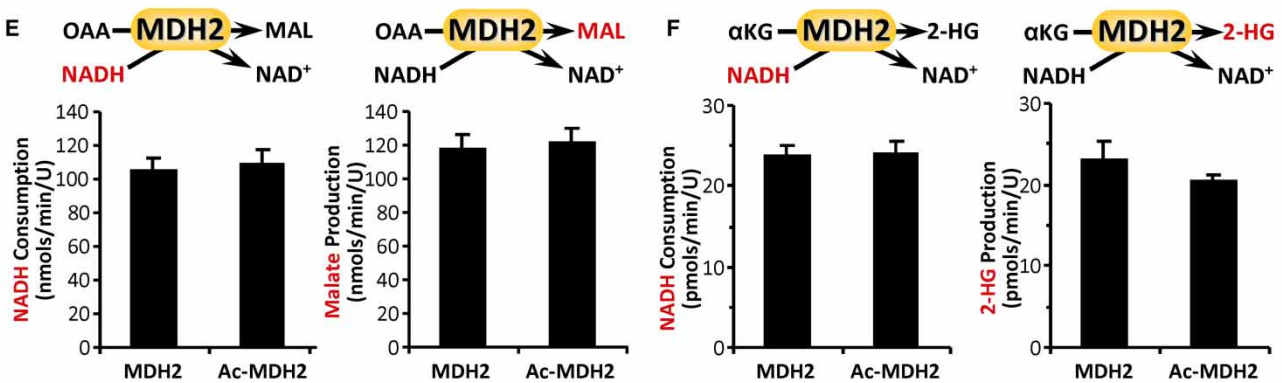

Figure 3. MDH2 acetylation and canonical vs. 2-HG-generating activity.

(**A**) Purified MDH2 was acetylated *in vitro* with acetyl-CoA (see Methods), followed by western blot probe with K-Ac antibody. Ponceau S-stained membrane below indicates protein loading. Images representative of at least four independent experiments. (**B**–**D**) Naïve and Ac-MDH2 were digested with trypsin and resultant peptides analyzed by mass spectrometry to identify acetylation sites. Seventy-six percent sequence coverage was obtained (**C**, red font) including the peptide containing the proposed K_{239} acetylation site (underlined). Sample spectra are shown in **B**, with panel **D** listing identified peptides corresponding to b^{n+} ions (red) and y^{n+} ions (blue). A difference of 42 mass units between y^{+1} ion in MDH2 vs. Ac-MDH2 (189.123–147.113) indicates acetylation. (**E**) Canonical activity of naive and acetylated MDH2 with OAA and NADH as substrates was measured spectrophotometrically as NADH consumption (left panel) or by LC–MS/MS as malate production (right panel). (**F**) Non-canonical activity of MDH2 with α-KG and NADH as substrates was measured spectrophotometrically as NADH consumption (left panel) or by LC–MS/MS as 2-HG production (right panel). All enzyme rate data are means ± SEM, $n = 4$–6. Reactions monitored are shown above each graph, with the metabolite measured shown in red font.

