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Protein Acetylation Microarray Reveals NuA4 Controls Key Metabolic Target Regulating Gluconeogenesis

Yu-yi Lin[1,3,9], **Jin-ying Lu**[1,2,4,5,9], **Junmei Zhang**[6], **Wendy Walter**[7], **Weiwei Dang**[7], **Jun Wan**[8], **Sheng-Ce Tao**[1,2], **Jiang Qian**[8], **Yingming Zhao**[6], **Jef D. Boeke**[1,3,*], **Shelley L. Berger**[7,*], and **Heng Zhu**[1,2,*]

1 *High Throughput Biology Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

2 *Departments of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

3 *Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

4 *Department of Laboratory Medicine, National Taiwan University Hospital*

5 *Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei 100, Taiwan*

6 *Department of Biochemistry, UT Southwestern Medical Center, Dallas, Texas 75390-9038*

7 *Gene Expression and Regulation Program, The Wistar Institute, Philadelphia, PA 19104*

8 *The Wilmer Eye Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

SUMMARY

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) conduct many critical functions through nonhistone substrates in metazoans, but only chromatin-associated nonhistone substrates are known in *Saccharomyces cerevisiae*. Using yeast proteome microarrays, we identified and validated many nonchromatin substrates of the essential nucleosome acetyltransferase of H4 (NuA4) complex. Among these, acetylation sites (Lys 19 and 514) of phosphoenolpyruvate carboxykinase (Pck1p) were determined by tandem mass spectrometry. Acetylation at Lys 514 was crucial for enzymatic activity and the ability of yeast cells to grow on non-fermentable carbon sources. Loss of Pck1p activity blocked the extension of yeast chronological life span caused by water starvation. In human hepatocellular carcinoma (HepG2) cells, human Pck1 acetylation and glucose production was dependent on *TIP60*, the human homolog of *ESA1*. Our results demonstrate a novel regulatory function for the NuA4 complex in glucose metabolism and life span by acetylating a critical metabolic enzyme.

INTRODUCTION

Acetylation and deacetylation of histone lysine residues are among the best characterized post-translational modifications, and control many chromatin-related processes (Kouzarides,

*Correspondence: E-mail: jboeke@jhmi.edu (J.D.B.), E-mail: berger@wistar.org (S.L.B.), E-mail: heng.zhu@jhmi.edu (H.Z.).
[9]These authors contributed equally to this work.

2007; Millar and Grunstein, 2006). There are over 20 histone acetyltransferases (HATs) and histone deacetylases (HDACs) in *S. cerevisiae* (Lee and Workman, 2007; Shahbazian and Grunstein, 2007); among which only Esa1p, the catalytic enzyme of NuA4 is essential for cell viability (Allard et al., 1999; Clarke et al., 1999; Smith et al., 1998). Esa1, with Epl1p and Yng2p forms the "piccolo NuA4" core that acetylates the N-terminal tails of histones H2A, H4 and Htz1p *in vivo* (Babiarz et al., 2006; Boudreault et al., 2003; Keogh et al., 2006; Millar et al., 2006). NuA4 is critical for regulating gene transcription, limiting the spread of silent heterochromatin, maintaining cell cycle progression and repairing DNA double-strand breaks. The NuA4 complex is highly conserved from yeast to humans (Auger et al., 2008); the TIP60 complex, the human homolog of NuA4, is important for efficient DNA damage repair and maintenance of apoptotic competence (Ikura et al., 2000). Several recent large-scale yeast genetic interaction screens have implied functional relationships between HATs/HDACs and many extranuclear proteins (Lin et al., 2008; Mitchell et al., 2008). Previous gene expression studies showed that although global downregulation of acetylation profoundly affected gene expression (Takahashi et al., 2006), dysfunction of the individual major HATs and HDACs resulted in only minor transcriptional alterations of distinct gene sets (Huisinga and Pugh, 2004; Robyr et al., 2002; Zhang et al., 2004), suggesting that these enzymes regulate specific extranuclear functions through mechanisms other than transcriptional control, such as (de) acetylation of nonhistone substrates. Most recently, several chromatin-associated proteins were found to be acetylated in yeast, including a cohesin subunit (Smc3p) acetylated by Eco1p (Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008), an RSC chromatin remodeling complex subunit (Rsc4p) acetylated by Gcn5p (Choi et al., 2008; VanDemark et al., 2007), and a subunit of NuA4 (Yng2p) acetylated by Esa1p (Lin et al., 2008). However, nonchromatin substrates of HATs/HDACs have not been reported in yeast despite the identification of many such acetylation substrates in higher organisms (Yang and Seto, 2008a; Yang and Seto, 2008b). Full understanding of the molecular functions of these enzymes requires the identification of nonchromatin substrates.

The enzymatic activities of HATs and HDACs are closely related to the metabolic condition of the cells, whereas acetylation and deacetylation of metabolic enzymes also plays a pivotal role in adaptation of the cells to changing energy status (Schwer and Verdin, 2008). In yeast, histone acetylation and global gene transcription is directly regulated by abundance of a key metabolic intermediate, acetyl-CoA, which in turn is determined by nucleocytoplasmic acetyl-CoA synthetase activity (Takahashi et al., 2006). In mammals, the cytoplasmic acetyl-CoA synthetase 1 (AceCS1) is deacetylated and activated by *SIRT1*, an NAD^+ dependent HDAC (Hallows et al., 2006). A large number of cytoplasmic and mitochondrial metabolic enzymes were recently reported to be subject to (de)acetylation in mammals (Kim et al., 2006), but the responsible (de)acetylases remain largely unknown.

The intersection of metabolism and protein (de)acetylation is further reflected by the links between sirtuins and life span regulation in yeast, worms, fruit flies, and possibly mammals (Guarente and Picard, 2005; Schwer and Verdin, 2008). In yeast, limiting the concentration of either glucose or amino acids in the medium can lengthen both replicative life span (RLS, defined as the number of mitotic divisions occurring in a mother cell before senescence) and chronological life span (CLS, defined as the period of time for a cell to survive in stationary phase) (Fabrizio et al., 2005; Lin et al., 2000; Powers et al., 2006). *SIR2* plays an antagonistic role in the extension of CLS under water starvation (Fabrizio et al., 2005). Although the detailed mechanism is largely unknown, the effect of *SIR2* on CLS is partly mediated by *SIR2*-dependent inactivation of alcohol dehydrogenase 2 (*ADH2*) that depletes extracellular ethanol, a proposed central mediator of chronological longevity in yeast (Fabrizio et al., 2005). Although ethanol was shown to be critical in this longevity paradigm, and the requirement for deacetylase activity in blocking extreme life span extension was demonstrated, no relevant Sir2 substrate(s) were identified.

Here, we report 13 novel substrates of the NuA4 complex identified by a protein acetylation microarray approach. In depth analysis of one substrate, Pck1p, provides the first evidence of a metabolic enzyme (de)acetylation state controlling the gluconeogenic flux in the yeast and possibly human cells. Remarkably, Pck1p acetylation is necessary and sufficient for extension of yeast chronological longevity under water starvation.

RESULTS

Identification of nonhistone NuA4 substrates using proteome microarrays

To fully understand the molecular functions of the NuA4 complex, we sought its substrates using yeast proteome microarrays (Lu et al., 2008; Zhu et al., 2001). First, we developed an *in vitro* acetylation reaction on a pilot microarray containing histones H3, H4, and bovine serum albumin (BSA). To optimize reaction conditions and surface chemistry (Tao et al., 2007), we performed the assay using both the NuA4 and Spt-Ada-Gcn5-acetyltransferase (SAGA) complexes and [^{14}C]-acetyl-CoA as a labeling reagent on three surfaces at various reaction temperatures and time. NuA4 and SAGA complexes showed preference for H4 and H3, respectively, as expected (Sterner and Berger, 2000) on the FAST (nitrocellulose) slide (Figure S1). No detectable signals were observed for BSA. We then carried out acetylation reactions in duplicate with NuA4 complex on proteome microarrays containing 5,800 yeast proteins (Zhu et al., 2001). We found that 91 proteins were readily acetylated by NuA4 (Figure 1A, and Table S1 for a full list of candidate substrates). Gene Ontology (GO) analysis indicated that these candidate substrates were enriched for nucleosome assembly and histone binding activities, and depleted for mitochondrial and membrane-associated proteins, as expected. However, significant proportions ($P < 10^{-4}$) of the candidate substrates are cytoplasmic proteins such as Pck1p. Therefore, we chose 20 candidates involved in regulating metabolism (Glk1p, Gph1p, Gsy2p, Pck1p, Sip2p, Sip5p, and Tap42p), transcription (Nnt1p and Ume1p), cell cycle progression (Cdc34p, Ctf4p, Rpt5p and Skp1p), RNA processing (Brx1p and Prp19p), and stress response (Atg3p, Atg11p, Hsp104p, Imp2′p and Plp2p) for further validation.

We tested the ability of the NuA4 complex to acetylate these candidate substrates using standard *in vitro* activity assays (Smith et al., 1998) and confirmed 13 substrates (Atg3p, Atg11p, Brx1p, Cdc34p, Gph1p, Hsp104p, Nnt1p, Pck1p, Prp19p, Rpt5p, Sip2p, Sip5p and Tap42p), as detected by immunoprecipitation (IP) with a monoclonal anti-pan-acetyl-lysine antibody (Li et al., 2007) (Figure 1B and Figure S2A), followed by immunoblotting with an anti-TAG antibody. Because *in vitro* enzymatic assays could suffer from promiscuity caused by non-physiologic conditions created in the experiments, we further validated the substrates with *in vivo* experiments performed in an *ESA1* Ts (*esa1-531*) mutant (Lin et al., 2008). We found that all the *in vitro* substrates were also acetylated in the wild type (WT) cells; their acetylated fractions dramatically diminished in *esa1-531* at nonpermissive temperature (Figure 1C and Figure S2B). Furthermore, the *in vivo* acetylation signals in the IP assay could be efficiently competed with lysine-acetylated BSA (Ac-K-BSA), indicating the specificity of the detected acetylation signals (Figure S3). Together, these data suggested that Gph1p, Pck1p, Sip2p, Sip5p, Tap42p, Nnt1p, Cdc34p, Rpt5p, Brx1p, Prp19p, Atg3p, Atg11p, and Hsp104p are *in vivo* substrates of Esa1p.

