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Lysine Succinylation Is a Frequently Occurring Modification in Prokaryotes and Eukaryotes and Extensively Overlaps with Acetylation

Brian T. Weinert,[1] Christian Schölz,[1] Sebastian A. Wagner,[1] Vytautas Iesmantavicius,[1] Dan Su,[1] Jeremy A. Daniel,[1] and Chunaram Choudhary[1,*]
[1]The NNF Center for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 3B, 2200 Copenhagen, Denmark
*Correspondence: chuna.choudhary@cpr.ku.dk
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SUMMARY

Recent studies have shown that lysines can be post-translationally modified by various types of acylations. However, except for acetylation, very little is known about their scope and cellular distribution. We mapped thousands of succinylation sites in bacteria (*E. coli*), yeast (*S. cerevisiae*), human (HeLa) cells, and mouse liver tissue, demonstrating widespread succinylation in diverse organisms. A majority of succinylation sites in bacteria, yeast, and mouse liver were acetylated at the same position. Quantitative analysis of succinylation in yeast showed that succinylation was globally altered by growth conditions and mutations that affected succinyl-coenzyme A (succinyl-CoA) metabolism in the tricarboxylic acid cycle, indicating that succinylation levels are globally affected by succinyl-CoA concentration. We preferentially detected succinylation on abundant proteins, suggesting that succinylation occurs at a low level and that many succinylation sites remain unidentified. These data provide a systems-wide view of succinylation and its dynamic regulation and show its extensive overlap with acetylation.

INTRODUCTION

Advances in high-resolution mass spectrometry (MS) and antibody-based affinity enrichment of acetylated lysine residues enabled the identification of thousands of acetylation sites (Choudhary et al., 2009; Kim et al., 2006), triggering a new appreciation of acetylation as a regulatory posttranslational modification (PTM). Acetylation, which depends on the metabolic intermediate acetyl-coenzyme A (acetyl-CoA), is reported to regulate multiple metabolic processes (Guan and Xiong, 2010; Hirschey et al., 2011). Similarly, several other lysine PTMs involving short-chain acyl-CoA metabolites are reported, such as malonylation (Peng et al., 2011; Xie et al., 2012), crotonylation (Montellier et al., 2012; Tan et al., 2011), propionylation (Chen et al., 2007), butyrylation (Chen et al., 2007), and succinylation (Lin et al., 2012). Such acylation could provide an elegant mechanism to coordinate metabolism and signaling by utilizing metabolic intermediates as sensors to regulate metabolism (Wellen and Thompson, 2012). Lysine succinylation was first shown to occur in the active site of homoserine trans-succinylase (Rosen et al., 2004), although this was assumed to indicate a reaction intermediate during the transfer of a succinyl group from succinyl-CoA to homoserine. Docosahexomic acid (DHA) was shown to promote the succinylation of lysine residues (Kawai et al., 2006), suggesting possible implications of DHA exposure in the CNS. Zhang et al. (2011) utilized an antibody-based enrichment strategy and MS-based proteomics to identify several dozens of succinylation sites in bacteria. Subsequent work identified several succinylation sites in animal tissues (Du et al., 2011) and on histones (Xie et al., 2012). DHA was implicated in the formation of succinylated lysines (Kawai et al., 2006), and sodium succinate was shown to promote succinylation in *E. coli* (Zhang et al., 2011). However, the mechanism of succinylation and the succinyl donor to lysine remains unknown, and a global analysis of lysine succinylation in diverse organisms is lacking.

Here, we developed a method based on antibody-based affinity enrichment of succinylated peptides followed by strong cation exchange (SCX) chromatography to identify thousands of lysine succinylation sites in bacteria (*E. coli*), budding yeast (*S. cerevisiae*), human cervical cancer (HeLa) cells, and mouse liver tissue. To obtain more detailed insights into succinylation, we used a quantitative MS approach to characterize succinylation in yeast strains deficient for the formation of succinyl-CoA or for its subsequent conversion to succinate. A comparative bioinformatic analysis of succinylation in these four organisms revealed several features of succinylation as a PTM and the biases inherent in the analysis of succinylation by MS. The data set, including all identified succinylation sites and their quantification, is provided in the Supplemental Information as a resource to the research community.

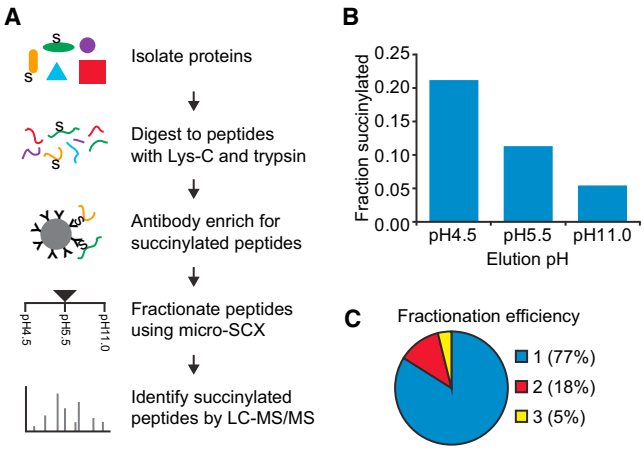

Figure 1. Enrichment of Succinylated Peptides from *E. coli* Lysate

(A) Experimental approach used to identify succinylated (S) peptides.

(B) Succinylated peptides are further enriched by low pH elution from a micro-SCX column. The column chart shows the fraction of succinylated peptides present in each elution fraction.

(C) Micro-SCX efficiently separates succinylated peptides. The pie chart shows the number of fractions in which any given succinylated peptide was identified.

See also Figure S1.

