Rerouting 'coenzyme A' biosynthesis
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CHAPTER 5

Extracellular 4’-Phosphopantetheine is a source for intracellular coenzyme A synthesis

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**SUMMARY**

The metabolic cofactor coenzyme A (CoA) gained renewed attention because of its roles in neurodegeneration, protein acetylation, autophagy and signal transduction. The long-standing dogma is that eukaryotic cells obtain CoA exclusively via the uptake of extracellular precursors, especially vitamin B5, which is intracellularly converted through five conserved enzymatic reactions into CoA. This study demonstrates an alternative mechanism that allows cells and organisms to adjust intracellular CoA levels by using exogenous CoA. Here CoA was hydrolyzed extracellularly by ectonucleotide pyrophosphatases to 4’-phosphopantetheine, a biologically stable molecule able to translocate through membranes via passive diffusion. Inside the cell, 4’-phosphopantetheine was enzymatically converted back to CoA by the bifunctional enzyme CoA synthase. Phenotypes induced by intracellular CoA deprivation were reversed when exogenous CoA was provided. Our findings answer long-standing questions in fundamental cell biology and have major implications for the understanding of CoA-related diseases and therapies.
INTRODUCTION

CoA was identified more than 60 years ago, and as a carrier of acyl groups it is essential for more than 100 metabolic reactions. It is estimated that CoA is an obligatory cofactor for 9% of known enzymatic reactions. CoA and acetyl-CoA influence levels of protein acetylation in various model organisms. Protein acetylation is an essential posttranslational modification catalyzed by acetyltransferases that use acetyl-CoA as their source. Acetyl-CoA levels also affect autophagy, and CoA promotes oocyte survival in *Xenopus laevis* by binding to and activating calcium/calmodulin-dependent protein kinase II (CaMKII). Together, intracellular concentrations of acetyl-CoA and CoA are critical to a broad range of cellular processes.

Current thinking about how cells and organisms obtain this indispensable molecule originates from experiments performed in the 1950’s, which showed how a specific sequence of enzymatic activities result in the formation of CoA *in vitro* when vitamin B5 is used as a substrate. These enzymes are, in order, pantothenate kinase (PANK), phosphopantothenoylcysteine synthetase (PPCS), phosphopantothenoylcysteine decarboxylase (PPCDC), phosphopantetheine adenyllyltransferase (PPAT) and dephospho-CoA kinase (DPCK) (Figure 1a). Later, genes encoding these enzymes were identified in a wide range of organisms. In some organisms, including *Drosophila melanogaster*, mice and humans, PPAT and DPCK enzyme activities are carried out by a single bifunctional protein, referred to as CoA synthase, or COASY. In vitro experiments have shown that in addition to vitamin B5, pantetheine can also be phosphorylated by pantothenate kinase activity, and the formed product, 4’-phosphopantetheine, can serve as a precursor for CoA. However, direct evidence that cells take up intact pantetheine and use it for CoA biosynthesis is still lacking.

In addition to renewed interest in the CoA molecule and its cellular roles, attention is being paid to the biosynthetic route because of its connection with specific forms of neurodegeneration. Two enzymes in the CoA de novo biosynthetic route, PANK (first step) and COASY (last two steps), are associated with a neurodegenerative disease known as Neurodegeneration with Brain Iron Accumulation (NBIA). Mutations in the gene encoding PANK2 (one of four human genes encoding PANK proteins) cause an NBIA disorder called pantothenate kinase-associated neurodegeneration (PKAN). Affected individuals experience progressive dystonia and accumulate iron in specific brain regions. Recently, individuals with mutations in the gene encoding COASY were identified and found to have similar clinical features and brain iron accumulation. This new NBIA disorder is referred to as COASY protein-associated neurodegeneration. This strongly suggests that impairment of the classic CoA biosynthetic route underlies progressive neurodegeneration in these patient groups. Currently there is no treatment available to halt or reverse the neurodegeneration in people with these CoA-related disorders.
CoA levels are decreased in a *Drosophila* model of PKAN, and the neurodegenerative phenotypes and decreased CoA levels seen in this model are rescued by the addition of pantethine to the food\(^7\). Pantethine addition also rescues a ketogenic diet-induced neurodegenerative phenotype in *Pank2*\(^2\) mice\(^2\). These studies demonstrate that in a pantothenate kinase-impaired background, CoA precursors other than vitamin B5 can alleviate neurodegenerative symptoms. How pantethine exerts its rescuing function (especially in the mouse study) is unclear because pantethine is highly unstable in serum and rapidly converted into vitamin B5 and cysteamine by pantetheinases\(^{20,21}\).