Pck1p Is acetylated by NuA4 and deacetylated by Sir2p at K514

More than half the confirmed *in vivo* substrates of NuA4 are enzymes (Atg3p, Gph1p and Pck1p) or effectors (Atg11p, Sip2p, Sip5p and Tap42p) involved in signaling pathways responsive to nutrient availability and energy status. To explore the possible link between NuA4 acetylation and metabolism, we focused on Pck1p for several reasons. First, it is a cytosolic non-chromatin substrate. Second, it is a well-studied enzyme (Baly et al., 1985;

Hanson and Reshef, 1997). Finally, it seemed to provide a potential insight into a new and poorly understood connection between acetylation and metabolism. Moreover, the important role of the human enzyme in diabetes (Burgess et al., 2007; Permutt and Hattersley, 2000) and prolonged life span in mice overexpressing *PCK1* (Hakimi et al., 2007) made it interesting. *PCK1* encodes the intricately regulated phosphoenolpyruvate carboxykinase (PEPCK), catalyzing the rate-limiting step in gluconeogenesis by converting oxaloacetate (OAA) and ATP to phosphoenolpyruvate (PEP), ADP, and CO_2. We applied tandem mass spectrometric (MS/MS) analysis and identified lysines 19 (K19) and 514 (K514) as potential acetylation sites for the NuA4 complex (Figure S4). A K514 arginine mutant (K514R) was generated to test association of acetylation of Pck1p with NuA4 activity. *In vitro* and *in vivo* assays showed that NuA4-dependent acetylation of overproduced Pck1p (Figure 2A–2C) or endogenously expressed (Figure 2D) was almost completely dependent on K514, suggesting that NuA4 acetylates Pck1p at K514. Conversely, acetylation at K19 is far less abundant than K514 *in vivo* (Figure 2E), and a K19R mutation did not affect efficient acetylation by NuA4 *in vitro* (Figure 2F).

Because acetylation is reversible, we applied a similar *in vivo* approach to search for Pck1p deacetylase. A substantial increase of acetylated Pck1p was observed in the *sir2*Δ mutant, but not in *hda1*Δ or *rpd3*Δ mutants (Figure 3A). *SIR2* encodes an NAD^+-dependent HDAC involved in chromatin silencing (Rusche et al., 2003), while the other two encode major HDACs governing global histone deacetylation and transcriptional control (Vogelauer et al., 2000). Moreover, affinity-purified Sir2p, but not Hda1p (Figure S5), effectively deacetylated purified Pck1p *in vitro* in an NAD^+-dependent reaction (Figure 3B). Acetylation of endogenous Pck1p increased significantly in the *sir2*Δ mutant (Figure 2D); in summary, Esa1p and Sir2p specifically catalyze reversible (de)acetylation at K514 of Pck1 *in vivo*.

PCK1 mRNA and protein are highly induced during a diauxic shift, *i.e.*, the glycolysis to gluconeogenesis shift as glucose is depleted (Schuller, 2003; Zaman et al., 2008). To determine whether (de)acetylation of Pck1p controls its induction, we compared endogenous protein levels of chromosomally tagged Pck1p in *esa1-531*, *sir2*Δ, *gcn5*Δ (the catalytic subunit of the SAGA complex), and *hda1*Δ mutants with the WT strain. Except for *gcn5*Δ, none of these mutations significantly affected Pck1p abundance (Figure 3C). Consistent with this finding, neither *K514R* nor *K514Q* mutations significantly affected Pck1p abundance (Figure 3C). We checked *PCK1* mRNA levels in HAT and HDAC deletion mutants during a diauxic shift, and observed that *PCK1* mRNA increased ~8-fold in *esa1-531* grown at nonpermissive temperature, and decreased slightly in *sir2*Δ (Figure 3D). Interestingly, *PCK1* mRNA increased 3-fold in *hda1Δ*, and decreased significantly in *gcn5* (Figure 3D), suggesting that Hda1p and Gcn5p repress and activate *PCK1* transcription via chromatin modification, respectively, on non-fermentable carbon sources (NFCSs). These results confirm previous functional genomics studies of yeast HDACs, in which Hda1p deacetylates (and probably suppresses) Hda1-affected subtelomeric domains in subtelomeric chromatin containing gluconeogenic genes (Robyr et al., 2002), and clarifies the previously unexplained finding that yeast SAGA is essential for growth on ethanol (van Voorst et al., 2006).

In addition, we observed that C-terminally GFP-tagged Pck1p localized to both nucleus and cytoplasm, independent of (de)acetylation status (Figure S6). Because Pck1p functions as a homotetramer *in vivo* (Ravanal et al., 2003), we also examined Pck1p oligomerization status by size exclusion chromatography; its oligomerization was normal in *esa1-531* and *sir2*Δ (Figure S7).

Acetylation of Pck1p controls enzymatic activity

To determine whether (de)acetylation of Pck1p was physiologically relevant, we monitored viability of various mutants on NFCSs. Gluconeogenesis is essential for yeast growth on

NFCSs, such as ethanol and glycerol, allowing replenishment of sugar phosphate stores important for biomass generation (Schuller, 2003). Deletion of *PCK1* is lethal when ethanol is the only available carbon source (Figure 4A). Adding glycerol and ethanol (GE) bypasses the need of Pck1p in the early stages of the pathway and rescues *pck1*Δ lethality (Figure 4A). Similar phenotypes were observed for *esa1-531* grown at a semi-permissive temperature but not at permissive temperature (Figure S8), and for the *pck1-K514R* mutant grown on either ethanol or GE, suggesting acetylation at K514 of Pck1p is crucial for function (Figure 4A). There was no additive effect on the growth defect in the *esa1-531/pck1-514R* double mutant on NFCSs (Figure S9). Consistent with this result, substitution of K514 with glutamine (*K514Q*) almost completely rescued the *esa1-531* growth defect on ethanol or GE (Figure 4A). Moreover, deletion of *SIR2* did not rescue *pck1-K514R* growth defects on ethanol or GE, suggesting that K514 is the only Pck1p lysine residue targeted by Sir2p (Figure S10). Overexpression of *SIR2* by introducing an extra copy of *SIR2* on a centromeric plasmid (*2X SIR2* in Figure S11A) does not significantly affect growth on glucose, ethanol or GE (Figure S11B). Although both *GCN5* and *HDA1* regulate *PCK1* at the mRNA level, only deletion of *GCN5* showed similar growth defects, consistent with its effect on Pck1p abundance (Figure 3C, 3E and 4B). Interestingly, both *pck1-K514Q* and *sir2*Δ enhanced growth on high concentrations of ethanol (Figure 4C), indicating the contribution of gluconeogenesis to ethanol tolerance in yeast.

These results prompted us to investigate whether acetylation directly affects Pck1p enzymatic activity. An enzyme assay (Figure 4D) was used to assess the ability of Pck1p to convert PEP and ADP to OAA and ATP, coupled with the conversion of OAA to malate by malate dehydrogenase; in this standard assay for Pck1p activity the latter activity is detected spectrophotometrically via NADH consumption (Baly et al., 1985). We observed that Pck1p purified from *esa1-531* was defective in the reaction under a wide range of ADP concentrations (Figure 4E and 4F). While *pck1-K514R* diminished Pck1p enzymatic activity (Figure 4E; the curves of *pck1-K514R* and *esa1-531* overlapped), *pck1-K514Q* did not cause significant changes in activity (Figure S12), but partially restored the impaired enzymatic activity of Pck1p purified from *esa1-531* grown at the non-permissive temperature (Figure 4E), indicating that acetylation at K514 of Pck1p is important in maintaining maximal activity. Consistent with the insignificant contribution of K19 to the overall Pck1p acetylation status, *pck1-K19R* minimally affected activity (Figure S13). Finally, the K_m and V_{max} of Pck1p purified from *esa1-531* differ substantially from those from WT cells (Figure 4F).

Loss of *PCK1* activity blocks chronological life span extension in water starvation

The enzyme-substrate relationship between Sir2p and Pck1p and Pck1p's ability to convert ethanol to glucose in postdiauxic cells suggest a possible connection between gluconeogenesis and longevity. To test this hypothesis, we investigated whether Pck1p is involved in CLS extension caused by water starvation. The CLS of various mutants in the BY4742 background was assessed by holding saturated cultures in SC medium containing 2% glucose, a standard culture condition to propagate yeast cells in the laboratory, and in water, a severe form of starvation, causing entry into a low metabolic state and a dramatic extension of CLS (Fabrizio et al., 2005; Fabrizio and Longo, 2003). In agreement with a previous study (Fabrizio et al., 2005), the chronological life span of a *sir2*Δ mutant was extended significantly compared to WT in water but not in SC medium containing glucose (Figure 5A). By contrast, knockout of *PCK1* dramatically reduced CLS in both water and glucose containing medium (Figure 5A). K514 acetylation and hence enzymatic activity of Pck1p was involved since the *pck1-K514R* mutation, like *pck1*Δ, shortened CLS both in water and glucose-containing medium; whereas the *pck1-K514Q* mutation, just like *sir2*Δ, extends CLS in water (Figure 5A). Moreover, deletion of *SIR2* did not alter the life span of *pck1*Δ, *pck1-K514R* and *pck1-K514Q* mutants, indicating *PCK1* was epistatic to *SIR2* in this pathway and implicating it as the key Sir2p

substrate controlling this form of longevity (Figure 5B). The *esa1-531* mutant has an even shorter CLS than *pck1*Δ in both 2% glucose and water at 30° C, a semi-permissive temperature; at the permissive temperature there is little difference (25° C, Figure S14). This finding suggests that additional NuA4 substrates besides Pck1p control yeast CLS. In contrast, Pck1p acetylation status had minimal effect on replicative life span of yeast cells grown in YPD (Figure S15), thus Pck1p is not involved in the replicative pathway.

On the basis of previous findings that uptake of extracellular ethanol mediates extension of CLS, and that extracellular ethanol is depleted faster in long-lived mutants such as *sir2*Δ (Fabrizio et al., 2005), we measured the ethanol concentration in the CLS conditioned medium. Interestingly, extracellular ethanol concentration was inversely proportional to CLS. Ethanol concentration remained high up to day 7 in short-lived mutants including *esa1-531* at semi-permissive temperature (30° C), *pck1*Δ and *pck1-K514R*; whereas in long-lived mutants including *sir2*Δ and *pck1-K514Q* extracellular ethanol was depleted as early as day 3 (Figure 5C). Similar to the effect on CLS, *PCK1* was epistatic to *SIR2* in the mechanism controlling consumption of extracellular ethanol, since deletion of *SIR2* resulted in only minimal changes of ethanol concentration in *pck1*Δ, *pck1-K514R* and *pck1-K514Q* mutants (Figure 5C).