RESULTS

Enrichment of Succinylated Peptides and Identification by MS

In order to map lysine succinylation sites in vivo, proteins were isolated from exponentially growing cells or, in the case of mouse liver tissue, from 16-week-old animals. Proteins were digested with endoprotease Lys-C and trypsin to yield tryptic peptides, and succinylated peptides were enriched using succinyllysine polyclonal antibody. The enrichment of succinylated peptides with succinyllysine antibody, and the verification of lysine succinylation by orthogonal methods, were validated in a previous study (Zhang et al., 2011). Peptides were further separated by microscale SCX chromatography. SCX microcolumns were prepared by packing a micropipette tip with Empore (3M) disks containing cation exchange beads immobilized in a Teflon meshwork, as described previously (Rappsilber et al., 2007). We further modified this protocol by using pH-based elution, previously described for use in strong anion exchange fractionation (Wiśniewski et al., 2009), and by including a high percentage of acetonitrile in the buffer system (for details, see Experimental Procedures). This method is particularly suited for the fractionation of posttranslationally modified peptides following antibody affinity enrichment. Peptides eluted from antibody beads with acidified water can be directly loaded onto the SCX microcolumn and eluted with a step-wise pH gradient. Fractionation can be performed with as many individual fractions as desired, providing flexibility in reducing sample complexity. The presence of a high concentration of acetonitrile in the buffer system helps to remove hydrophobic contaminants, such as detergents, and prevents loss of peptides through hydrophobic interactions. In addition, the procedure is rapid and the small scale and simplicity of the system reduces sample handling. Following peptide separation into three fractions, the samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and peptide sequences were identified with a maximum false discovery rate of 1% using MaxQuant (Cox et al., 2011) (Figure 1A).

Widespread Lysine Succinylation in *E. coli*

In order to optimize our proteomics workflow and to obtain a detailed view of succinylation, we performed succinyllysine enrichment from *E. coli*. Fractionation of affinity-enriched peptides by micro-SCX resulted in further enrichment of succinylated peptides at low pH elutions (Figure 1B). Succinylation of lysine neutralizes its positive charge and introduces a negative charge. For this reason, the SCX-based chromatography is highly suited for the selective enrichment of succinylated peptides, which helps to further increase the relative abundance and detection of these peptides by MS. Fractionation of succinylated peptides by micro-SCX was efficient, with 77% of peptides occurring in only one fraction and 95% of peptides occurring in one or two fractions (Figure 1C). We identified 2,572 lysine succinylation sites occurring on 990 proteins (Table S1). Succinylation occurred on diverse proteins involved in nearly all aspects of cellular functions (Figure S1A), indicating that succinylation is a widespread and frequently occurring PTM in bacteria. We found succinylation on all 14 proteins that were previously shown to be succinylated (Zhang et al., 2011), including 72% (50/69) of the previously identified sites (Table S1). Furthermore, we found an additional 89 succinylation sites on these 14 proteins, indicating extensive modification of these proteins by succinylation and demonstrating improved sensitivity of our method for global succinylation analysis.

The mitochondrial sirtuin SIRT5 has an atypical acyl-binding domain that allows this enzyme to catalyze the desuccinylation of lysine residues (Du et al., 2011). The only known sirtuin in *E. coli*, CobB, has a similar sequence in the conserved acyl-binding domain, suggesting that CobB may be able to catalyze desuccinylation of lysines (Du et al., 2011). To test this, we used stable isotope labeling with amino acids in cell culture (SILAC) (Ong et al., 2002) to quantify relative differences in succinylation between wild-type and isogenic *cobB* mutant cells (*cobB*Δ). We quantified succinylation at more than 2,000 sites (Table S2) and found that succinylation was not globally altered by loss of CobB (Figure S1B). We expected that loss of a desuccinylase activity would result in an increased abundance of lysine succinylation. However, the distribution of succinylation site ratios that were corrected for differences in protein abundance between wild-type and *cobB*Δ cells was centered on a median value of 0.90, demonstrating that succinylation was not globally elevated in the mutant cells. In contrast, we recently showed that loss of *cobB* caused increased acetylation at hundreds of sites (Weinert et al., 2013). Based on these results, we conclude that CobB does not regulate succinylation levels globally, although there may exist sites that are regulated in a site-specific manner but not detected in our analysis.

Succinylation in Eukaryotes

To understand the scope of succinylation in eukaryotes, we used the above-described approach to identify lysine succinylation

Table 1. Succinylation Sites Identified in This Study Compared with Previous Studies

	Identified in This Study		Previously Known		
Species	Sites	Proteins	Sites	Proteins	Reference
E. coli	2,572	990	69	14	Zhang et al., 2011
S. cerevisiae	1,345	474	7	4	Xie et al., 2012
***H. sapiens* (HeLa)**	2,004	738	13	4	Du et al., 2011
***M. musculus* (liver)**	2,140	750	7	3	Du et al., 2011

sites in budding yeast (*S. cerevisiae*), human cervical cancer (HeLa) cells, and mouse liver tissue (Tables S3, S4, and S5). As in *E. coli*, fractionation by micro-SCX resulted in further enrichment of succinylated peptides in low pH fractions in all samples analyzed (Figure S2A). We found 1,345 sites in yeast, 2,004 sites in HeLa cells, and 2,140 sites in mouse liver (Table 1), greatly expanding the number of known succinylation sites in these organisms. We examined the amino acid sequences flanking succinylation sites in order to determine if particular amino acids were biased to occur adjacent to succinylated lysines. Filled logo motifs were generated for succinylation sites in all four organisms tested (Figure S2B). The sequence logos do not reveal a strong bias for a particular amino acid; however, aspartic acid, glutamic acid, and lysine occurred most frequently at the +1 position, while leucine and alanine occurred frequently at the −1 position. These biases may reflect a genuine preference for these amino acids or may result from the sequence bias of antibodies that were used to selectively enrich succinylated peptides, as was previously seen for acetyllysine enrichment (Henriksen et al., 2012). In order to determine whether succinylation was biased to occur within particular secondary structural domains on proteins, we used the secondary structure assignments from the UniProt database, when present, to compare succinylated and nonsuccinylated lysines. We found a moderate bias for succinylation to occur in helical regions and a moderate bias against coiled regions in all four organisms (Figure S2D).

Subcellular Distribution of Succinylated Proteins

Succinyl-CoA and succinate are primarily formed in the mitochondria during the tricarboxylic acid (TCA) cycle or via odd-numbered fatty acid oxidation. However, succinate can traverse the mitochondrial membrane and can be formed in the cytoplasm as a side product from α-ketoglutarate-dependent enzymes (Hausinger, 2004). Although acetyl-CoA exists in separate mitochondrial and nonmitochondrial pools (Takahashi et al., 2006; Wellen et al., 2009), the subcellular distribution and compartmentalization of succinyl-CoA, to our knowledge, is not known. Using localization data from GFP-tagged proteins (Huh et al., 2003) we found that only 8% of succinylation sites occurred on mitochondrial proteins in yeast (Figure 2A). In order to localize human and mouse proteins to different subcellular compartments, we analyzed the association of succinylated proteins with the UniProt keywords "mitochondrion," "cytoplasm," and "nucleus." Only proteins that could be associated with a keyword were analyzed. In HeLa cells and mouse liver, the proportion of succinylation occurring on mitochondrial proteins was significantly larger than in yeast, with the greatest proportion found in mouse liver, possibly due to the abundance of mitochondria in this tissue (Figure 2B). This finding was verified by examining proteins designated as mitochondrial in the MitoCarta (Pagliarini et al., 2008) database (Figure S3A). The occurrence of frequent lysine succinylation outside of mitochondria suggests that succinyl-CoA, succinate, or another succinyl-metabolite drives succinylation in the cytoplasm and nucleus.