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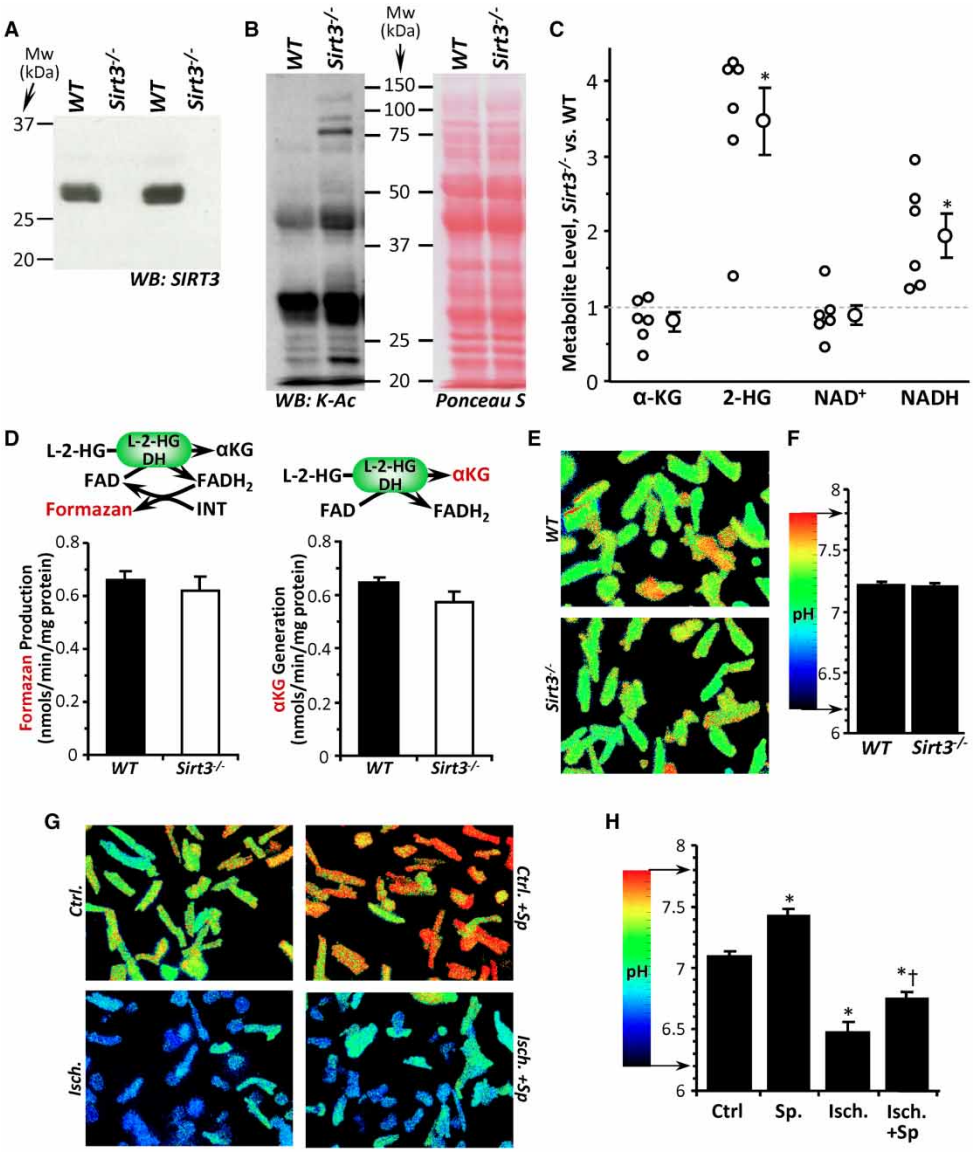

Figure 4. Acetylation, 2-HG generation and pH in *Sirt3*$^{-/-}$ mice, and the effects of SIRT inhibition on cytosolic pH.
(**A**) Western blot shows the absence of SIRT3 protein in cardiac tissue from *Sirt3*$^{-/-}$ mice (vs. WT). (**B**) Western blot shows protein acetylation (pan K-Ac antibody) in cardiac mitochondria from WT and *Sirt3*$^{-/-}$ mice. Ponceau S-stained membrane indicates protein loading. Blots are representative of at least four independent experiments. (**C**) LC–MS/MS-based metabolite profiling of WT vs. *Sirt3*$^{-/-}$ cardiac tissue. Data are expressed as the metabolite level in *Sirt3*$^{-/-}$ relative to paired WT samples, with individual data points ($n = 6$ pairs) alongside means ± SEM. *$P < 0.05$ between WT and *Sirt3*$^{-/-}$. (**D**) L-2-HG dehydrogenase (L-2-HGDH) activity in cardiac mitochondria from WT and *Sirt3*$^{-/-}$ mice was measured spectrophotometrically via formazan-linked assay (left panel) or by LC–MS/MS as α-KG formation (right panel). Enzyme rate data are means ± SEM, $n = 5$. Reactions monitored are shown above each graph, with the metabolite measured shown in red font. (**E** and **F**) Cytosolic pH measurements in primary cultured adult cardiomyocytes from WT and *Sirt3*$^{-/-}$ mice. Alongside representative images of cellular pH (**E**), graph shows quantitation of pH from four independent cell preparations (**F**). Data are means ± SEM. (**G** and **H**) Cytosolic pH measurements in primary cultured adult cardiomyocytes from WT mice subjected to simulated ischemia (see Methods), with or without the SIRT1 inhibitor Sp. Alongside representative images of cellular pH (**G**), graph shows quantitation of pH from four to six independent cell preparations (**H**). Data are means ± SEM. *$P < 0.05$ vs. control (Ctrl.). †$P < 0.05$ vs. ischemia alone (Isch.).