We further examined the resistance of these mutants to oxidative stress resultingfrom exposure to high level of H_2O_2, generating reactive oxygen species (Lin et al., 2002; Scherz-Shouval and Elazar, 2007). Consistent with a previous study (Fabrizio et al., 2005), a *sir2*Δ mutant in stationary phase (day 3 cells) relative to the exponential growth phase (day 1 cells) was more resistant to H_2O_2 stress (Figure S16). Although *pck1*Δ was less resistant overall, the sensitivity of *pck1-K514R* and *pck1-K514Q* to oxidative stress was similar to the WT (Figure S16) regardless of the growth phase, suggesting that (de)acetylation status and enzymatic activity of Pck1p play minimal role in this function.

Acetylation of human Pck1 and glucose secretion depends on *TIP60* activity

Two isozymes of PEPCK exist in mammalian cells: cytosolic (*PCK1*) and mitochondrial (*PCK2*) (Hanson and Reshef, 1997). The mammalian Pck1 (PEPCK-C) catalyzes the first committed step of gluconeogenesis in liver and kidney (Rognstad, 1979), and induces glyceroneogenesis in liver and adipose tissue (Reshef et al., 2003); the physiologic role of Pck2 (PEPCK-M) has not been well established (Hanson and Reshef, 1997). Both mammalian isozymes show low sequence identity with yeast Pck1p (Figure S17), and utilize GTP instead of ATP (Utter and Kolenbrander, 1972). However, both ATP- and GTP-dependent enzymes share similar active site sequence consensus motifs, suggesting conserved catalytic mechanism (Matte et al., 1997).

To examine this hypothesis, plasmid-borne full-length coding regions of both human PEPCK genes were introduced into the yeast *pck1*Δ mutant, and growth of transformants on NFCSs was examined. As illustrated in Figure 6A, only human *PCK1* but not *PCK2* could partially complement the function of yeast *PCK1*, suggesting that human Pck1 shares gluconeogenic mechanism with yeast Pck1p. To further investigate the role of acetylation in controlling human Pck1 enzymatic activity, we used short hairpin RNAs (shRNAs) to efficiently knockdown *TIP60* and *SIRT1*, the human homologs of yeast *ESA1* and *SIR2*, respectively, in human hepatocellular carcinoma (HepG2) cells (Figure S18A–B). Transcription of human *PCK1* depends on *SIRT1* in primary hepatocytes (Rodgers et al., 2005). Consistent with this, knockdown of *SIRT1* reduced the endogenous human Pck1 level (~50%) in HepG2 cells (Figure 6B). By IP, we found that human Pck1 was acetylated in HepG2 cells (Figure 6C), but not in human embryonic kidney 293T cells (data not shown). Whereas knockdown of *TIP60* did not affect the endogenous human Pck1 level (Figure 6B), it dramatically diminished the acetylation fraction of human Pck1 compared with GFP knockdown control (Figure 6C). However, knockdown of *SIRT1* showed no detectable effect on human Pck1 acetylation (Figure

6C) suggesting a distinct deacetylation mechanism in mammalian cells. Treatment with HDAC inhibitors such as trichostatin A (TSA, a class I/II HDAC inhibitor) or nicotinamide (NAM, a class III HDAC inhibitor) (Neugebauer et al., 2008) increased the acetylation fraction of human Pck1 in HepG2 cells (Figure 6D). Consistent with the hypothesis that acetylation controls enzymatic activity of human Pck1 *in vivo*, the amount of glucose secreted into glucose-free medium significantly declined (~40%) in *TIP60* knockdown HepG2 cells, but knockdown of *SIRT1* had no significant effect (Figure 6E).

DISCUSSION

We report a proteome-wide search for nonhistone substrates of the essential yeast NuA4 complex. We have validated 13 *in vivo* substrates among 20 candidates, 7 of which are cytoplasmic proteins involved in metabolic pathways responding to extracellular nutrient sources and intracellular energy status. It is noteworthy that several functions (*e.g.*, gluconeogenesis and autophagy) of NuA4 metabolic protein substrates are induced by limiting extracellular nutrient sources (Schuller, 2003; Takeshige et al., 1992; Zaman et al., 2008). Considering that acetylation of Pck1p by NuA4 is important for its gluconeogenic activity, it is reasonable to speculate that NuA4 might act as a central hub to help yeast cells meet the challenge of nutrient deprivation by acetylating these nonhistone substrates, and activating a metabolic network.

Several functions are important in the extension of yeast life span under calorie restriction or starvation, including stress response (Fabrizio et al., 2005), autophagy (Powers et al., 2006), decreased ribosome biogenesis and protein translation (Kaeberlein et al., 2005), respiration (Lin et al., 2002), and changes in carbon metabolism (Ashrafi et al., 2000). The target of rapamycin (TOR) signaling pathway is regarded as a central negative regulator of these functions (Kaeberlein et al., 2005; Powers et al., 2006) to block extension of life span. Other genes such as *SIR2* might also be important because of additive effects on life span extension when both the TOR pathway and *SIR2* function are disrupted compared to either single mutation (Fabrizio et al., 2005). Our finding that acetylation control of Pck1p gluconeogenic activity to convert ethanol to glucose is crucial for longevity extension provides evidence that NuA4 might be a central positive regulator that opposes TOR in CLS extension under water starvation. NuA4 might further modulate TOR function by acetylating Tap42p, a downstream effector in TOR pathway (Duvel et al., 2003) uncovered as a substrate in this study. It also provides a new insight into how *SIR2* plays an antagonistic role in extending CLS. Considering that transcription of *PCK1* is repressed by even trace amounts of glucose (as low as 0.001% in the medium) (Yin et al., 2000), the lack of effect on RLS by deleting *PCK1* or altering the acetylation status of its encoding protein is unsurprising (Figure S15). However, NuA4 might regulate RLS through acetylating Nnt1p, the yeast putative nicotinamide N-methyltransferase with a role in rDNA silencing and life span determination (Anderson et al., 2003). Interestingly, mice that overexpress *PCK1* in skeletal muscle live longer and are more energetic at an older age (Hakimi et al., 2007), suggesting a potential role of *PCK1* in regulating longevity in mammals.

Gluconeogenesis is extensively and elaborately regulated to maintain blood glucose level within a narrow range during fasting to ensure a steady and continuous glucose supply in mammals (Burgess et al., 2007). As a crucial enzyme in gluconeogenesis, mammalian *PCK1* transcription is tightly controlled by various transcription factors in response to varied physiological stimuli, including dietary carbohydrate content, nutritional status and various hormones (Beale et al., 2004; Hanson and Reshef, 1997). Fasting also induces *PCK1* expression in liver and adipose tissue (Hagopian et al., 2003; Hagopian et al., 2008; Raab et al., 2005). Failure of this exquisite transcriptional control mechanism causes dysregulated overexpression of *PCK1* in liver and inappropriately high levels of hepatic gluconeogenesis in all forms of

diabetes (Burgess et al., 2007; Permutt and Hattersley, 2000). Previous studies have focused mainly on transcriptional regulation of *PCK1*. However, our result suggests that HATs and HDACs not only regulate *PCK1* transcription (by Gcn5p and Hda1p) in yeast, but also regulate Pck1p activity post-translationally both in yeast and humans (Figure 7). Whereas acetyl-CoA synthetase activates acetate produced by non-fermentable sugar metabolism to acetyl-CoA during diauxic shift in yeast (Haurie et al., 2001; van den Berg et al., 1996), fasting activates expression of *SIRT1* in mammals, which in turn promotes the production of acetyl-CoA by deacetylating and activating AceCS1 (Schwer and Verdin, 2008). The elevated intracellular acetyl-CoA concentration favors the transcription of *PCK1* and the stimulatory acetylation of Pck1p. Our results suggest that acetylation of Pck1p increases its enzymatic activity through mechanisms other than regulating macromolecular abundance, subcellular localization or quaternary structure of the protein. Based on the crystal structure of the *Escherichia coli* ortholog (Delbaere et al., 2004), which shares high level of amino acid sequence homology with yeast Pck1p (Figure S19), acetylation at K514 might cause a conformational change of the C-terminal mononucleotide-binding domain in favor of substrate binding or catalysis of the phosphoryl-transfer reaction. Despite sharing minimal primary structural identity with yeast Pck1p (Ravanal et al., 2003) (Figure S17), we found that human Pck1 is also activated by *TIP60*-dependent acetylation. Unlike yeast Pck1p, which is deacetylated exclusively by Sir2p, human Pck1p might be deacetylated by multiple deacetylases. Upregulation of Pck1p transcription and activity, and allosteric modulation of the activity of pyruvate carboxylase (another key enzyme of the gluconeogenesis pathway) by acetyl-CoA (Pronk et al., 1996) might together provide a “triple-switch” to ensure a prompt adaptation of the gluconeogenic flux to energy status in yeast and possibly human cells (Figure 7).

In summary, we have found novel extracellular functions of yeast NuA4 complex in regulating gluconeogenesis and chronological life span. PEPCK gluconeogenic activity, etiologically important in human diabetes, is acetylated in both yeast and humans. Characterization of the structural basis of this acetylation control mechanism, as well as identification of the HDACs responsible for reversing the acetylation of human Pck1 in human liver will be crucial for further clarifying how blood glucose level is maintained within a remarkably narrow range. Besides Pck1p, understanding how other metabolic protein substrates are regulated by NuA4 in response to starvation and their possible roles in extension of chronological life span will also be an important next step. Lastly a similar approach using a human proteome microarray will help identify the HATs/HDACs responsible for the increasing number of acetylated human proteins.