Our analysis indicated a significant difference in the subcellular distribution of lysine succinylation between yeast and mammals (Figures 2A and 2B). However, the detection of succinylation sites is biased to occur on more abundant proteins (see below), which are more readily detected in the MS. Since mitochondrial proteins are more abundant in HeLa cells and mouse liver, a greater proportion of succinylation sites are identified on these proteins. When accounting for these differences in protein abundance, succinylation is similarly biased to occur on mitochondrial proteins in all three eukaryotic organisms examined (Figure S3B). Thus, differences in the abundance of mitochondria between these samples likely account for the greater proportion of succinylation sites found on mitochondrial proteins in HeLa cells and mouse liver.

Overlap between Lysine Succinylation and Acetylation

Lysine residues can be modified by a plethora of PTMs, including ubiquitylation, sumoylation, methylation, biotinylation, acetylation, and other forms of acylation. Of these modifications, acetylation has been extensively mapped in diverse organisms. We compared the succinylation sites identified in our study to previously determined acetylation sites in *E. coli* (Weinert et al., 2013) and *S. cerevisiae* (Henriksen et al., 2012) and to acetylation sites that we identified in the same HeLa cells and in mouse liver tissue used in this study (Tables S6 and S7). We found that 66% of *E. coli*, 56% of yeast, 27% of human, and 57% of mouse succinylation sites were acetylated at the same position (Figure 2C; Tables S3, S4, and S5). The small overlap found in HeLa cells likely occurs because succinylation was detected ∼2-fold more often on mitochondrial proteins than acetylation sites, while in mouse liver succinylation was detected only ∼1.3-fold more often on mitochondrial proteins, and a similar fraction of yeast succinylation and acetylation occurred on mitochondrial proteins (Figure 2D). Thus, the greater overlap between acetylation and succinylation in yeast and mouse liver is likely due to the more similar subcellular distribution of these modifications in these two samples. In HeLa cells and mouse liver tissue, the proportion of acetylation occurring on mitochondrial proteins is significantly lower than the proportion of succinylation occurring on mitochondrial proteins (Figure 2D). In contrast, a similar proportion of yeast acetylation and succinylation occurs on mitochondrial proteins. These observations show that acetylation and succinylation occur frequently at the same sites; however, the subcellular distribution of these modifications is different in mammals compared to yeast. This likely reflects the mitochondrial origin of succinyl-CoA, which drives succinylation (shown below), while acetyl-CoA exists in distinct mitochondrial and nonmitochondrial pools (Takahashi et al., 2006; Wellen et al., 2009).

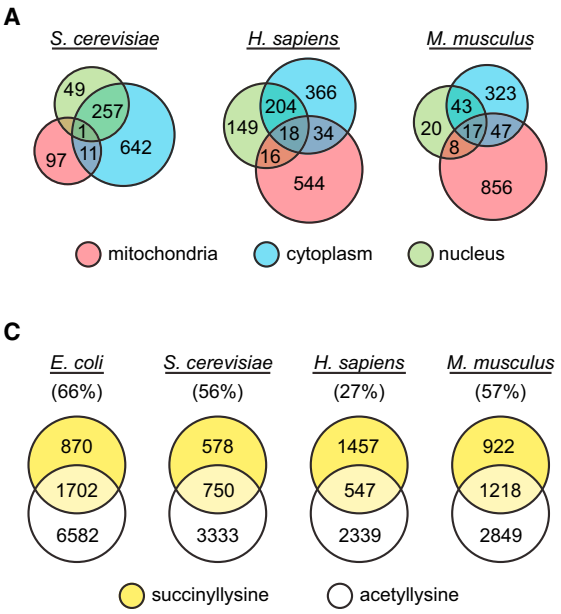

Figure 2. Subcellular Distribution of Lysine Succinylation Sites and Their Overlap with Acetylation

(A) The Venn diagrams show the subcellular distribution of succinylation sites determined in *S. cerevisiae* using their previously published subcellular localizations (Huh et al., 2003) and by associating *H. sapiens* and *M. musculus* proteins with the UniProt keywords "mitochondrion," "cytoplasm," and "nucleus."

(B) The fraction of mitochondrial succinylation is significantly higher in human cells and in mouse liver. The column graph shows the fraction of sites occurring on mitochondrial proteins in *S. cerevisiae* (*Sc*), *H. sapiens* (*Hs*), and *M. musculus* (*Mm*). Statistical significance was determined by Fisher exact test ($^*p < 1 \times 10^{-20}$).

(C) The Venn diagrams show the overlap between succinylation and acetylation sites. The fraction of succinylation sites that were also acetylated is indicated in parenthesis.

(D) The subcellular distribution of acetylation and succinylation differs in mammals. The fraction of succinylation or acetylation occurring on mitochondrial proteins was determined as in (A). Statistical significance was determined by Fisher exact test ($^*p < 1 \times 10^{-20}$). N.S., not significant.

See also Figures S2 and S3.

Succinylation Depends on Succinyl-CoA Formed in Mitochondria

In order to identify the succinyl donor to lysine throughout the cell, we used a SILAC-based quantitative approach to determine whether enzymes that metabolize succinyl-CoA and succinate during the TCA cycle affect lysine succinylation. In yeast, α-ketoglutarate is converted to succinyl-CoA by the mitochondrial α-ketoglutarate dehydrogenase complex, composed of Kgd1, Kgd2, and Lpd1. Kgd1 is required for α-ketoglutarate dehydrogenase activity and is under control of catabolite repression, whereby growth on galactose-containing media induces *kgd1* gene expression (Repetto and Tzagoloff, 1989). Succinyl-CoA is further converted to succinate by succinyl-CoA ligase, composed of Lsc1 and Lsc2. Lsc1 is required for succinyl-CoA ligase activity and, similar to Kgd1, is induced by growth on galactose-containing media (Przybyla-Zawislak et al., 1998).