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Biochemical Journal (2017) 474 2829–2839
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Finally, to uncover the basis for Sp sensitivity in the hypoxic increase in 2-HG ([14] and Figure 1A), we measured the effects of Sp on ischemia-induced acidification of primary mouse cardiomyocytes. As shown in Figure 4G,H, Sp elicited a slight alkalinization alone and blunted the acidification caused by ischemia. These data suggest that the role of SIRT1 in the elevation of 2-HG in hypoxia/ischemia is likely at a position upstream of acidification.

Discussion and conclusions

The conclusions of the present study are as follows: (i) both canonical enzyme activities and 2-HG generation by Ac-LDH and Ac-MDH2 were similar to their corresponding naive enzymes; (ii) IDH2 (either naïve or acetylated) did not produce 2-HG, although Ac-IDH2 showed higher canonical activity (isocitrate → α-KG); (iii) 2-HG was elevated in *Sirt3*$^{-/-}$ hearts, concurrent with higher NADH levels; and (iv) the ability of the SIRT1 inhibitor Sp to blunt 2-HG elevation in hypoxia/ischemia may be due to an upstream role for SIRT1 in regulating acidosis. Overall, these data suggest that direct acetylation of the major identified 2-HG-generating enzymes is not a mechanism that regulates 2-HG. Although our utilization of a range of experimental systems (HEK293 cells, purified enzymes, isolated mitochondria, pharmacologic inhibitors, *Sirt3*$^{-/-}$ mice and isolated cardiomyocytes) would normally be considered a caveat, the broad agreement of results obtained across these systems suggests conserved mechanisms.

Our observation that IDH2 acetylation by a physiologically relevant mechanism (acetyl-CoA) increases its canonical activity (Figure 2B) is an interesting result. Since acetyl-CoA is the upstream substrate for the Krebs cycle, it is possible that acetyl-CoA-mediated IDH2 acetylation represents a novel feed-forward activation mechanism for the cycle. The importance of this relative to the already well-characterized regulation of this cycle by numerous allosteric mechanisms (e.g. Ca^{2+}, NADH and phosphorylation) [37–39] remains to be seen. Furthermore, although we show here that IDH2 can be acetylated non-enzymatically, it has recently been noted that considerable variance can exist between the reactivity of specific lysines for *in vitro* acetylation vs. their *in vivo* enzymatic modification [40]. In this regard, it is not known whether the combination of acetylation events that occur under *in vitro* conditions may adequately model the *in vivo* situation, where mitochondrial protein acetylation is regulated not only by deacetylases such as SIRT3, but also by acetyl-transferases such as GCN5L1 [41].

While the application of quantitative proteomic labeling techniques to determine site-specific stoichiometry of lysine acetylation [42] may be informative, nevertheless our *in vitro* acetylation method yielded ∼75% global lysine site occupancy (for MDH2), suggesting that the lack of an effect of acetylation observed herein was not simply due to low site occupancy of lysines.

A consistent finding in the current study was contrast with previous observations using acetyl-mimetic mutations in dehydrogenase enzymes [17,26,32]. Specifically, acetyl-mimetic K_5Q of LDH-A, $K_{413}Q$ of IDH2 and $K_{239}Q$ of MDH2 were all shown to result in lower activities for these enzymes. In contrast, we found that acetylation stimulated IDH2 activity, while having no effect on LDH or MDH2. The acetylation methods used herein were physiologically relevant [18], and acetylation was verified by western blot, mass spectrometry, reversibility with a recombinant SIRT and assessment of lysine occupancy by the TNBS assay. Interpreting mutational studies can be problematic when mutations result in loss of function, and in the lysine acetylation field, there are vanishingly few examples of acetyl-mimetic mutation resulting in the stimulation of activity. As such, we speculate that the results of some mutational studies reporting enzyme inhibition may be artifactual, due to unexpected effects of mutations on protein folding or other enzyme properties. Indeed, it was shown that the acetyl-mimetic mutants K_3Q and $K_{118}Q$ of MDH isoform 1 had no effect on activity, while the corresponding deacetylation mimetics K_3R and $K_{118}R$ were inhibitory [43]. Thus, in particular for MDH, the relationship between acetylation and enzyme activity is not clear.