EXPERIMENTAL PROCEDURES

Acetylation reactions on yeast proteome microarrays

A 200 μL reaction mixture containing 50 mM sodium butyrate, 5 mM DTT and 5 mM PMSF, 0.025 μCi [^{14}C]-Acetyl-CoA (GE Healthcare, 0.05 mCi/mL, 8 mCi/mmol), with or without 4 μg of NuA4, was prepared in reaction buffer (250 mM Tris-HCl, pH7.5, 25% glycerol, 0.5 mM EDTA, and 250 mM KCl). A yeast proteome microarray (Zhu et al., 2001) was incubated with the reaction mixture at 30°C for 1 h, and then subjected to three 15 min washes with 50 mM $NaHCO_3$-Na_2CO_3 (3:1) (pH9.3). The acetylation signals were then visualized by autoradiography after exposure to X-ray film for 20 d, scanned and saved as TIFF files. The TIFF images were processed by assigning an artificial green color to each brightened spot and analyzed using GenePix software to determine relative acetylation levels. After subtraction of backgrounds, protein spots with median signal intensity greater than an arbitrary cutoff value of 3,000 (determined based on visual inspection) were chosen as candidate substrates of NuA4 for further validation studies.

In vivo acetylation

GST-tagged Pck1p driven by the *GAL1* promoter was purified from WT, *esa1-531*, *pck1-K514R* and the *esa1-531pck1-K514R* double mutant after protein induction at 37°C (non-permissive temperature) for 2 h, or from WT, *hda1Δ*, *rpd3Δ*, and *sir2Δ* strains after protein induction at 30°C for 4 h, immunoprecipitated by incubating with 30 μL protein A-sepharose beads (GE Healthcare) conjugated with 6 μg of mouse monoclonal anti-acetylated-lysine (Cell Signaling, 9681S) in the presence of a cocktail of HDAC inhibitors (100 μM Trichostatin A, 50 mM nicotinamide, and 50 mM sodium butyrate) at 4°C for 3 h. To detect acetylation of endogenous C-terminally Myc-tagged Pck1p from WT, *esa1-531*, *pck1-K514R* and *sir2Δ* mutants, whole cell extracts were precleared by incubating with 30 μL protein A-sepharose beads at 4°C for 90 min, followed by IP as described above. After extensive washes with 500 μL of washing buffer (50 mM HEPES, pH 7.0, and 100 mM NaCl) at 4°C for four times (10 min each time), the immunoprecipitate was denatured in 20 μL of 2xSDS sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with rabbit polyclonal anti-GST antibody (Chemicon, AB3282) or mouse monoclonal anti-Myc antibody (Santa Cruz, SC-40) for purified or endogenous Pck1p, respectively. 150 μg of acetylated BSA (Ambion, AM26141) was used for competition of binding, and an equal amount of unacetylated BSA (New England BioLabs, B9001S) was used in parallel samples as control.

Pck1p enzymatic activity assay

The activity of purified Pck1p was assayed as described previously (Baly et al., 1985). In brief, enzymatic activity of Pck1p was assayed using a coupled system in which the oxaloacetate produced in the reaction was reduced to malate by NADH in the presence of malate dehydrogenase. The NADH consumption rate was measured spectrophotometrically at 340 nm. Equal amount (50 μg) of purified GST-tagged enzyme was added to a 1 mL reaction mixture (10 mM potassium phosphoenolpyruvate, 10 mM $NaHCO_3$, 2 mM of $MnCl_2$, 1 mM ADP, 12 mM sodium aspartate, 80 mM Tris-HCl, pH 8.8, 14 units malate dehydrogenase, and 0.15 mM NADH) for each reaction. Samples without NADH served as a blank control, and samples without Pck1p as a negative control.

Chronological life span and ethanol measurement

Chronological life span of yeast cells grown in either synthetic complete medium (with a 4-fold excess of the supplements histidine, leucine, tryptophan and uracil) containing 2% glucose, 2% glycerol and ethanol, or water was measured as described (Fabrizio and Longo, 2007). In either culture condition, colony-forming units (CFUs) were measured every 48 h. The number of CFUs on day 3 was considered the initial survival (100%). The ethanol concentration in medium collected from day 1, 3, 5 and 7 yeast cultures was analyzed with a colorimetric ethanol assay kit (BioAssay Systems, DIET-500).

Mammalian cell culture and treatment

HepG2 cells were cultured in minimal essential medium (MEM, GIBCO) containing 10% fetal bovine serum (GIBCO), 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The shRNA (obtained from The RNAi Consortium human lentiviral shRNA library) targeting GFP, *TIP60* (TRCN0000020314, TRCN0000020315 and TRCN0000020316) or *SIRT1* (TRCN0000018979, TRCN0000018980 and TRCN0000018981) were used for transfection of the packaging HEK 293T cells with helper vectors using Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Medium containing lentiviral particles and 8 μg/mL polybrene (Sigma-Aldrich) was used to infect HepG2 cells. Infected HepG2 cells were then selected in MEM in the presence of 1.25 μg/mL puromycin (Sigma-Aldrich). Knockdown efficiency of *TIP60* and *SIRT1* was assayed by immunoblotting and RT-PCR. When treated

with histone deacetylase inhibitors (HDACi), TSA (100 μM; Sigma-Aldrich) or NAM (5 mM; Sigma-Aldrich) was added in the culture medium 6 hours prior to harvest cells for preparation of whole-cell extracts.

Glucose production

The amount of glucose secreted by HepG2 cells in media was assayed as described previously (Yoon et al., 2001). HepG2 cells expressing shRNAs of GFP control, *TIP60* or *SIRT1* were cultured in six-well plates (1.4×10^6 cells per well) in MEM with 10% FBS. The medium was replaced with 2 mL of glucose production buffer consisting of glucose-free MEM (pH 7.4), without phenol red, supplemented with 2 mM sodium pyruvate and 20 mM sodium lactate. After a 3 h incubation, half of the glucose production buffer was collected and the glucose concentration was measured with a colorimetric glucose assay kit (Sigma-Aldrich, GAGO20). The readings were normalized to the total protein content determined from the whole cell extracts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Allard S, Utley RT, Savard J, Clarke A, Grant P, Brandl CJ, Pillus L, Workman JL, Cote J. NuA4, an essential transcription adaptor/histone H4 acetyltransferase complex containing Esa1p and the ATM-related cofactor Tra1p. Embo J 1999;18:5108–5119. [PubMed: 10487762]

Anderson RM, Bitterman KJ, Wood JG, Medvedik O, Sinclair DA. Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae. Nature 2003;423:181–185. [PubMed: 12736687]

Ashrafi K, Lin SS, Manchester JK, Gordon JI. Sip2p and its partner snf1p kinase affect aging in S. cerevisiae. Genes Dev 2000;14:1872–1885. [PubMed: 10921902]

Auger A, Galarneau L, Altaf M, Nourani A, Doyon Y, Utley RT, Cronier D, Allard S, Cote J. Eaf1 is the platform for NuA4 molecular assembly that evolutionarily links chromatin acetylation to ATP-dependent exchange of histone H2A variants. Mol Cell Biol 2008;28:2257–2270. [PubMed: 18212047]

Babiarz JE, Halley JE, Rine J. Telomeric heterochromatin boundaries require NuA4-dependent acetylation of histone variant H2A.Z in Saccharomyces cerevisiae. Genes Dev 2006;20:700–710. [PubMed: 16543222]

Baly DL, Keen CL, Hurley LS. Pyruvate carboxylase and phosphoenolpyruvate carboxykinase activity in developing rats: effect of manganese deficiency. J Nutr 1985;115:872–879. [PubMed: 4009297]

Beale EG, Hammer RE, Antoine B, Forest C. Disregulated glyceroneogenesis: PCK1 as a candidate diabetes and obesity gene. Trends Endocrinol Metab 2004;15:129–135. [PubMed: 15046742]

Ben-Shahar TR, Heeger S, Lehane C, East P, Flynn H, Skehel M, Uhlmann F. Eco1-dependent cohesin acetylation during establishment of sister chromatid cohesion. Science 2008;321:563–566. [PubMed: 18653893]

Boudreault AA, Cronier D, Selleck W, Lacoste N, Utley RT, Allard S, Savard J, Lane WS, Tan S, Cote J. Yeast enhancer of polycomb defines global Esa1-dependent acetylation of chromatin. Genes Dev 2003;17:1415–1428. [PubMed: 12782659]

Burgess SC, He T, Yan Z, Lindner J, Sherry AD, Malloy CR, Browning JD, Magnuson MA. Cytosolic phosphoenolpyruvate carboxykinase does not solely control the rate of hepatic gluconeogenesis in the intact mouse liver. Cell Metab 2007;5:313–320. [PubMed: 17403375]

Choi JK, Grimes DE, Rowe KM, Howe LJ. Acetylation of Rsc4p by Gcn5p is essential in the absence of histone H3 acetylation. Mol Cell Biol. 2008

Clarke AS, Lowell JE, Jacobson SJ, Pillus L. Esa1p is an essential histone acetyltransferase required for cell cycle progression. Mol Cell Biol 1999;19:2515–2526. [PubMed: 10082517]

Delbaere LT, Sudom AM, Prasad L, Leduc Y, Goldie H. Structure/function studies of phosphoryl transfer by phosphoenolpyruvate carboxykinase. Biochim Biophys Acta 2004;1697:271–278. [PubMed: 15023367]

Duvel K, Santhanam A, Garrett S, Schneper L, Broach JR. Multiple roles of Tap42 in mediating rapamycin-induced transcriptional changes in yeast. Mol Cell 2003;11:1467–1478. [PubMed: 12820961]

Fabrizio P, Gattazzo C, Battistella L, Wei M, Cheng C, McGrew K, Longo VD. Sir2 blocks extreme life-span extension. Cell 2005;123:655–667. [PubMed: 16286010]

Fabrizio P, Longo VD. The chronological life span of Saccharomyces cerevisiae. Aging Cell 2003;2:73–81. [PubMed: 12882320]

Fabrizio P, Longo VD. The chronological life span of Saccharomyces cerevisiae. Methods Mol Biol 2007;371:89–95. [PubMed: 17634576]

Guarente L, Picard F. Calorie restriction--the SIR2 connection. Cell 2005;120:473–482. [PubMed: 15734680]

Hagopian K, Ramsey JJ, Weindruch R. Caloric restriction increases gluconeogenic and transaminase enzyme activities in mouse liver. Exp Gerontol 2003;38:267–278. [PubMed: 12581790]

Hagopian K, Ramsey JJ, Weindruch R. Enzymes of glycerol and glyceraldehyde metabolism in mouse liver: effects of caloric restriction and age on activities. Biosci Rep 2008;28:107–115. [PubMed: 18429748]