We designed an experimental approach to test whether lysine succinylation was affected by growth on galactose-containing media and whether loss of *kgd1* or *lsc1* further affected lysine succinylation (Figures 3A and 3B). In both experiments, growth on galactose caused a ~1.7-fold increase in global lysine succinylation while mitochondrial proteins were more robustly affected with a ~2.7- to 4.7-fold increase in succinylation (Figure 3C; Table S8). Loss of *kgd1* caused a highly significant 4-fold reduction in global succinylation levels (relative to wild-type cells grown on galactose) and a 6-fold reduction in mitochondrial succinylation. In contrast, loss of *lsc1* caused a significant ~3-fold increase in succinylation of all proteins and of mitochondrial proteins. It is striking that the entire distribution of SILAC ratios was altered, indicating that nearly all detectable succinylation sites were affected by these manipulations. Growth on galactose, which is known to induce the enzymes that catalyze the formation of succinyl-CoA in the TCA cycle, caused increased succinylation, suggesting that this increase occurred due to increased concentration of succinyl-CoA in cells. Similarly, loss of *lsc1* blocks the conversion of succinyl-CoA to succinate and would likely cause accumulation of succinyl-CoA. We found increased succinylation in *lsc1*Δ cells, further suggesting that succinylation depends on succinyl-CoA concentration. Although we have not assayed succinyl-CoA concentration in this work, loss of *kgd1* causes reduced succinate levels in yeast (Arikawa et al., 1999). Since succinate is downstream of succinyl-CoA in the TCA cycle, *kgd1*Δ cells should have reduced levels of succinyl-CoA as well. Importantly, our data show that succinyl-CoA formed through this pathway affects succinylation levels throughout the cell, indicating that succinyl-CoA is the succinyl donor to lysine and that succinyl-CoA may traverse the mitochondrial membrane or be generated outside of mitochondria.

Succinylation Abundance

In order to determine the relationship between protein abundance and succinylation in our samples, we determined relative protein abundances using an intensity-based absolute quantification (iBAQ) method (Schwanhäusser et al., 2011). This method provides a good estimate of relative protein abundances for these samples since protein abundance is determined using the same samples from which succinylated peptides were enriched, thus controlling for differences introduced during sample preparation. We found succinylation more frequently on abundant proteins (Figure 4A), suggesting that, given adequate depth of coverage, we would detect succinylation on most proteins. This bias suggests that succinylation occurs at a low stoichiometry, as we are more likely to detect succinylation

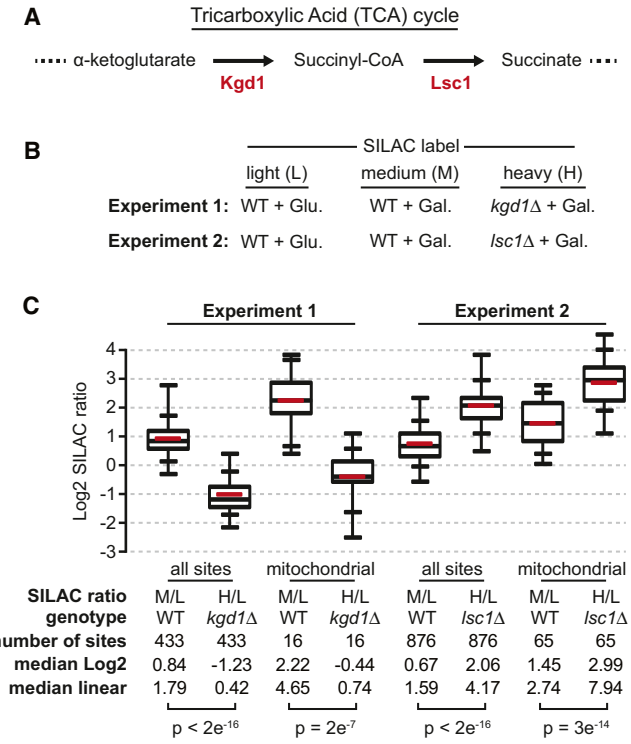

Figure 3. Lysine Succinylation Depends on Succinyl-CoA Formed through the Tricarboxylic Acid Cycle

(A) Schematic overview of the conversion of α-ketoglutarate to succinate during the tricarboxylic acid (TCA) cycle. Enzymes investigated in this study are shown in red type.

(B) Diagram indicating the quantitative mass spectrometry approach using stable isotope labeling with amino acids in cell culture (SILAC). The genotype (wild-type [WT]) and growth condition (glucose [Glu.] or galactose [Gal.]) are indicated for each of the SILAC labeling conditions in each experiment.

(C) The generation of succinyl-CoA affects lysine succinylation. Box plots show the distributions of SILAC ratios for succinylation sites. The box indicates the middle 50% of the distribution, inner hatch marks indicate 82%, and outer hatch marks 94% of the distribution; the median is indicated by the black line and the average by the red line. Significant differences between distributions were calculated by Wilcoxon test.

See also Figure S5.

if proteins are more abundant. Consistent with this idea, the frequency of observed succinylation (number of sites per 100 amino acids) is correlated with protein abundance in all four organisms (Figure 4B). This comparison indicates that we detect more succinylation sites on abundant proteins, suggesting that succinylation may target most of the accessible lysine residues in cells and that protein abundance is the determining factor in its detection. This correlation is notable considering that the lysine content, and accessibility of these lysines, is likely to vary between proteins. In contrast, the frequency of observed phosphorylation is poorly correlated with protein abundance in yeast, HeLa cells, and mouse liver (Figure S4). While we detected many succinylation sites on highly abundant proteins, we did not detect succinylated peptides without antibody enrichment, even though we readily detect peptides from proteins that are more than two orders of magnitude less abundant (Figure 4A). Together, these observations suggest that most succinylation occurs at a low stoichiometry.