An important caveat in interpreting our findings is subcellular compartmentation. In cardiomyocytes, SIRT1 is cytosolic [15,44], and as shown in Figure 4H, the SIRT1 inhibitor Sp results in alkalinization of the cardiomyocyte cytosol. Sp also blunts the hypoxic increase in 2-HG (Figure 1A). However, acetylation of cytosolic LDH does not affect its 2-HG generation. Thus, we conclude that SIRT1 may play a role in regulating hypoxic cellular 2-HG at a point upstream of acidification. In contrast, SIRT3 is mitochondrial and its genetic ablation had no effect on cytosolic pH and no effect on L-2-HGDH activity. Furthermore, no effect of acetylation on 2-HG generation was seen for the mitochondrial enzymes IDH2 and MDH2. Thus, in the case of *Sirt3*$^{-/-}$, we speculate that elevated 2-HG originates at the level of reductive stress (excess NADH), possibly due to the inhibition of mitochondrial respiratory complex I [23,35]. Although it was recently reported that mitochondrial

complex II (succinate ubiquinone oxidoreductase) is down-regulated in *Sirt3*$^{-/-}$ mice [45], it is unclear how such a phenomenon might be mechanistically linked to reductive stress or 2-HG generation.

Interestingly, the *Sirt3*$^{-/-}$ mouse exhibits acetylation of cytosolic LDH [17], and we showed that SIRT1 inhibition resulted in acetylation of numerous mitochondrial enzymes [15]. Thus, there appear to be signaling mechanisms by which inactivation (genetic or pharmacologic) of an SIRT in one cell compartment can affect acetylation in another compartment. Indeed, it was recently shown that SIRT1 can translocate from cytosol to mitochondria [46]. Furthermore, in the context of compartmentation, it has also been reported that 2-HG can be transported into cardiomyocytes [47]. As such, it is intriguing that we found a small but non-significant increase in 2-HG levels in the serum of *Sirt3*$^{-/-}$ mice (1.68 ± 0.29 fold vs. control, mean ± SEM, $n = 6$, $P = 0.078$, paired *t*-test). This finding suggests an alternative hypothesis: the elevated 2-HG level observed in *Sirt3*$^{-/-}$ hearts may originate elsewhere in the body. However, regardless of the compartment, it is clear from our results that direct regulation of 2-HG-generating enzymes by SIRT1- or SIRT3-mediated deacetylation is unlikely to be a mechanism regulating 2-HG levels.

It was recently proposed that the conversion of α-KG into 2-HG is a quantitatively significant mechanism by which 'cells handle a mounting pool of reducing equivalents' such as excess NADH [36]. 2-HG was also proposed to represent a 'redox reservoir' to enable cells to handle reductive stress [36]. However, the canonical activities of LDH and MDH2 are at least three orders of magnitude greater than the rates at which these enzymes reduce α-KG to 2-HG [2,6]. In hypoxia and reductive stress, the 2-HG reaction is quantitatively insignificant as a consumer of NADH, relative to the role of LDH ($\text{pyruvate} + \text{NADH} \rightarrow \text{lactate} + \text{NAD}^+$). In hypoxia, 2-HG generation is also several orders of magnitude smaller than another important redox sink, namely the generation of succinate from fumarate by reversal of respiratory complex II [48–50]. Furthermore, 2-HG is not a true redox reservoir, because the reverse reaction to regenerate α-KG from 2-HG (i.e. the reaction catalyzed by L-2-HGDH) uses FAD as an electron acceptor and is not known to regenerate NADH. These quantitative limitations on the role of 2-HG as a redox sink do not diminish the potential importance of 2-HG as a hypoxic-signaling molecule [6,51,52].

Finally, the recent finding that 2-HG can inhibit α-ketoglutarate dehydrogenase (α-KGDH) activity [47] suggests that 2-HG and the TCA cycle may exhibit a complex cross-talk, wherein inhibition of α-KGDH would elevate α-KG levels, providing more substrate for 2-HG generation, resulting in a feed-forward activation for 2-HG generation. In conclusion, while direct enzyme acetylation does not appear to be an important 2-HG regulatory mechanism, the role of other protein post-translational modifications and signaling pathways that regulate this metabolite may be worthy of further investigation.