The aim of the current study was to determine whether there are alternate routes by which cells and organisms can obtain CoA. We found that extracellular CoA levels influence intracellular CoA levels both *in vitro* and *in vivo*, although the CoA molecule was not biologically stable and cells did not take up CoA directly. We present evidence that ectonucleotide pyrophosphatases (ENPPs) hydrolyzed CoA into 4’-phosphopantetheine. In contrast to pantetheine\(^{21}\), 4’-phosphopantetheine was stable in serum, was taken up by cells via passive diffusion and was intracellularly reconverted into CoA. Via this route, exogenous CoA rescued CoA-deprived phenotypes at the cellular, developmental, organismal and behavioral levels. We show that CoA rescue was independent of the first three classic CoA biosynthetic steps (involving PANK, PPCS and PPCDC, respectively) and depended on the last bifunctional enzyme, COASY. Our data demonstrate the existence of an alternate mechanism by which cells and organisms can influence intracellular CoA levels through the use of an extracellular CoA source with 4’-phosphopantetheine as the key intermediate.

**RESULTS**

**CoA supplementation rescues CoA-depleted phenotypes**

To learn whether cells are able to obtain CoA from sources other than classic *de novo* biosynthesis (Figure 1a), we first sought to determine whether extracellular sources of CoA can serve as a supply for intracellular CoA. For this, we used RNA interference (RNAi) to induce PANK depletion to block the *de novo* biosynthesis route and to create a CoA-depleted phenotype. Subsequently we tested the rescue potential of exogenous CoA. PANK depletion by RNAi in cultured *Drosophila* S2 cells (Figure 1b) was associated with reductions in cell count.

*Figure 1: CoA supplementation rescues PANK impaired phenotypes.*

**a.** Canonical *de novo* CoA biosynthesis pathway. Vitamin B5 (pantothenate) is taken up and intracellularly converted to CoA by the enzymes PANK, PPCS, PPCDC, and COASY. (PANK - Pantothenate Kinase; PPCS - Phosphopantothenoylcysteine synthetase; PPCDC - Phosphopantothenoylcysteine decarboxylase; COASY - CoA synthase). **b.** Relative *Drosophila* S2 cell count of control (100%) and dPANK/Fbl RNAi treated cells. ‘Insert’ - western blot of dPANK/Fbl protein levels in control and dPANK/Fbl RNAi treated cells, tubulin as loading control. Data represent mean ± SD (n = 3), two-tailed unpaired Student’s t-test
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Figure 1: CoA supplementation rescues PANK impaired phenotypes.

a. Canonical de novo CoA biosynthesis pathway. Vitamin B5 (pantothenate) is taken up and intracellularly converted to CoA by the enzymes PANK, PPCS, PPCDC, and COASY. (PANK - Pantothenate Kinase; PPCS - Phosphopantothenoylcysteine synthetase; PPCDC - Phosphopantothenoylcysteine decarboxylase; COASY - CoA synthase).

b. Relative Drosophila S2 cell count of control (100%) and dPANK/fbl RNAi treated cells. 'Insert' - western blot of dPANK/Fbl protein levels in control and dPANK/fbl RNAi treated cells, tubulin as loading control. Data represent mean ± SD (n = 3), two-tailed unpaired Student's t-test was used for statistical analysis (***P ≤ 0.001).

c. Relative cell count of control (100%) and dPANK/fbl RNAi treated cells in the presence of increasing concentrations of CoA. Data represent mean ± SD (n = 4).

d-f. Immunofluorescence showing protein acetylation levels in control (d) and dPANK/fbl RNAi treated cells without (e) and with CoA (f). Anti-acetylated-Lysine antibodies (green), Rhodamin-Phallloidin (red, F-actin) and DAPI (blue, DNA) were used. Scale bar indicates 20µm.

g. Relative cell count of control (100%) and HoPan treated HEK293 cells with and without CoA. Data represent mean ± SD (n = 3), two-tailed unpaired Student’s t-test was used (*P ≤ 0.05, **P ≤ 0.01).