Phosphoglycerate kinase (Pgk1) is one of the most abundant proteins in yeast according to Ghaemmaghami et al. (2003) and the iBAQ method used in this study. When we enriched for succinylated peptides from yeast lysate, we identified 17 succinylation sites on Pgk1 (Table S3). However, when we analyzed purified, GFP-tagged Pgk1, we identified only a single succinylated peptide from this protein, even though we identified unmodified peptides covering 95% of the protein sequence (Figure 4C). If we then enriched for succinylated peptides from the same protein preparation, we identified 14 succinylated peptides from Pgk1 and five succinylated peptides from GFP (Figure 4C; Table S9). Nonsuccinylated peptides from Pgk1 and GFP were median ~100-fold more intense than succinylated peptides from these proteins, even though the succinylated peptides had been enriched by ~100-fold before MS analysis (Figure 4D), indicating that succinylation occurred at a very low level. Succinylation of GFP suggests that a foreign protein can be targeted by succinylation, and the similar median succinylated peptide intensities from Pgk1 and GFP suggest that succinylation occurs to a similar degree on both proteins.

Nonenzymatic Succinylation

A low level of succinylation, together with the global succinylation dynamics in yeast grown on galactose, in *kgd1*Δ cells and in *lsc1*Δ cells, suggested that succinylation may occur by a nonenzymatic mechanism dependent on succinyl-CoA concentration. A previous study showed that acetyl-CoA can nonenzymatically acetylate lysines in vitro (Paik et al., 1970). In order to test whether succinyl-CoA can nonenzymatically succinylate proteins, we mixed increasing concentrations of succinyl-CoA with BSA and egg white albumin (ovalbumin). Increased succinylation relative to untreated control samples was assayed by MS using TMT isobaric mass tags for peptide quantification. We found that succinyl-CoA caused increased succinylation in a concentration-dependent manner at all identified succinylation sites (Figure S5). Although we cannot completely rule out the presence of a succinyl-transferase activity in these reactions, it is likely that succinylation occurred by direct chemical modification of lysine residues as BSA and ovalbumin were isolated from two different extracellular sources yet both showed a similar reactivity to succinyl-CoA. Our reactions were performed at or below physiological pH, suggesting that nonenzymatic succinylation may occur by exposure to succinyl-CoA in cells. However, it is impossible to rule out the potential existence of enzymatic succinyl-transferase activities in cells and their contribution to lysine succinylation in vivo.

Succinylation of Metabolic Proteins in Mouse Liver

Mitochondrial proteins were highly succinylated in mouse liver. As much as 70% of succinylation sites occurred on mitochondrial proteins, and mitochondrial proteins were more frequently multiply succinylated than nonmitochondrial proteins (Figure S6A). A total of 32% (348/1,097) of the proteins designated as mitochondrial in the MitoCarta database (Pagliarini et al., 2008) were succinylated. We performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

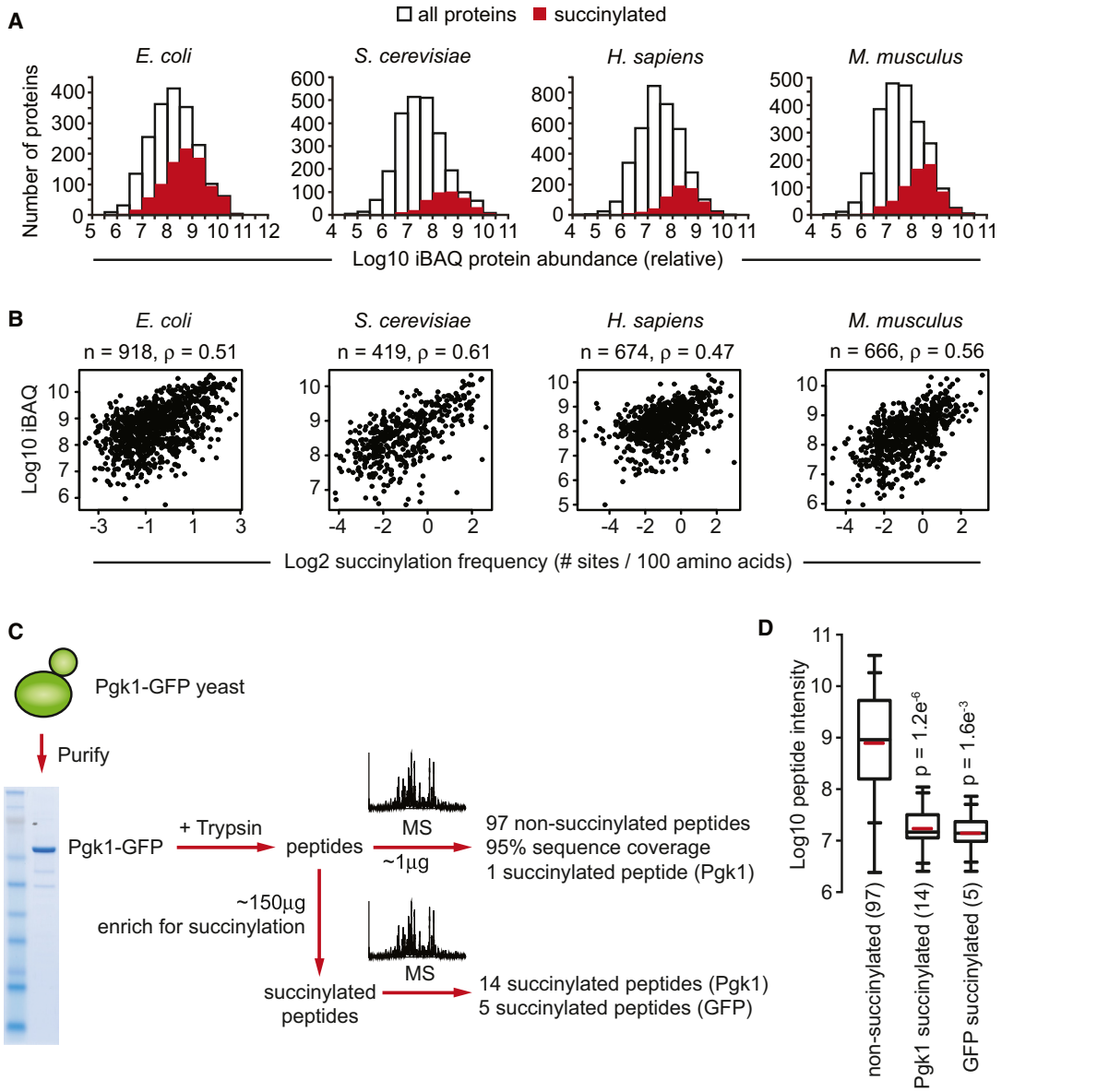

Figure 4. Protein Abundance Is a Determining Factor in the Detection of Succinylation Sites

(A) Detection of succinylation is biased to occur on abundant proteins. The histograms show experimentally determined relative protein abundance distributions for the samples used to analyze succinylation in this study.