Hakimi P, Yang J, Casadesus G, Massillon D, Tolentino-Silva F, Nye CK, Cabrera ME, Hagen DR, Utter CB, Baghdy Y, et al. Overexpression of the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) in skeletal muscle repatterns energy metabolism in the mouse. J Biol Chem 2007;282:32844–32855. [PubMed: 17716967]

Hanson RW, Reshef L. Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annu Rev Biochem 1997;66:581–611. [PubMed: 9242918]

Haurie V, Perrot M, Mini T, Jeno P, Sagliocco F, Boucherie H. The transcriptional activator Cat8p provides a major contribution to the reprogramming of carbon metabolism during the diauxic shift in Saccharomyces cerevisiae. J Biol Chem 2001;276:76–85. [PubMed: 11024040]

Huisinga KL, Pugh BF. A genome-wide housekeeping role for TFIID and a highly regulated stress-related role for SAGA in Saccharomyces cerevisiae. Mol Cell 2004;13:573–585. [PubMed: 14992726]

Ikura T, Ogryzko VV, Grigoriev M, Groisman R, Wang J, Horikoshi M, Scully R, Qin J, Nakatani Y. Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell 2000;102:463–473. [PubMed: 10966108]

Kaeberlein M, Powers RW 3rd, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S, Kennedy BK. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science 2005;310:1193–1196. [PubMed: 16293764]

Keogh MC, Mennella TA, Sawa C, Berthelet S, Krogan NJ, Wolek A, Podolny V, Carpenter LR, Greenblatt JF, Baetz K, Buratowski S. The Saccharomyces cerevisiae histone H2A variant Htz1 is acetylated by NuA4. Genes Dev 2006;20:660–665. [PubMed: 16543219]

Kim SC, Sprung R, Chen Y, Xu Y, Ball H, Pei J, Cheng T, Kho Y, Xiao H, Xiao L, et al. Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. Mol Cell 2006;23:607–618. [PubMed: 16916647]

Kouzarides T. Chromatin modifications and their function. Cell 2007;128:693–705. [PubMed: 17320507]

Lee KK, Workman JL. Histone acetyltransferase complexes: one size doesn't fit all. Nat Rev Mol Cell Biol 2007;8:284–295. [PubMed: 17380162]

Li Y, Yokota T, Gama V, Yoshida T, Gomez JA, Ishikawa K, Sasaguri H, Cohen HY, Sinclair DA, Mizusawa H, Matsuyama S. Bax-inhibiting peptide protects cells from polyglutamine toxicity caused by Ku70 acetylation. Cell Death Differ 2007;14:2058–2067. [PubMed: 17885668]

Lin SJ, Defossez PA, Guarente L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. Science 2000;289:2126–2128. [PubMed: 11000115]

Lin SJ, Kaeberlein M, Andalis AA, Sturtz LA, Defossez PA, Culotta VC, Fink GR, Guarente L. Calorie restriction extends Saccharomyces cerevisiae lifespan by increasing respiration. Nature 2002;418:344–348. [PubMed: 12124627]

Lin YY, Qi Y, Lu JY, Pan X, Yuan DS, Zhao Y, Bader JS, Boeke JD. A comprehensive synthetic genetic interaction network governing yeast histone acetylation and deacetylation. Genes Dev 2008;22:2062–2074. [PubMed: 18676811]

Lu JY, Lin YY, Qian J, Tao SC, Zhu J, Pickart C, Zhu H. Functional dissection of a HECT ubiquitin E3 ligase. Mol Cell Proteomics 2008;7:35–45. [PubMed: 17951556]

Matte A, Tari LW, Goldie H, Delbaere LT. Structure and mechanism of phosphoenolpyruvate carboxykinase. J Biol Chem 1997;272:8105–8108. [PubMed: 9139042]

Millar CB, Grunstein M. Genome-wide patterns of histone modifications in yeast. Nat Rev Mol Cell Biol 2006;7:657–666. [PubMed: 16912715]

Millar CB, Xu F, Zhang K, Grunstein M. Acetylation of H2AZ Lys 14 is associated with genome-wide gene activity in yeast. Genes Dev 2006;20:711–722. [PubMed: 16543223]

Mitchell L, Lambert JP, Gerdes M, Al-Madhoun AS, Skerjanc IS, Figeys D, Baetz K. Functional dissection of the NuA4 histone acetyltransferase reveals its role as a genetic hub and that Eaf1 is essential for complex integrity. Mol Cell Biol 2008;28:2244–2256. [PubMed: 18212056]

Neugebauer RC, Sippl W, Jung M. Inhibitors of NAD+ dependent histone deacetylases (sirtuins). Curr Pharm Des 2008;14:562–573. [PubMed: 18336301]

Permutt MA, Hattersley AT. Searching for type 2 diabetes genes in the post-genome era. Trends Endocrinol Metab 2000;11:383–393. [PubMed: 11042470]

Powers RW 3rd, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S. Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes Dev 2006;20:174–184. [PubMed: 16418483]

Pronk JT, Yde Steensma H, Van Dijken JP. Pyruvate metabolism in Saccharomyces cerevisiae. Yeast 1996;12:1607–1633. [PubMed: 9123965]

Raab RM, Bullen J, Kelleher J, Mantzoros C, Stephanopoulos G. Regulation of mouse hepatic genes in response to diet induced obesity, insulin resistance and fasting induced weight reduction. Nutr Metab (Lond) 2005;2:15. [PubMed: 15985155]

Ravanal MC, Goldie H, Cardemil E. Thermal stability of phosphoenolpyruvate carboxykinases from Escherichia coli, Trypanosoma brucei, and Saccharomyces cerevisiae. J Protein Chem 2003;22:311–315. [PubMed: 13678294]

Reshef L, Olswang Y, Cassuto H, Blum B, Croniger CM, Kalhan SC, Tilghman SM, Hanson RW. Glyceroneogenesis and the triglyceride/fatty acid cycle. J Biol Chem 2003;278:30413–30416. [PubMed: 12788931]

Robyr D, Suka Y, Xenarios I, Kurdistani SK, Wang A, Suka N, Grunstein M. Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. Cell 2002;109:437–446. [PubMed: 12086601]

Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature 2005;434:113–118. [PubMed: 15744310]

Rognstad R. Rate-limiting steps in metabolic pathways. J Biol Chem 1979;254:1875–1878. [PubMed: 422559]

Rusche LN, Kirchmaier AL, Rine J. The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu Rev Biochem 2003;72:481–516. [PubMed: 12676793]

Scherz-Shouval R, Elazar Z. ROS, mitochondria and the regulation of autophagy. Trends Cell Biol 2007;17:422–427. [PubMed: 17804237]

Schuller HJ. Transcriptional control of nonfermentative metabolism in the yeast Saccharomyces cerevisiae. Curr Genet 2003;43:139–160. [PubMed: 12715202]

Schwer B, Verdin E. Conserved metabolic regulatory functions of sirtuins. Cell Metab 2008;7:104–112. [PubMed: 18249170]

Shahbazian MD, Grunstein M. Functions of site-specific histone acetylation and deacetylation. Annu Rev Biochem 2007;76:75–100. [PubMed: 17362198]

Smith ER, Eisen A, Gu W, Sattah M, Pannuti A, Zhou J, Cook RG, Lucchesi JC, Allis CD. ESA1 is a histone acetyltransferase that is essential for growth in yeast. Proc Natl Acad Sci U S A 1998;95:3561–3565. [PubMed: 9520405]

Sterner DE, Berger SL. Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev 2000;64:435–459. [PubMed: 10839822]

Takahashi H, McCaffery JM, Irizarry RA, Boeke JD. Nucleocytosolic acetyl-coenzyme a synthetase is required for histone acetylation and global transcription. Mol Cell 2006;23:207–217. [PubMed: 16857587]

Takeshige K, Baba M, Tsuboi S, Noda T, Ohsumi Y. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. J Cell Biol 1992;119:301–311. [PubMed: 1400575]

Tao SC, Chen CS, Zhu H. Applications of protein microarray technology. Comb Chem High Throughput Screen 2007;10:706–718. [PubMed: 18045082]

Unal E, Heidinger-Pauli JM, Kim W, Guacci V, Onn I, Gygi SP, Koshland DE. A molecular determinant for the establishment of sister chromatid cohesion. Science 2008;321:566–569. [PubMed: 18653894]

van den Berg MA, de Jong-Gubbels P, Kortland CJ, van Dijken JP, Pronk JT, Steensma HY. The two acetyl-coenzyme A synthetases of Saccharomyces cerevisiae differ with respect to kinetic properties and transcriptional regulation. J Biol Chem 1996;271:28953–28959. [PubMed: 8910545]

van Voorst F, Houghton-Larsen J, Jonson L, Kielland-Brandt MC, Brandt A. Genome-wide identification of genes required for growth of Saccharomyces cerevisiae under ethanol stress. Yeast 2006;23:351–359. [PubMed: 16598687]

VanDemark AP, Kasten MM, Ferris E, Heroux A, Hill CP, Cairns BR. Autoregulation of the rsc4 tandem bromodomain by gcn5 acetylation. Mol Cell 2007;27:817–828. [PubMed: 17803945]

Vogelauer M, Wu J, Suka N, Grunstein M. Global histone acetylation and deacetylation in yeast. Nature 2000;408:495–498. [PubMed: 11100734]

Yang XJ, Seto E. Lysine acetylation: codified crosstalk with other posttranslational modifications. Mol Cell 2008a;31:449–461. [PubMed: 18722172]

Yang XJ, Seto E. The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. Nat Rev Mol Cell Biol 2008b;9:206–218. [PubMed: 18292778]

Yin Z, Hatton L, Brown AJ. Differential post-transcriptional regulation of yeast mRNAs in response to high and low glucose concentrations. Mol Microbiol 2000;35:553–565. [PubMed: 10672178]

Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford J, Kahn CR, Granner DK, et al. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature 2001;413:131–138. [PubMed: 11557972]

Zaman S, Lippman SI, Zhao X, Broach JR. How Saccharomyces Responds to Nutrients. Annu Rev Genet. 2008

Zhang H, Richardson DO, Roberts DN, Utley R, Erdjument-Bromage H, Tempst P, Cote J, Cairns BR. The Yaf9 component of the SWR1 and NuA4 complexes is required for proper gene expression, histone H4 acetylation, and Htz1 replacement near telomeres. Mol Cell Biol 2004;24:9424–9436. [PubMed: 15485911]