h. Western blot and quantification of histone acetylation levels in control and HoPan treated HEK293 cells in presence and absence of CoA. GAPDH was used as loading control. Data indicate mean ± SD (n = 3), two-tailed unpaired Student’s t-test was used (*P ≤ 0.05).
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(Figure 1b,c) and histone acetylation (Figure 1d,e), as previously demonstrated\(^4\). Addition of CoA to the medium of the cultured cells rescued the cell count in a concentration-dependent manner (Figure 1c) and restored the histone acetylation phenotype (Figure 1f). Next, we asked whether this rescue would also be observed in other cell types and systems with impaired CoA biosynthesis. Treating \textit{Drosophila} S2 cells with the chemical PANK inhibitor Hopantenate (HoPan)\(^{22}\) also induced decreases in cell count (Supplementary Results, Supplementary Figure 1a) and histone acetylation levels (Supplementary Figure 1b,c). This HoPan-induced phenotype was also rescued by direct CoA supplementation to the cell medium (Supplementary Figure 1a,d). Next we studied the effects of HoPan in mammalian HEK293 cells to address the possibility that the beneficial effects of exogenous CoA are specific to insect cells. When HEK293 cells were treated with HoPan, they showed a phenotype similar to that of treated \textit{Drosophila} S2 cells, with decreased cell count and impaired histone acetylation. When CoA was added to the culture medium, both the decreased cell count (Figure 1g) and the impaired histone acetylation (Figure 1h) were rescued. These \textit{in vitro} results confirmed the ability of exogenous CoA to rescue phenotypes induced by impaired PANK activity in diverse cellular systems.

To test the effect of CoA supplementation \textit{in vivo}, we used homozygous \textit{Caenorhabditis elegans} pantothenate kinase (\textit{pnk-1}) mutants\(^4\), which showed decreased motility (Figure 2a, Supplementary Figure 2a) and a decreased lifespan (Figure 2b). Addition of CoA to the food of these mutants improved these phenotypes significantly (Figure 2a,b and Supplementary Figure 2a-e). Furthermore, when a \textit{Drosophila w} \textit{1118} control fly line was treated with HoPan, larval lethality was induced, and a decreased rate of eclosion (emerging from the pupal case) was observed (Figure 2c). This HoPan-induced phenotype was fully rescued by the addition of CoA to the food of the larvae (Figure 2d).

These data demonstrate that supplementation with CoA rescued the phenotypes arising from impaired \textit{de novo} CoA biosynthesis in diverse eukaryotic cell types and organisms.

**External supply of CoA influences intracellular CoA**

The rescue effect we observed could occur in several ways. Intracellular CoA levels could have been restored, or rescue could have been achieved independent of the restoration of CoA levels in the cells. In the latter case, intracellular levels of CoA would not be restored by exogenous CoA. To investigate this, we developed a sensitive HPLC method consisting of precolumn thiol-specific derivatization of samples with ammonium 7-fluorobenzofurazan-4-sulfonate followed by chromatographic separation by gradient elution on a C18 column and fluorescence detection (Online Methods). The HPLC CoA analysis showed that intracellular CoA levels were significantly reduced in extracts of HoPan-treated S2 and HEK293 cells, and the addition of CoA to the culture medium restored the intracellular concentration of CoA.
Figure 2: CoA rescues impaired PANK phenotypes of C. elegans and Drosophila.

a. Motility (bends per 30 sec) was determined in C. elegans pk-1 mutants and wild-types with and without CoA treatment. Error bars indicate ± SD (n = 45), analysed with two-tailed unpaired Student’s t-test (**P ≤ 0.001).

b. Lifespan analysis of C. elegans pk-1 mutants with (n = 90) and without (n = 96) CoA treatment compared to wild-types with (n = 92) and without (n = 83) CoA treatment. Survival curves were significant (P < 0.001), analyzed with Log-rank (Mantel-Cox) test, between untreated and CoA treated pk-1 mutants.

c. Eclosion rate of adult control flies (set as 100%) and flies treated with increasing concentrations HoPan, added to the food during development. Data indicates mean ± SD (n = 3), two-tailed unpaired Student’s t-test was used (*P ≤ 0.05, **P ≤ 0.01).

d. Relative eclosion rate of adult control flies and flies treated with 2.5mM HoPan, added to the food during development, in the presence of increasing concentrations of CoA. Data represent mean ± SD (n = 3).

e. Intracellular CoA levels measured with HPLC in Drosophila S2 control cells (100%) and cells treated with HoPan alone or with HoPan and CoA.

f. Intracellular CoA levels measured with HPLC in HEK293 control cells (100%) and HoPan treated cells with and without CoA. Data (in e,f) represent mean ± SD (n = 3), analysed with two-tailed unpaired Student’s t-test (**P ≤ 0.01).
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(Figure 2e,f). These results suggested that extracellular CoA exerted its beneficial effects in CoA-depleted cells by increasing and thereby ‘normalizing’ intracellular CoA concentrations.