Abbreviations

Ac-IDH2, acetylated IDH2; Ac-MDH2, acetylated MDH2; 2-HG, 2-hydroxyglutarate; AGC, automatic gain control; FDR, false discovery rate; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; DTT, Dithiothreitol; HRP, horseradish peroxidase; IAA, indole-3-acetic acid; L-2-HGDH, L-2-HG dehydrogenase; IDH, isocitrate dehydrogenase; IPC, ischemic preconditioning; K-Ac, anti-acetyl-lysine; α-KG, α-ketoglutarate; α-KGDH, α-ketoglutarate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; OAA, oxaloacetic acid; Sp, splitomicin; TFA, trifluoroacetic acid; TNBS, trinitrobenzene sulfonic acid; WT, wild type.

Author Contribution

S.M.N. performed experiments and drafted the manuscript. Y.T.W., J.Z., K.N., X.S., K.W., S.G., and J.M. performed experiments. P.S.B. experimental design and wrote the manuscript.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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References

1 Gross, S., Cairns, R.A., Minden, M.D., Driggers, E.M., Bittinger, M.A., Jang, H.G. et al. (2010) Cancer-associated metabolite 2-hydroxyglutarate accumulates in acute myelogenous leukemia with isocitrate dehydrogenase 1 and 2 mutations. *J. Exp. Med.* **207**, 339–344 doi:10.1084/jem.20092506

2 Rzem, R., Vincent, M.-F., Van Schaftingen, E. and Veiga-da-Cunha, M. (2007) L-2-Hydroxyglutaric aciduria, a defect of metabolite repair. *J. Inherit. Metab. Dis.* **30**, 681–689 doi:10.1007/s10545-007-0487-0

3 Intlekofer, A.M., Dematteo, R.G., Venneti, S., Finley, L.W.S., Lu, C., Judkins, A.R. et al. (2015) Hypoxia induces production of L-2-hydroxyglutarate. *Cell Metab.* **22**, 304–311 doi:10.1016/j.cmet.2015.06.023

4 Oldham, W.M., Clish, C.B., Yang, Y. and Loscalzo, J. (2015) Hypoxia-mediated increases in L-2-hydroxyglutarate coordinate the metabolic response to reductive stress. *Cell Metab.* **22**, 291–303 doi:10.1016/j.cmet.2015.06.021

5 Armitage, E.G., Kotze, H.L., Allwood, J.W., Dunn, W.B., Goodacre, R. and Williams, K.J. (2015) Metabolic profiling reveals potential metabolic markers associated with hypoxia inducible factor-mediated signalling in hypoxic cancer cells. *Sci. Rep.* **5**, 15649 doi:10.1038/srep15649

6 Nadtochiy, S.M., Schafer, X., Fu, D., Nehrke, K., Munger, J. and Brookes, P.S. (2016) Acidic pH is a metabolic switch for 2-hydroxyglutarate generation and signaling. *J. Biol. Chem.* **291**, 20188–20197 doi:10.1074/jbc.M116.738799

7 Intlekofer, A.M., Wang, B., Liu, H., Shah, H., Carmona-Fontaine, C., Rustenburg, A.S. et al. (2017) L-2-Hydroxyglutarate production arises from noncanonical enzyme function at acidic pH. *Nat. Chem. Biol.* **13**, 494–500 doi:10.1038/nchembio.2307

8 Teng, X., Emmett, M.J., Lazar, M.A., Goldberg, E. and Rabinowitz, J.D. (2016) Lactate dehydrogenase C produces *S*-2-hydroxyglutarate in mouse testis. *ACS Chem. Biol.* **11**, 2420–2427 doi:10.1021/acschembio.6b00290

9 Anderson, K.A., Green, M.F., Huynh, F.K., Wagner, G.R. and Hirschey, M.D. (2014) Snapshot: mammalian sirtuins. *Cell* **159**, 956–956.e1 doi:10.1016/j.cell.2014.10.045

10 Huang, J.-Y., Hirschey, M.D., Shimazu, T., Ho, L. and Verdin, E. (2010) Mitochondrial sirtuins. *Biochim. Biophys. Acta* **1804**, 1645–1651 doi:10.1016/j.bbapap.2009.12.021

11 Correia, M., Perestrelo, T., Rodrigues, A.S., Ribeiro, M.F., Pereira, S.L., Sousa, M.I. et al. (2017) Sirtuins in metabolism, stemness and differentiation. *Biochim. Biophys. Acta* **1861**, 3444–3455 doi:10.1016/j.bbagen.2016.09.008