(B) The frequency of detected succinylation sites is correlated with protein abundance. The scatterplots show the relationship between protein abundance (Log10 iBAQ) and succinylation frequency (number of sites per 100 amino acids). The number (n) of proteins analyzed and the Spearman's correlation coefficient (ρ) is shown.

(C) The diagram illustrates the analysis of purified Pgk1-GFP by mass spectrometry (MS).

(D) Succinylated peptides are significantly less intense. The box plots compare peptide intensity distributions for the indicated classes of peptides shown in (C). Nonsuccinylated peptide intensities from both Pgk1 and GFP were determined without enrichment. Succinylated peptide intensities were determined after ~100-fold enrichment of succinylated peptides, as shown in (C). The number of peptides examined is shown in parentheses. Significance (p value) was determined by Wilcoxon test comparing succinylated peptide intensities to nonsuccinylated peptide intensities.

See also Figures S4, S5, and Table S9.

and found that many metabolic pathways were significantly enriched within the group of succinylated proteins (Figure S6B). We further illustrated the occurrence of succinylation and acetylation in three key metabolic pathways: the TCA, the urea cycle, and fatty acid oxidation (Figure 5). Nearly every enzyme in the TCA cycle and in the urea cycle was succinylated and the majority of sites were also found to be acetylated. These results mirror the observation of widespread acetylation of metabolic proteins from human liver tissue (Zhao et al., 2010), suggesting that both of these acylations may play a role in regulating metabolic

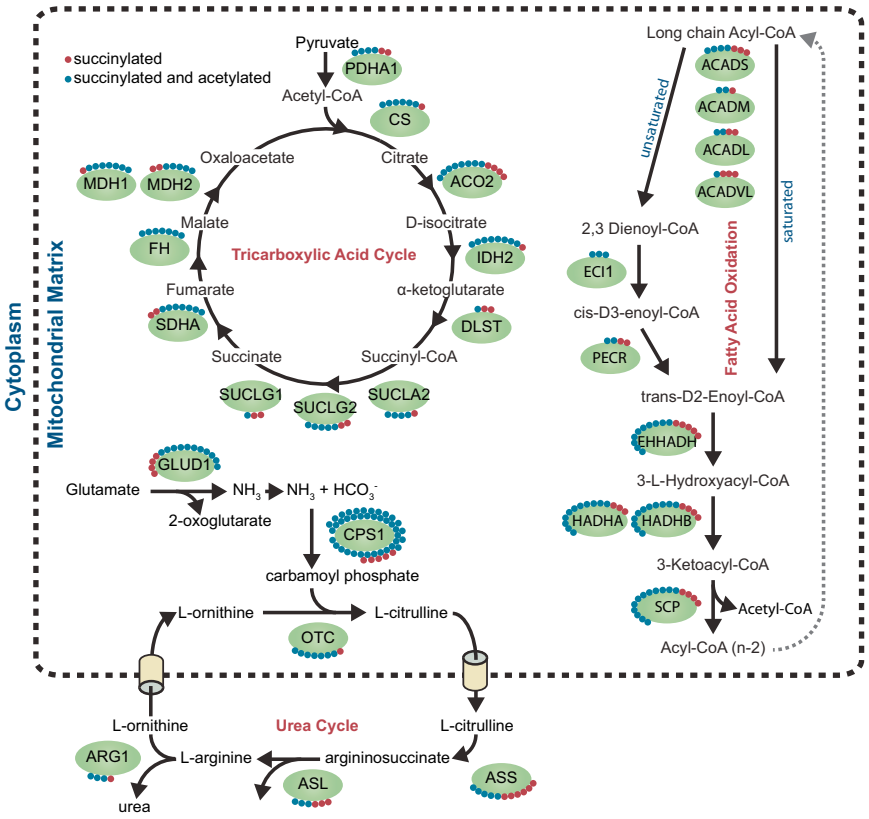

Figure 5. Succinylation of Enzymes Involved in the Tricarboxylic Acid Cycle, the Urea Cycle, and Fatty Acid Oxidation

Succinylated enzymes identified in mouse liver tissue are shown in green ovals, sites that were found to be both acetylated and succinylated are shown as blue circles, sites only found to be succinylated are shown as red circles. The enzymes shown are pyruvate dehydrogenase E1 component subunit alpha (PDHA1), citrate synthase (CS), aconitate hydratase (ACO2), isocitrate dehydrogenase (IDH2), dihydrolipoamide S-succinyltransferase (DLST), succinyl-CoA ligases G1, G2, and A1 (SUCLG1, SUCLG2, and SUCLA1), succinate dehydrogenase (SDHA), fumarate hydratase (FH), malate dehydrogenase 1 and 2 (MDH1 and MDH2), glutamate dehydrogenase (GLUD1), carbamoyl phosphate synthetase I (CPS1), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), arginase 1 (ARG1), short-, medium-, long-, and very-long-chain-specific acyl-CoA dehydrogenases (ACADS, ACADM, ACADL, and ACADVL), enoyl-CoA delta isomerase 1 (ECI1), peroxisomal *trans*-2-enoyl-CoA reductase (PECR), enoyl-CoA hydratase/3,2-*trans*-enoyl-CoA isomerase,3-hydroxyacyl-CoA dehydrogenase (EHHADH), long-chain enoyl-CoA hydratase, long-chain 3-hydroxyacyl-CoA dehydrogenase alpha and beta (HADHA and HADHB), and nonspecific lipid-transfer protein (SCP).

processes. However, as noted above, the preponderance of lysine succinylation on mitochondrial proteins and metabolic pathways in mouse liver is most likely due to a technical bias to detect succinylation sites on the most abundant proteins, which are predominantly mitochondrial in mouse liver.