In serum, CoA is degraded to stable 4’-phosphopantetheine

The mechanism behind this alternative CoA route was not known. The observations shown in Figures 1 and 2 indicated that either (1) CoA entered cells directly, although such a transport process has not been described, or (2) CoA was converted to an intermediate product that entered the cell and was converted back to CoA in a PANK-independent manner. Previous research showed that CoA is not stable in liver extracts and degrades to 50% at -20°C after a week\(^2\); however, the stability of CoA in an extracellular environment such as an aqueous or standard cell culture medium is unknown. Moreover, these early reports did not identify specific degraded or converted products. We measured the stability of CoA in PBS, serum-free medium, medium containing fetal calf serum (FCS) and FCS during a 3-h incubation. HPLC analysis showed that CoA was relatively stable in PBS and serum free medium, with >95% of the initial concentration still present after 3 h (Supplementary Figures 3 and 4a). However, in the presence of FCS, CoA was rapidly degraded (Figure 3a; Supplementary Figure 4b), and after 3 h of incubation, only 10% of the initial concentration was detectable (Supplementary Figure 3, 4b). Detailed stability analysis of CoA in PBS and FCS at different time points showed that 90% of CoA was degraded after only 30 min in FCS (Figure 3a). The disappearance of CoA coincided with the appearance of one unknown thiol-containing product in the HPLC chromatogram, which migrated at 18.273 min and remained stable over the full 3-h time course (Figure 3b, Supplementary Figure 4b). Because this extra peak had to be a thiol-containing molecule, we speculated that it might be a product of CoA degradation, specifically, dephospho-CoA, 4’-phosphopantetheine or pantetheine\(^2\). In contrast to dephospho-CoA and pantetheine, 4’-phosphopantetheine is not commercially available, so we chemically synthesized this compound (Supplementary Note) in order to complete our analysis. HPLC analysis and comparison with standards demonstrated that the thiol-containing degradation product of CoA was neither dephospho-CoA nor pantetheine (Supplementary Figure 4), but its retention time exactly matched that of the 4’-phosphopantetheine standard (Figure 3c,d and Supplementary Figure 4b,c). These results indicated that CoA was converted into 4’-phosphopantetheine in serum and was stable. This is in contrast to pantetheine, which is not stable in serum (Supplementary Figure 5a)\(^2\). We further investigated the conversion of CoA in mouse serum and in human serum. In sera from both species, including serum derived from people with PKAN (Supplementary Figure 5b), we found that CoA was converted to 4’-phosphopantetheine (Figure 3e and Supplementary Figure 5c).

To investigate whether this conversion also occurred in vivo, we fed CoA to Drosophila larvae, and obtained L1 and L2-stage larval extracts after 2 and 3 d of feeding, respectively. HPLC analysis showed that the addition of CoA resulted in increased levels of 4’-phosphopantetheine.
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Figure 3: CoA is converted into stable 4’-phosphopantetheine (PPanSH) in vitro, in serum, and in vivo in Drosophila and mice.

a. Stability profile of CoA determined by HPLC analysis in PBS (time 0 hrs in PBS is 100%) and in fetal calf serum over the course of 6 hrs. Data represent mean ± SD (n = 3). b-c. HPLC chromatogram profile of CoA incubated for 3 hrs in (b) PBS and in (c) fetal calf serum (FCS). d. Retention time of standard PPanSH is identical to the observed conversion product of CoA in fetal calf serum. e. CoA was added to mouse serum and concentrations of CoA and PPanSH in mouse serum over 6 hrs were determined by HPLC analysis. Data indicate mean ± SD (n = 3). f. Relative PPanSH levels in Drosophila L1 and L2 stage larvae determined by HPLC analysis under untreated conditions (100%) and after feeding CoA. Data indicate mean ± SD (n = 3), two-tailed unpaired Student’s t-test was used (**P ≤ 0.01, ***P ≤ 0.001). g. Concentration of CoA and PPanSH in mouse serum determined by HPLC analysis, 30 min after in vivo injecting various amounts of CoA intravenously. Data represent mean (n = 2), in g; Solid thick bars without error bars indicate no PPanSH or CoA was detected.