12 van de Ven, R.A.H., Santos, D. and Haigis, M.C. (2017) Mitochondrial sirtuins and molecular mechanisms of aging. *Trends Mol. Med.* **23**, 320–331 doi:10.1016/j.molmed.2017.02.005

13 Ye, X., Li, M., Hou, T., Gao, T., Zhu, W.-g. and Yang, Y. (2017) Sirtuins in glucose and lipid metabolism. *Oncotarget* **8**, 1845–1859 doi:10.18632/oncotarget.12157

14 Nadtochiy, S.M., Urciuoli, W., Zhang, J., Schafer, X., Munger, J. and Brookes, P.S. (2015) Metabolomic profiling of the heart during acute ischemic preconditioning reveals a role for SIRT1 in rapid cardioprotective metabolic adaptation. *J. Mol. Cell. Cardiol.* **88**, 64–72 doi:10.1016/j.yjmcc.2015.09.008

15 Nadtochiy, S.M., Redman, E., Rahman, I. and Brookes, P.S. (2011) Lysine deacetylation in ischaemic preconditioning: the role of SIRT1. *Cardiovasc. Res.* **89**, 643–649 doi:10.1093/cvr/cvq287

16 Parker, B.L., Shepherd, N.E., Trefely, S., Hoffman, N.J., White, M.Y., Engholm-Keller, K. et al. (2014) Structural basis for phosphorylation and lysine acetylation cross-talk in a kinase motif associated with myocardial ischemia and cardioprotection. *J. Biol. Chem.* **289**, 25890–25906 doi:10.1074/jbc.M114.556035

17 Hebert, A.S., Dittenhafer-Reed, K.E., Yu, W., Bailey, D.J., Selen, E.S., Boersma, M.D. et al. (2013) Calorie restriction and SIRT3 trigger global reprogramming of the mitochondrial protein acetylome. *Mol. Cell* **49**, 186–199 doi:10.1016/j.molcel.2012.10.024

18 Wagner, G.R. and Payne, R.M. (2013) Widespread and enzyme-independent N^{ε}-acetylation and N^{ε}-succinylation of proteins in the chemical conditions of the mitochondrial matrix. *J. Biol. Chem.* **288**, 29036–29045 doi:10.1074/jbc.M113.486753

19 Still, A.J., Floyd, B.J., Hebert, A.S., Bingman, C.A., Carson, J.J., Gunderson, D.R. et al. (2013) Quantification of mitochondrial acetylation dynamics highlights prominent sites of metabolic regulation. *J. Biol. Chem.* **288**, 26209–26219 doi:10.1074/jbc.M113.483396

20 Cayot, P. and Tainturier, G. (1997) The quantification of protein amino groups by the trinitrobenzenesulfonic acid method: a reexamination. *Anal. Biochem.* **249**, 184–200 doi:10.1006/abio.1997.2161

21 Nadtochiy, S.M., Madukwe, J., Hagen, F. and Brookes, P.S. (2014) Mitochondrially targeted nitro-linoleate: a new tool for the study of cardioprotection. *Br. J. Pharmacol.* **171**, 2091–2098 doi:10.1111/bph.12405

22 Guo, S., Olm-Shipman, A., Walters, A., Urciuoli, W.R., Devito, S., Nadtochiy, S.M. et al. (2012) A cell-based phenotypic assay to identify cardioprotective agents. *Circ. Res.* **110**, 948–957 doi:10.1161/CIRCRESAHA.111.263715

23 Porter, G.A., Urciuoli, W.R., Brookes, P.S. and Nadtochiy, S.M. (2014) SIRT3 deficiency exacerbates ischemia-reperfusion injury: implication for aged hearts. *Am. J. Physiol. Heart Circ. Physiol.* **306**, H1602–H1609 doi:10.1152/ajpheart.00027.2014

24 Sol, E.M., Wagner, S.A., Weinert, B.T., Kumar, A., Kim, H.-S., Deng, C.-X. et al. (2012) Proteomic investigations of lysine acetylation identify diverse substrates of mitochondrial deacetylase sirt3. *PLoS ONE* **7**, e50545 doi:10.1371/journal.pone.0050545