Zhang J, Shi X, Li Y, Kim BJ, Jia J, Huang Z, Yang T, Fu X, Jung SY, Wang Y, et al. Acetylation of Smc3 by Eco1 is required for S phase sister chromatid cohesion in both human and yeast. Mol Cell 2008;31:143–151. [PubMed: 18614053]

Zhu H, Bilgin M, Bangham R, Hall D, Casamayor A, Bertone P, Lan N, Jansen R, Bidlingmaier S, Houfek T, et al. Global analysis of protein activities using proteome chips. Science 2001;293:2101–2105. [PubMed: 11474067]

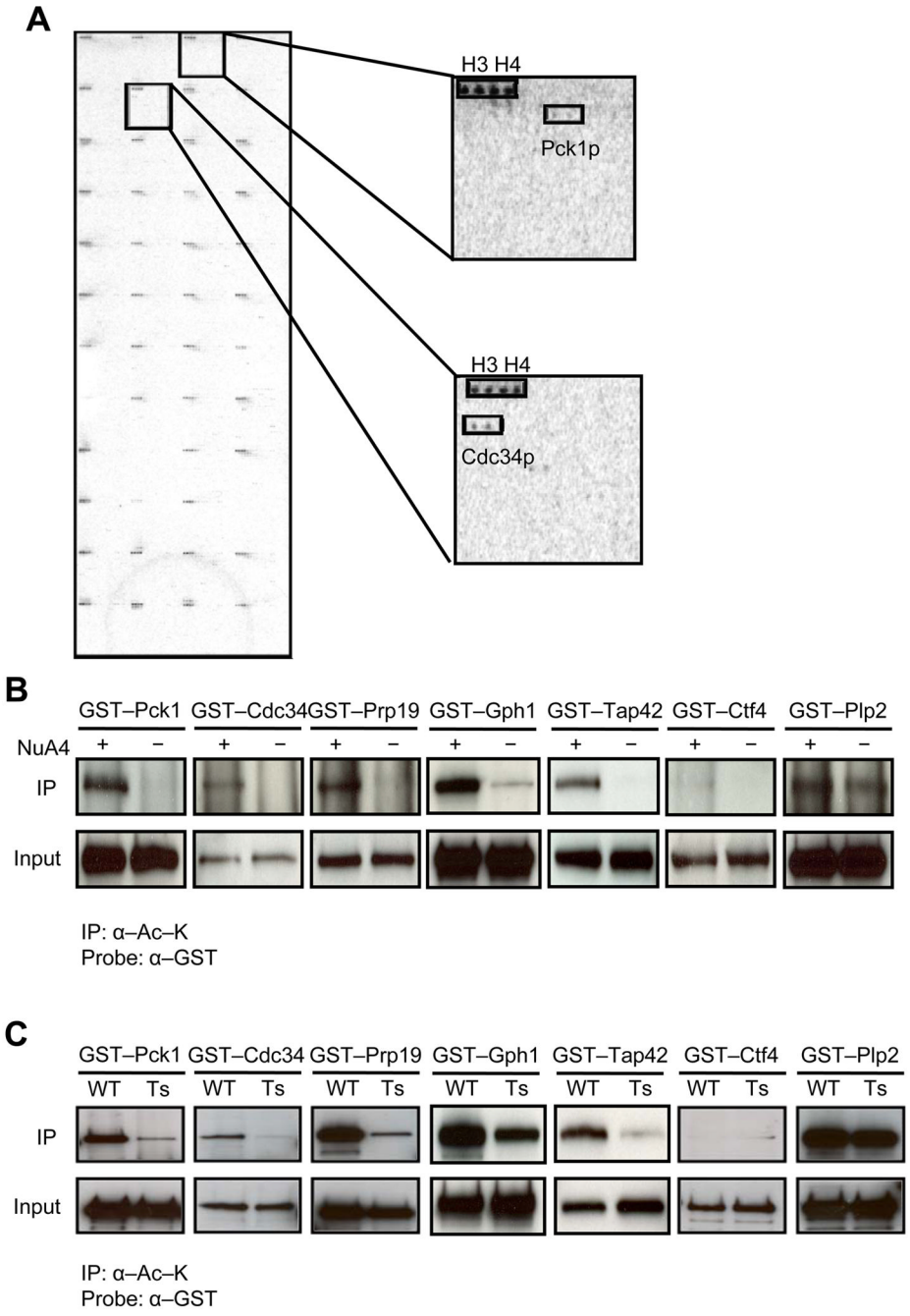

Figure 1. A protein acetylation microarray dentifies yeast Nua4 substrates

(A) Acetylation reactions on a yeast proteome microarray fabricated on the FAST surface. A total of 91 proteins were acetylated by the NuA4 complex. (B) *In vitro* validation of seven representative candidate substrates. GST fusion proteins were purified from the *esa1-531* mutant grown at non-permissive temperature and incubated with/without (+/−) the NuA4 complex *in vitro*. The reaction complex was immunoprecipitated with anti-pan-acetyl-lysine (α-Ac-K) followed by immunoblotting with anti-glutathione-S-transferase (α-GST). (C) Identification of five *in vivo* substrates (Pck1p, Cdc34p, Prp19p, Gph1p and Tap42p) of NuA4 complex. Overexpressed GST fusion proteins were purified from WT and *esa1-531* mutant

grown at non-permissive temperature, and immunoprecipitated with α-Ac-K followed by immunoblotting with α-GST.

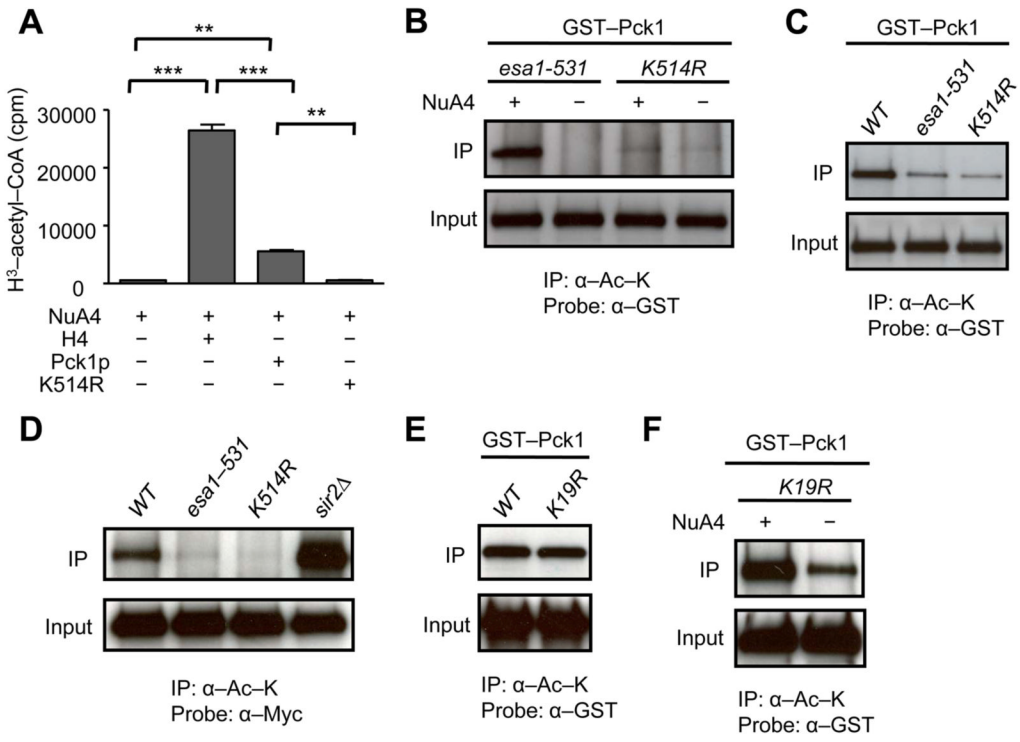

Figure 2. *In vitro* and *in vivo* detection of acetylated K514 of Pck1p

(A and B) Substitution of K514 with arginine (K514R) diminished *in vitro* acetylation of Pck1p by the NuA4 complex. A conventional HAT activity assay revealed that Pck1p, but not Pck1p-K514R (both purified from overexpression strains), was acetylated *in vitro* by the NuA4 complex. The results are presented in a bar graph with error bars indicating ± 1 s.e.m from three biological replicates. Double asterisk $P < 0.01$ and triple asterisk $P < 0.001$ (Student's *t*-test). (B) Prominent *in vitro* acetylation of purified Pck1p purified from the *esa1-531* mutant grown at non-permissive temperature, but not Pck1p-K514R, by the NuA4 complex. See Figure 1B legend for assay protocols. (C) Overexpressed Pck1p was acetylated at K514 *in vivo* by the NuA4 complex. See Figure 1C legend for assay protocols. (D) *In vivo* acetylation of endogenous Pck1p in WT, *esa1-531*, *pck1-K514R* and *sir2*Δ mutants was assessed using the protocols described in Figure 1C. (E) *In vivo* acetylation of overexpressed Pck1p in wild type and *pck1-K19R* mutant. Acetylation at K19 contributes minimally to the entire acetylation fraction of Pck1p. (F) The NuA4 complex efficiently acetylated purified Pck1p overexpressed in *pck1-K19R* mutant *in vitro*.