in both L1 (>20 fold) and L2 (>60 fold) larvae (Figure 3f). To investigate whether this conversion also occurred in higher organisms, we injected different concentrations of CoA intravenously into adult mice and collected plasma from the mice 30 min and 6 h after injection. HPLC analysis
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in combination with mass spectrometry revealed the presence of low levels of endogenous 4′-phosphopantetheine in fresh mouse serum (Supplementary Figure 6a-c) and showed that the injected CoA was rapidly converted to 4′-phosphopantetheine after 30 min (Figure 3g). Moreover, mass spectrometry showed that elevated levels of 4′-phosphopantetheine were still present in the plasma 6 h after CoA injection (Supplementary Figure 6d).

These data indicated that CoA is converted into 4′-phosphopantetheine in vitro and in vivo. Furthermore, these results suggested that 4′-phosphopantetheine could be the principal molecule taken up by CoA-depleted cells and converted into CoA intracellularly, and that this could result in the rescue of CoA-depleted phenotypes.

Conversion of CoA mediated by ENPPs

Next we asked which factors might be responsible for the conversion of CoA into 4′-phosphopantetheine in serum. To identify candidate enzymes, we preconditioned serum from various species (fetal calf, mouse and human) and assessed CoA conversion into 4′-phosphopantetheine. First, we studied the effect of heat inactivation of the serum. HPLC analysis showed that heating the serum at 56°C for 30 min completely abolished the conversion of CoA to 4′-phosphopantetheine (Figure 4a), which indicated the involvement of enzymes or proteins in the process. The conversion of CoA to 4′-phosphopantetheine requires the hydrolysis of a phosphoanhydride bond, which is typically catalyzed by (pyro)phosphatases or hydrolases. The majority of enzymes in the known family of (pyro)phosphatases and hydrolases depend on metal ions for their activity. To test these candidates, we added EDTA to serum to chelate metal ions. Treatment of serum with EDTA completely prevented the formation of 4′-phosphopantetheine (Figure 4b). This strongly suggested that metal ions were required for the CoA conversion. The most likely hydrolase or (pyro)phosphatase candidates that possess the ability to convert CoA and are metal-ion dependent for their activity are nudix hydrolases, alkaline phosphatases and ENPPs. These candidate enzymes are also known for their ability to hydrolyze adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP). Therefore, we tested the conversion of CoA into 4′-phosphopantetheine in serum after the addition of excess ATP and ADP. Both competitively blocked the conversion in all sera tested, further underscoring the involvement of one of these enzymes (Figure 4c). Alkaline phosphatase and ENPPs are excreted by cells and are present in serum. Nudix hydrolases are intracellular hydrolases of CoA; however, an additional possible extracellular role for this class of hydrolases cannot be excluded.

Next, we used sodium fluoride (NaF), and levamisole to inhibit nudix hydrolases and alkaline phosphatase, respectively, and we also used two different ENPP inhibitors, suramin and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS). Our data showed that suramin and DIDS were able to efficiently abolish the degradation of CoA into
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**Figure 4: Conversion of CoA into stable 4'-phosphopantetheine (PPanSH) in serum is mediated by heat unstable and metal-activated enzymes**