Carbamoyl phosphate synthase 1 (CPS1) has reduced activity in SIRT5 mice (Nakagawa et al., 2009), and one of three previously identified succinylation sites was affected by loss of SIRT5 (Du et al., 2011). In mouse liver we found the greatest number of succinylation sites on CPS1 (44 sites), including all three previously known sites. CPS1 was acetylated at 52 sites, 39 of which were also succinylated. These results are not surprising, since we determined that CPS1 was the fifth most abundant protein in our liver samples and it is also relatively large, containing 1,500 amino acids. CPS1 plays an important role in initiating the urea cycle by catalyzing the synthesis of carbamoyl phosphate from ammonia and bicarbonate. We found that every enzyme present in the urea cycle is succinylated in mouse liver tissue (Figure 5). Previous studies showed that ornithine transcarboxylase (OTC), another urea cycle enzyme, is regulated by acetylation at lysine 88 (Yu et al., 2009) and that the mitochondrial deacetylase SIRT3 regulates acetylation at this site in response to calorie restriction (Hallows et al., 2011). We found that OTC is succinylated at lysine 88 in mouse liver, indicating that this critical lysine residue is targeted by both succinylation and acetylation. Glutamate dehydrogenase (GLUD1) was one of the most highly succinylated proteins with 15 sites. GLUD1 catalyzes the oxidative deamination of glutamate to α-ketoglutarate and ammonia, thereby providing a source of ammonia to enter the urea cycle via CPS1. GLUD1 was previously shown to be regulated by SIRT4-dependent ADP-ribosylation in pancreatic β cells (Haigis et al., 2006). The observation that SIRT3, SIRT4, and SIRT5 have roles in regulating OTC, GLUD1, and CPS1, respectively, raises the interesting possibility that multiple PTMs and mitochondrial sirtuins regulate the urea cycle. Our data suggest that succinylation may play a role in affecting the activity of these proteins and regulating the urea cycle, as was previously suggested for CPS1 (Du et al., 2011).

We found a striking overlap between acetylated and succinylated lysines overall (Figure 2C) and in mitochondrial metabolic pathways specifically (Figure 5). We found that succinylation occurred at many sites that were previously shown to be regulated by acetylation, including homologous lysines 172, 344, and 579 on 3,2-*trans*-enoyl-CoA isomerase,3-hydroxyacyl-CoA dehydrogenase (EHHADH) and malate dehydrogenase 2 (MDH2) lysines 185, 301, 307, and 314 (Zhao et al., 2010); malate dehydrogenase 1 (MDH1) lysines 118 and 298 (Kim et al., 2012), long-chain-specific acyl-CoA dehydrogenase (ACADL) lysine 42 (Hirschey et al., 2010); and super oxide dismutase (SOD2) lysines 68, 122, and 130 (Chen et al., 2011; Tao et al., 2010). The occurrence of succinylation at these positions raises the possibility of these enzymes being affected by both modifications and furthermore complicates the functional analysis of either modification by mutation, which would abrogate both acetylation and succinylation, as well as other possible lysine modifications.

DISCUSSION

In this study, we provide a detailed view of the scope of lysine succinylation in four different, evolutionarily diverse organisms. The identification of more than 2,500 succinylation sites in *E. coli* is striking because, in contrast to canonical regulatory PTMs, such as phosphorylation, the complexity of succinylation appears to be greater in bacteria than in eukaryotes. Our results indicate that during the evolution from bacteria to eukaryotes, the relative (in terms of proteome complexity) frequency of succinylation appears to have decreased. In eukaryotic cells, the frequency of succinylation in bacterially derived mitochondria is greatest. This is in stark contrast to protein phosphorylation (Weinert et al., 2011) and points to an interesting similarity between succinylation and acetylation in bacteria and mitochondria.

In yeast cells, succinylation depended on succinyl-CoA formed through the TCA cycle, indicating that succinyl-CoA is the succinyl donor to lysine and that succinyl-CoA concentration affects succinylation levels globally. Our data suggested that most lysine succinylation occurs at low levels, and a technical bias to detect succinylation on abundant proteins suggested that succinylation likely occurs at many more sites than were identified in this study. These observations suggested that succinylation may occur nonenzymatically, and we showed that succinyl-CoA could succinylate lysine residues on purified proteins in vitro, suggesting that nonenzymatic succinylation by succinyl-CoA may occur frequently in cells. These data are interesting as a lysine succinyltransferase has not been identified and the mechanism of lysine succinylation remains unknown. Previous studies have shown that changes in acetyl-CoA levels can affect lysine acetylation (Takahashi et al., 2006; Wellen et al., 2009) as well as protein N-terminal acetylation (Yi et al., 2011). Some acetyltransferases have an affinity for acetyl-CoA that is similar to physiological acetyl-CoA concentrations, suggesting that fluctuations in acetyl-CoA levels may regulate acetylation (Cai et al., 2011). A similar mechanism may regulate enzyme-catalyzed succinylation in cells and would be consistent with our observations. Furthermore, site-specific, enzyme-catalyzed succinylation may occur in parallel with nonenzymatic succinylation, complicating the functional categorization of sites identified by MS. Our results in yeast showed that succinylation occurred at a low level and was globally affected by succinyl-CoA metabolism, suggesting a common, possibly nonenzymatic mechanism of modification. However, yeast appears to lack a lysine desuccinylase and the mechanisms and regulation of succinylation in organisms that contain the regulatory desuccinylase SIRT5 may be more complex.

Our results show extensive overlap between succinylation and acetylation, raising the possibility of crosstalk between these PTMs. However, it remains to be determined whether different acylations have unique regulatory roles or if they perform redundant functions. Also, it would be interesting for future studies to investigate the relative stoichiometries of various acylations occurring on the same lysines. We provided a glance of succinylation in evolutionarily diverse organisms, and these data provide insights into succinyl-CoA metabolism and succinylation.

EXPERIMENTAL PROCEDURES

Cell Culture and Lysate Preparation

Details concerning the origin of cells and tissues and the preparation of protein lysates for MS analysis can be found in the Extended Experimental Procedures. All mouse work was performed according to national and international guidelines approved by the Danish Animal Ethical Committee and the review board at the Faculty of Health and Medical Sciences, University of Copenhagen.

Succinyllysine Enrichment and SCX-Based Peptide Fractionation

Peptides were mixed with 200 μl succinyllysine polyclonal rabbit antibody (PTM Biolabs) and ~50 μl of rec-Protein G Sepharose (Invitrogen) or with 100 μl of preconjugated succinyllysine antibody resin (PTM Biolabs). Reactions were incubated overnight at 4°C on a rotating wheel. The immunoprecipitates were washed three times at 4°C with IP-A buffer (50 mM MOPS [pH 7.2] and 50 mM NaCl) and once with H_2O. Immuno-enriched peptides were eluted with 3 × 100 μl acidified H_2O (0.2% trifluoroacetic acid [TFA]) and loaded directly onto a strong cation exchange (SCX) microtip column prepared as described (Rappsilber et al., 2007; Wiśniewski et al., 2009). The SCX microtip was conditioned with 100 μl 0.1% TFA, 50% acetonitrile, 100 μl pH 8.5 elution buffer (see below), and 100 μl 0.1% TFA, 50% acetonitrile. After loading, the column was washed with an additional 100 μl 0.1% TFA, 50% acetonitrile and peptides were eluted by stepwise 100 μl aliquots of elution buffer (20 mM acetic acid, 20 mM boric acid, 20 mM phosphoric acid, 40% acetonitrile) at pH 4.5, pH 5.5, and pH 8.5 or pH 11. Peptide eluates were briefly evaporated to remove acetonitrile and then loaded onto C18 stage-tips as described previously (Rappsilber et al., 2007).