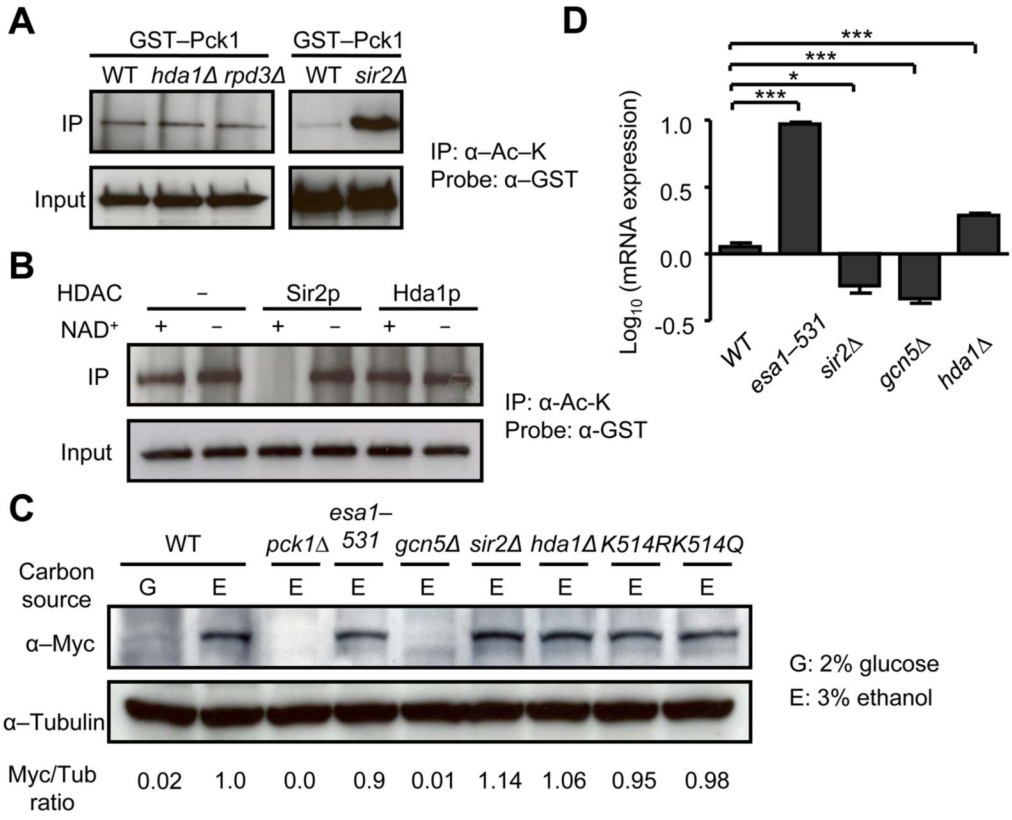

Figure 3. Sir2p deacetylates Pck1p K514

(A and B) Pck1p is deacetylated *in vivo* and *in vitro* by Sir2p. Overexpressed Pck1p was purified from WT, *hda1Δ*, *rpd3Δ*, or *sir2Δ* mutants and immunoprecipitated using anti-pan-acetyl-lysine monoclonal antibody as described in Figure 1E. Acetylated Pck1p increased significantly in *sir2Δ* strain when compared to WT, *hda1Δ*, and *rpd3Δ*. Purified Sir2-TAP, but not Hda1-TAP, efficiently deacetylated Pck1p *in vitro* in an NAD^+-dependent manner.

(C) Endogenous protein levels of Pck1p were detected by immunoblot analysis of the whole cell extracts derived from cells expressing C-terminally Myc-tagged Pck1p.

(D) Levels of *PCK1* mRNA were detected by RT-PCR in WT, *esa1-531*, *sir2Δ*, *gcn5Δ*, and *hda1Δ* strains. The results are presented in a bar graph with error bars indicating ± 1 s.e.m from three biological replicates. Single and triple asterisk indicates $P < 0.05$ and $P < 0.001$ (Student's *t*-test), respectively. Primers used in the RT-PCR reactions are provided in Table S3.

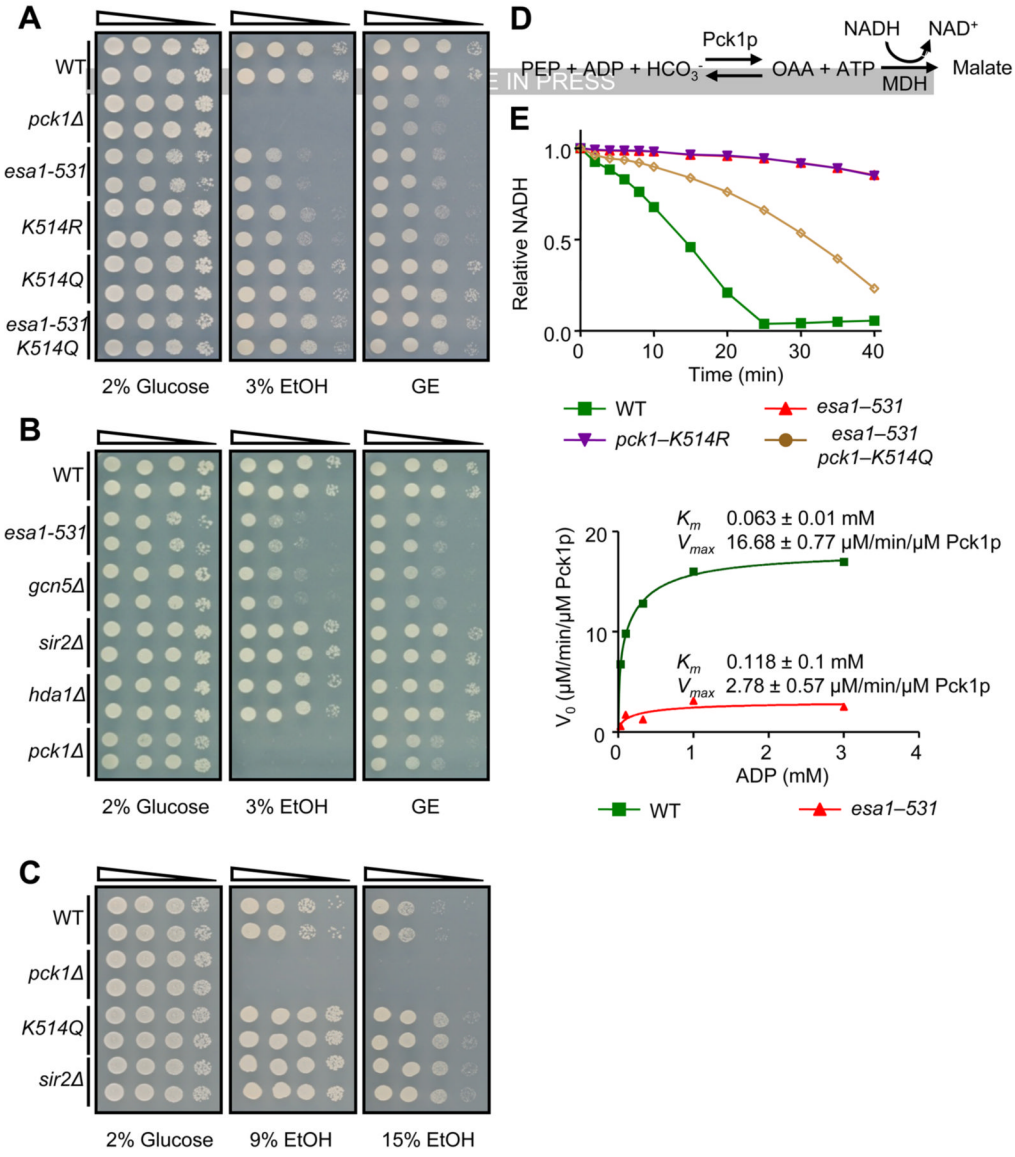

Figure 4. Pck1p acetylation controls activity

(A) Growth of WT, *pck1Δ*, *esa1-531*, *pck1-K514R* (*K514R*), *pck1-K514Q* (*K514Q*) and *esa1-531 pck1-K514Q* (*esa1-531 K514Q*) double mutant strains was examined on SC medium plates containing 2% glucose, 3% ethanol or 2% glycerol + 2% ethanol (GE). (B) Growth of WT, *esa1-531*, *gcn5Δ*, *sir2Δ*, *hda1Δ*, and *pck1Δ* strains was monitored in the same conditions as described above. (C) Growth of WT, *pck1Δ*, *pck1-K514Q* (*K514Q*) and *sir2*Δ was compared on SC plates containing 2% glucose, 6% ethanol, or 15% ethanol. (D) Pck1p activity was assayed in a coupled reaction, in which oxaloacetate (OAA) formed from phosphoenol pyruvate (PEP) was reduced to malate by NADH in the presence of malate dehydrogenase (MDH). Rate of NADH oxidation was measured at 340 nm. (E) Enzymatic activity of Pck1p purified from WT, *esa1-531*, *pck1-K514R*, and *esa1-531 pck1-K514Q* mutants was measured by consumption of NADH as a function of time. The curves of *esa1-531* and *pck1-K514R* overlapped with each other. (F) K_m and V_{max} values of Pck1p purified from WT and *esa1-531* were determined based on Michaelis-Menten Kinetics.