25 Rardin, M.J., Newman, J.C., Held, J.M., Cusack, M.P., Sorensen, D.J., Li, B. et al. (2013) Label-free quantitative proteomics of the lysine acetylome in mitochondria identifies substrates of SIRT3 in metabolic pathways. *Proc. Natl Acad. Sci. U.S.A.* **110**, 6601–6606 doi:10.1073/pnas.1302961110

26 Zhao, D., Zou, S.-W., Liu, Y., Zhou, X., Mo, Y., Wang, P. et al. (2013) Lysine-5 acetylation negatively regulates lactate dehydrogenase A and is decreased in pancreatic cancer. *Cancer Cell* **23**, 464–476 doi:10.1016/j.ccr.2013.02.005

27 Breitenstein, A., Stein, S., Holy, E.W., Camici, G.G., Lohmann, C., Akhmedov, A. et al. (2011) Sirt1 inhibition promotes in vivo arterial thrombosis and tissue factor expression in stimulated cells. *Cardiovasc. Res.* **89**, 464–472 doi:10.1093/cvr/cvq339

28 Chapalamadugu, K.C., Panguluri, S.K., Bennett, E.S., Kolliputi, N. and Tipparaju, S.M. (2015) High level of oxygen treatment causes cardiotoxicity with arrhythmias and redox modulation. *Toxicol. Appl. Pharmacol.* **282**, 100–107 doi:10.1016/j.taap.2014.10.019

29 Stavrou, E.X., Fang, C., Merkulova, A., Alhalabi, O., Grobe, N., Antoniak, S. et al. (2015) Reduced thrombosis in Klkb1$^{-/-}$ mice is mediated by increased Mas receptor, prostacyclin, Sirt1, and KLF4 and decreased tissue factor. *Blood* **125**, 710–719 doi:10.1182/blood-2014-01-550285

30 Stein, S., Lohmann, C., Schäfer, N., Hofmann, J., Rohrer, L., Besler, C. et al. (2010) SIRT1 decreases Lox-1-mediated foam cell formation in atherogenesis. *Eur. Heart J.* **31**, 2301–2309 doi:10.1093/eurheartj/ehq107