SILAC Labeling of *E. coli* and Yeast

Wild-type BW25113 and isogenic mutant *cobBΔ* (JW1106) strains (Baba et al., 2006) were obtained from the *E. coli* Genetic Resource Center (http://cgsc.biology.yale.edu). For SILAC labeling, cells were grown in M9 minimal media supplemented 0.2% glucose and with 100 μg/ml lysine (SILAC "light") or $^{13}C_6{}^{15}N_2$-lysine (Cambridge Isotope Laboratories) (SILAC "heavy"). Wild-type *S. cerevisiae* cells (BY4742, MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0; Open Biosystems), *lsc1Δ* (BY4742, *lsc1*::KanMX; Open Biosystems), and *kgd1Δ* (BY4742, *kgd1*::KanMX; Open Biosystems) were cultured in synthetic complete media (US Biologicals) supplemented with lysine (SILAC "light"), $^2H_4$ -lysine (SILAC "medium"), or $^{13}C_6{}^{15}N_2$-lysine (SILAC "heavy"). Cells were harvested during the midexponential phase of growth and lysate was prepared and succinylated peptides were enriched as described above.

Nonenzymatic Succinylation and Labeling with TMT Isobaric Mass Tags

BSA (Sigma) and egg white albumin (ovalbumin) (Sigma) were prepared in PBS (pH 7.2) at 10 mg/ml. A total of 1 mg protein (0.1 ml) was mixed with 1/10 vol of freshly prepared succinyl-CoA in H_2O to yield the final concentrations shown in Figure 5. Reactions were incubated at 30°C for 3 hr and stopped by addition of 5 vol −20°C acetone, which precipitated the protein. Protein precipitates were resuspended in 0.2 ml 100 mM triethyl ammonium bicarbonate (TEAB) and digested for 16 hr by addition of 1/100 (w/w) trypsin protease (Sigma). Labeling with TMTsixplex isobaric mass tags (Thermo Scientific) was performed according to the manufacturer's instructions. Quantification of TMT mass tags may be inaccurate if additional peptides are coisolated with the targeted peptide ion (Ting et al., 2011). In order to avoid this effect, we restricted the quantification of TMT mass tags to MS/MS scans in which the targeted parent ion constituted a minimum of 90% of the total ion current.

MS, Peptide Identification, and Computational Analysis

Peptide fractions were analyzed by online nanoflow LC-MS/MS using a Proxeon easy nLC system (Thermo Scientific) connected to a Q-Exactive (Thermo Scientific) mass spectrometer. The Q-Exactive was operated using Xcalibur 2.2 in the data-dependent mode to automatically switch between MS and MS/MS acquisition as described elsewhere (Kelstrup et al., 2012; Michalski et al., 2011). Peptides were fragmented using higher energy C-trap dissociation (HCD) fragmentation (Olsen et al., 2007). Raw data files

were processed using MaxQuant software (development v1.2.7.1; http://www.maxquant.org/) as described previously (Cox et al., 2011). Parent ion (MS) and fragment (MS2) spectra were searched against the *Saccharomyces* Genome Database genome release r63, January 5, 2010, and *E. coli*, human, and mouse data were searched against the UniProt species-specific fasta files from the April 2012 release. The search was performed using the integrated Andromeda search engine and both forward and reversed (decoy) versions of the databases (Cox et al., 2011). Peptides were identified by a target-decoy based strategy (Elias and Gygi, 2007), allowing a maximum false discovery rate of <1%. We applied further strict criteria on MS2 identification by requiring a PEP score < 0.01 and an Andromeda peptide score > 45, resulting in estimated FDRs that were below 1% (Table S10). The confidence of any given peptide identification is indicated by the PEP and Andromeda scores; high-confidence identifications have lower PEP scores and higher Andromeda scores (for more details, see Cox and Mann, 2008; Cox et al., 2011). Mass recalibration was performed using high confidence identifications based on an initial "first search" using a 20 parts per million (ppm) mass tolerance for parent ion masses and 20 ppm (HCD) for fragment ions. Spectra were subsequently searched with a mass tolerance of 6 ppm for parent ions and 20 ppm (HCD) for fragment ions, with strict trypsin specificity, and allowing up to 2 missed cleavage sites. Cysteine carbamidomethylation was searched as a fixed modification, whereas N-acetyl protein and oxidized methionine were searched as variable modifications. Succinyllysine adds a 100.0160 mass shift that is readily distinguishable from acetylation (42.0106), malonylation (86.0004), propionylation (56.0262), and crotonylation (69.0340), since the orbitrap analyzer used for fragment scans has a ppm-range mass accuracy. Methylmalonylation has an identical mass as succinylation and is not distinguishable in the MS, but antibody enrichment should specifically select for succinylated lysine as we were unable to detect substantial enrichment of other acylations (data not shown) and we observed altered succinylation levels upon manipulation of the enzymes regulating succinyl-CoA formation in yeast, suggesting that we primarily detect succinylation by this method. MS raw data are available upon request.

Data analysis

Gene Ontology (GO) and KEGG pathway association and enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Huang da et al., 2009). Filled logo motifs were generated using iceLogo (Colaert et al., 2009). Relative protein abundances (intensity-based absolute quantification [iBAQ]) were determined using MaxQuant. iBAQ values are calculated by taking the summed peptide intensities for a given protein and dividing by the number of theoretically observable tryptic peptides (Schwanhäusser et al., 2011). Overlap between succinylation sites and previously published acetylation sites was determined by comparing 13-amino-acid sequence windows (modification site ± six amino acids). Secondary structure biases were determined using the secondary structure assignments found in the UniProt database (http://www.uniprot.org/), when present.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and ten tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.07.024.

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