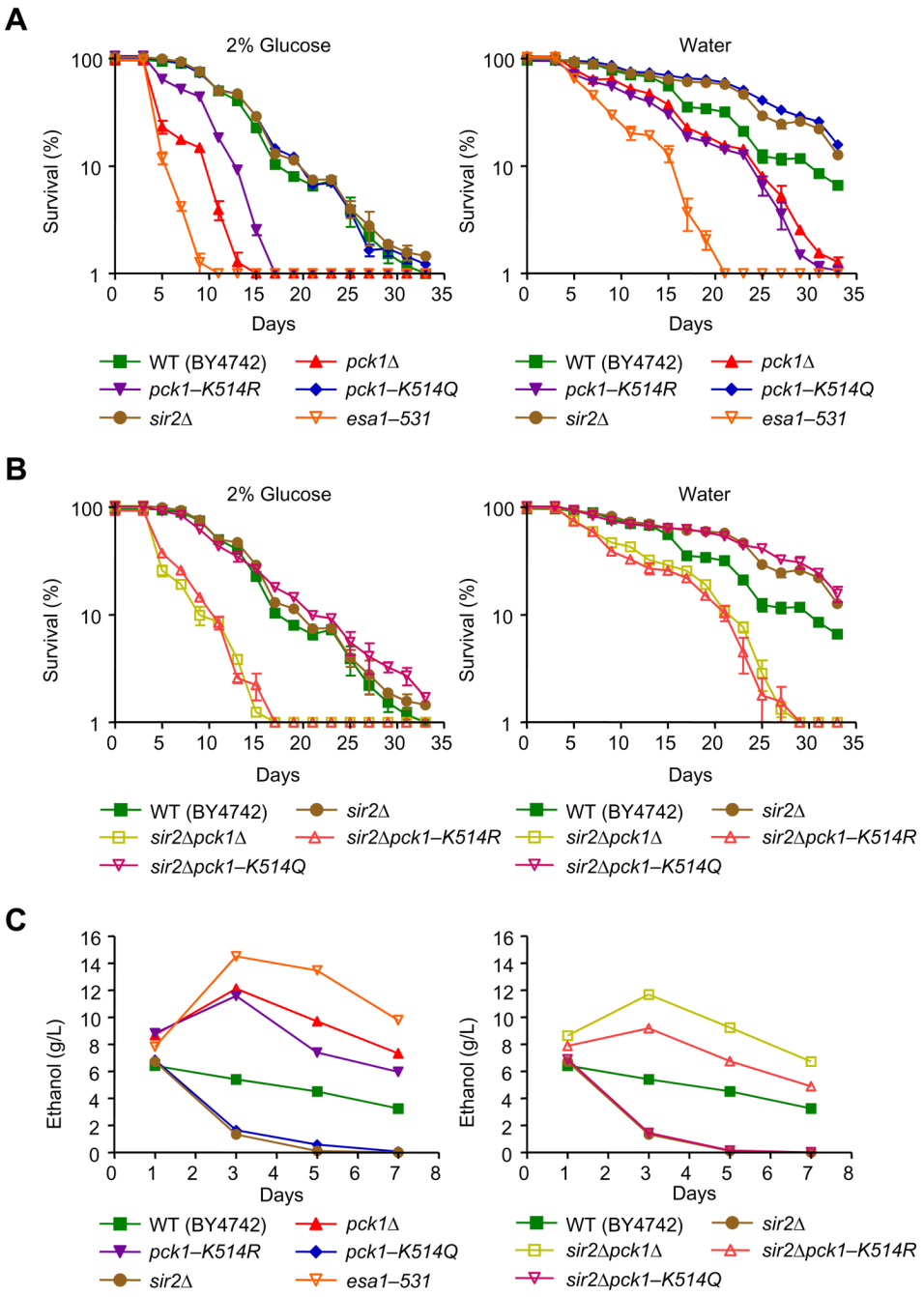

Figure 5. *PCK1* activity extends chronological life span under water starvation

(A, B) Chronological survival of wild type (WT, BY4742), *esa1-531*, *pck1*Δ, *sir2*Δ, *pck1-K514R*, *pck1-K514Q*, *sir2*Δ *pck1*Δ, *sir2*Δ *pck1-K514R* and *sir2*Δ *pck1-K514Q* in liquid synthetic complete medium containing either 2% glucose or water at 30°C was assessed. Error bars indicate ± 1 s.e.m from three biological replicates.

(D) Ethanol concentration in cell-free media from day 1, 3, 5 and 7 cultures of wild type (WT, BY4742), *esa1-531*, *pck1*Δ, *sir2*Δ, *pck1-K514R*, *pck1-K514Q*, *sir2*Δ *pck1*Δ, *sir2*Δ *pck1-K514R* and *sir2*Δ *pck1-K514Q* at 30°C was measured. Error bars indicate ± 1 s.e.m from three biological replicates.

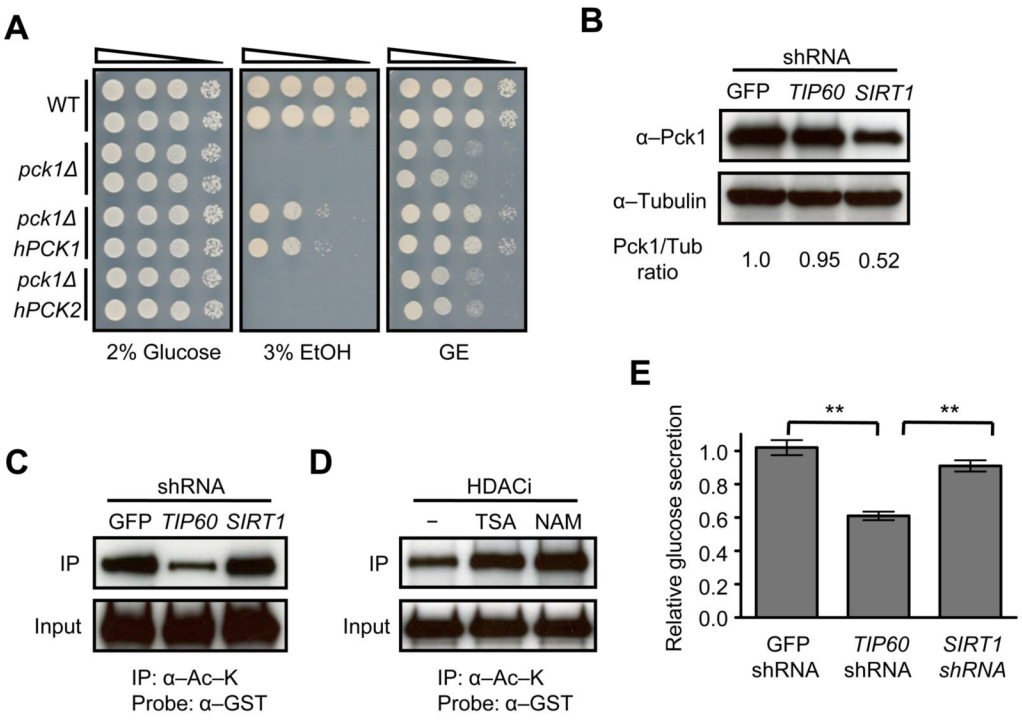

Figure 6. TIP60-dependent acetylation of human Pck1 and glucose production

(A) Human *PCK1* (*hPCK1*) but not *PCK2* (*hPCK2*) rescued the growth defect of yeast *pck1*Δ mutant on ethanol and GE. Growth was monitored as described in Figure 4A.

(B) Endogenous human Pck1 level decreased in HepG2 cells when *SIRT1* was silenced.

(C) Knockdown of *TIP60* diminished the *in vivo* acetylation of human Pck1 in HepG2 cells.

(D) Treatment of histone deacetylase inhibitors (HDACi) such as trichostatin A (TSA) or nicotinamide (NAM) increased the *in vivo* acetylation of human Pck1 in HepG2 cells.

(E) Glucose output of HepG2 cells was dependent on *TIP60*. The results are presented in a bar graph with error bars indicating ± 1 s.e.m from three biological replicates. Double asterisk indicates $P < 0.01$ (Student's *t*-test).

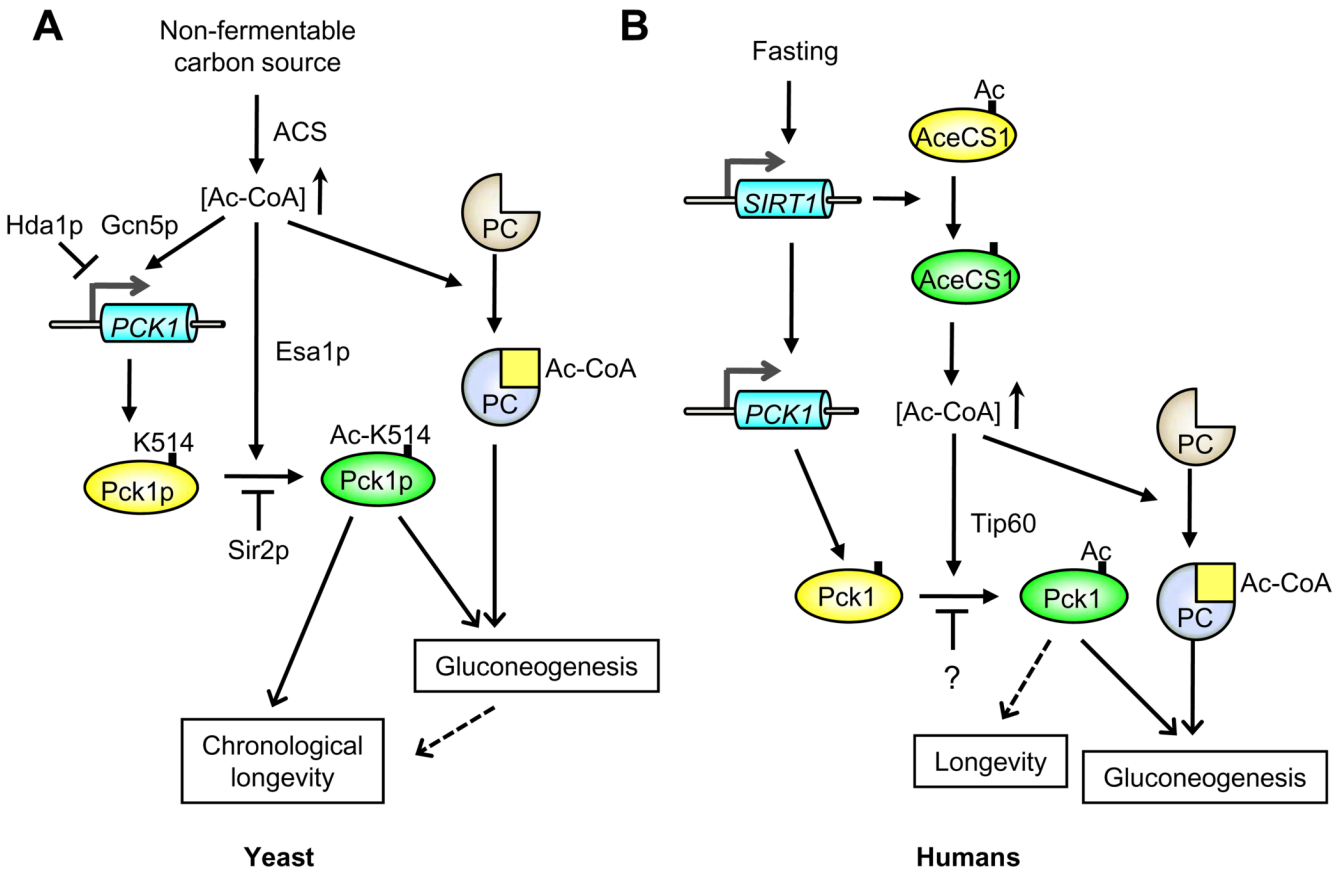

Figure 7. Schematic model for regulation of Pck1p activity in yeast and humans

A "triple-switch" (transcription of *PCK1*, acetylation of Pck1p and allosteric modification of PC) in the gluconeogenesis pathway controls prompt adaptation of a metabolic flux to energy status in yeast (A) and humans (B). The change of metabolic flux also mediates extension of chronological longevity under water starvation in yeast cells. ACS, acetyl-coenzyme A synthetase. AceCS1, acetyl-coenzyme A synthetase 1. PC, pyruvate carboxylase. Solid lines indicate the paths that have been proved by previous and the present study; dashed lines indicate those paths that were hypothesized as part of the present study.