31 Stein, S., Schäfer, N., Breitenstein, A., Besler, C., Winnik, S., Lohmann, C. et al. (2010) SIRT1 reduces endothelial activation without affecting vascular function in ApoE$^{-/-}$ mice. *Aging* **2**, 353–360 doi:10.18632/aging.100162
32 Yu, W., Dittenhafer-Reed, K.E. and Denu, J.M. (2012) SIRT3 protein deacetylates isocitrate dehydrogenase 2 (IDH2) and regulates mitochondrial redox status. *J. Biol. Chem.* **287**, 14078–14086 doi:10.1074/jbc.M112.355206
33 Wise, D.R., Ward, P.S., Shay, J.E.S., Cross, J.R., Gruber, J.J., Sachdeva, U.M. et al. (2011) Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of α-ketoglutarate to citrate to support cell growth and viability. *Proc. Natl Acad. Sci. U.S.A.* **108**, 19611–6 doi:10.1073/pnas.1117773108
34 Rzem, R., Achouri, Y., Marbaix, E., Schakman, O., Wiame, E., Marie, S. et al. (2015) A mouse model of L-2-hydroxyglutaric aciduria, a disorder of metabolite repair. *PLoS ONE* **10**, e0119540 doi:10.1371/journal.pone.0119540
35 Ahn, B.-H., Kim, H.-S., Song, S., Lee, I.H., Liu, J., Vassilopoulos, A. et al. (2008) A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. *Proc. Natl Acad. Sci. U.S.A.* **105**, 14447–14452 doi:10.1073/pnas.0803790105
36 Loscalzo, J. (2016) Adaptions to hypoxia and redox stress: essential concepts confounded by misleading terminology. *Circ. Res.* **119**, 511–513 doi:10.1161/CIRCRESAHA.116.309394
37 Hofmeyr, J.H.S., Rohwer, J.M. and Snoep, J.L. (2006) Conditions for effective allosteric feedforward and feedback in metabolic pathways. *IEE Proc. Syst. Biol.* **153**, 327–331 doi:10.1049/ip-syb:20060019
38 Bouskila, M., Hunter, R.W., Ibrahim, A.F.M., Delattre, L., Peggie, M., van Diepen, J.A. et al. (2010) Allosteric regulation of glycogen synthase controls glycogen synthesis in muscle. *Cell Metab.* **12**, 456–466 doi:10.1016/j.cmet.2010.10.006
39 Tanaka, T., Sue, F. and Morimura, H. (1967) Feed-forward activation and feed-back inhibition of pyruvate kinase type L of rat liver. *Biochem. Biophys. Res. Commun.* **29**, 444–449 doi:10.1016/0006-291X(67)90477-9
40 Baeza, J., Smallegan, M.J. and Denu, J.M. (2015) Site-specific reactivity of nonenzymatic lysine acetylation. *ACS Chem. Biol.* **10**, 122–128 doi:10.1021/cb500848p
41 Scott, I., Webster, B.R., Li, J.H. and Sack, M.N. (2012) Identification of a molecular component of the mitochondrial acetyltransferase programme: a novel role for GCN5L1. *Biochem. J.* **443**, 655–661 doi:10.1042/BJ20120118
42 Baeza, J., Dowell, J.A., Smallegan, M.J., Fan, J., Amador-Noguez, D., Khan, Z. et al. (2014) Stoichiometry of site-specific lysine acetylation in an entire proteome. *J. Biol. Chem.* **289**, 21326–21338 doi:10.1074/jbc.M114.581843
43 Kim, E.Y., Kim, W.K., Kang, H.J., Kim, J.-H., Chung, S.J., Seo, Y.S. et al. (2012) Acetylation of malate dehydrogenase 1 promotes adipogenic differentiation via activating its enzymatic activity. *J. Lipid Res.* **53**, 1864–1876 doi:10.1194/jlr.M026567
44 Tanno, M., Sakamoto, J., Miura, T., Shimamoto, K. and Horio, Y. (2007) Nucleocytoplasmic shuttling of the NAD$^+$-dependent histone deacetylase SIRT1. *J. Biol. Chem.* **282**, 6823–6832 doi:10.1074/jbc.M609554200
45 Parodi-Rullán, R.M., Chapa-Dubocq, X., Rullán, P.J., Jang, S. and Javadov, S. (2017) High sensitivity of SIRT3 deficient hearts to ischemia-reperfusion is associated with mitochondrial abnormalities. *Front. Pharmacol.* **8**, 275 doi:10.3389/fphar.2017.00275
46 Thompson, J.W., Dave, K.R., Saul, I., Narayanan, S.V. and Perez-Pinzon, M.A. (2013) Epsilon PKC increases brain mitochondrial SIRT1 protein levels via heat shock protein 90 following ischemic preconditioning in rats. *PLoS ONE* **8**, e75753 doi:10.1371/journal.pone.0075753
47 Karlstaedt, A., Zhang, X., Vitrac, H., Harmancey, R., Vasquez, H., Wang, J.H. et al. (2016) Oncometabolite D-2-hydroxyglutarate impairs α-ketoglutarate dehydrogenase and contractile function in rodent heart. *Proc. Natl Acad. Sci. U.S.A.* **113**, 10436–10441 doi:10.1073/pnas.1601650113
48 Chouchani, E.T., Pell, V.R., Gaude, E., Aksentijević, D., Sundier, S.Y., Robb, E.L. et al. (2014) Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature* **515**, 431–435 doi:10.1038/nature13909
49 Sanadi, D.R. and Fluharty, A.L. (1963) On the mechanism of oxidative phosphorylation. VII. The energy-requiring reduction of pyridine nucleotide by succinate and the energy-yielding oxidation of reduced pyridine nucleotide by fumarate. *Biochemistry* **2**, 523–528 doi:10.1021/bi00903a023
50 Hochachka, P.W., Owen, T.G., Allen, J.F. and Whittow, G.C. (1975) Multiple end products of anaerobiosis in diving vertebrates. *Comp. Biochem. Physiol. B* **50**, 17–22 doi:10.1016/0305-0491(75)90292-8
51 Su, X., Wellen, K.E. and Rabinowitz, J.D. (2016) Metabolic control of methylation and acetylation. *Curr. Opin. Chem. Biol.* **30**, 52–60 doi:10.1016/j.cbpa.2015.10.030
52 Xu, W., Yang, H., Liu, Y., Yang, Y., Wang, P., Kim, S.-H. et al. (2011) Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α-ketoglutarate-dependent dioxygenases. *Cancer Cell* **19**, 17–30 doi:10.1016/j.ccr.2010.12.014