- a. CoA was incubated in heat-inactivated fetal calf serum, mouse serum and human serum for 3 hrs and CoA stability was measured using HPLC analysis.
- b. CoA stability was determined in fetal calf serum, mouse serum and human serum pre-treated with EDTA (10mM) and CoA levels were measured after 3 hrs using HPLC analysis.
- c. CoA was incubated in fetal calf serum, mouse serum and human serum pre-treated with ATP or ADP (both 10mM) and CoA levels were measured after 3 hrs.
- d. CoA stability was determined in fetal calf serum, mouse serum and human serum pre-treated with sodium fluoride (NaF), levamisole, suramin or 4,4'-diisothiocyanatostilbene-2,2’ disulphonic acid (DIDS) (all 10mM) and CoA levels were measured after 3 hrs. Data in all the above represent mean ± SD (n = 3), two-tailed unpaired Student’s t-test was used for statistical analysis to compare indicated subsets (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). In all the above experiments CoA was added to the indicated sera to a final concentration of 10µM, and percentages relative to CoA stability for 3 hrs in PBS (100%) are indicated (see Online methods for detailed protocol).
4'-phosphopantetheine in all the sera, unlike levamisole and NaF, which showed only mild or no inhibition of CoA degradation into 4'-phosphopantetheine, respectively (Figure 4d). NaF did not influence CoA degradation in serum, which indicated that nudix hydrolases either were not present or did not degrade CoA in serum. These experiments implicated ENPPs as the most likely class of enzymes to hydrolyze CoA into 4'-phosphopantetheine in serum. Moreover, in all of the CoA serum stability experiments mentioned above, there was an inverse correlation between levels of CoA and 4'-phosphopantetheine (Supplementary Figure 7), which supported the idea that CoA degradation into 4'-phosphopantetheine was mediated by ENPPs.

**Rescues of CoA-depleted phenotypes**

Our data so far showed that PANK impairment induced a decrease in CoA levels, and they also predicted a decrease in 4'-phosphopantetheine levels. Furthermore, they suggested that the addition of 4'-phosphopantetheine to CoA-depleted cells would rescue the induced phenotypes. HPLC analysis of HoPan-treated Drosophila S2 cells indeed showed reduced levels of 4'-phosphopantetheine, and external supplementation with either CoA or 4'-phosphopantetheine significantly increased intracellular levels of 4'-phosphopantetheine (Figure 5a). Moreover, when 4'-phosphopantetheine was added to Drosophila S2 cells treated with HoPan (Figure 5b) or dPANK/fbl RNAi (Figure 5c), the CoA-depleted phenotype was again rescued. 4'-Phosphopantetheine supplementation also rescued the histone acetylation defect in Drosophila S2 cells treated with dPANK/fbl RNAi (Supplementary Figure 8a-c) or HoPan (Supplementary Figure 8d-f). Finally, we tested the rescue effect of 4'-phosphopantetheine in HoPan-treated mammalian HEK293 cells and found that it also rescued the HoPan-induced reductions in cell count (Supplementary Figure 8g), intracellular CoA levels (Supplementary Figure 8h) and histone acetylation levels (Supplementary Figure 8i). Next we investigated whether intact 4'-phosphopantetheine entered cells and whether it was subsequently converted into CoA. First we treated intact cultured Drosophila S2 cells with stable isotope-labeled 4'-phosphopantetheine under various conditions and used mass spectrometry analysis to measure the levels of stable isotope-labeled CoA and 4'-phosphopantetheine (Supplementary Figure 9a-d) in extracts from the harvested cells. When labeled 4'-phosphopantetheine was added to the cell culture medium, labeled CoA was detected in harvested cell extracts (Figure 5d). In the presence of HoPan, CoA levels were decreased and replenished in the form of labeled CoA when labeled 4'-phosphopantetheine was added. These data demonstrated that exogenously provided 4'-phosphopantetheine was able to enter cells and was intracellularly converted into CoA under normal culturing conditions and conditions of impaired CoA biosynthesis after treatment with HoPan. Next we investigated the characteristics of the passage of 4'-phosphopantetheine over the cell membrane. First, we incubated S2 cells cultured at 25°C (the normal culturing temperature for these cells) and 4°C with labeled 4'-phosphopantetheine. The cultured cells showed intracellular presence of
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Our data showed that CoA from external sources could be used to replenish intracellular CoA levels through its hydrolysis product 4'-phosphopantetheine and subsequent conversion back to CoA. The enzyme most likely to be involved in this conversion is the last bifunctional enzyme of the classic CoA synthesis pathway.

Figure 5: External supplementation of 4'-phosphopantetheine (PPanSH) rescues CoA-deprived phenotypes

a. Measurement of intracellular PPanSH levels by HPLC analysis in control Drosophila S2 cells (100%) and cells treated with HoPan (0.5mM) with and without addition of CoA or PPanSH (100µM). b. Drosophila S2 cell count was determined in control cells (100%) and HoPan (0.5mM) treated cells under conditions of increasing PPanSH concentrations. c. Cell count was determined in control (100%) and dPANK/fbl RNAi treated Drosophila S2 cells with and without addition of PPanSH (100µM) to the medium. d. S2 cells, with and without HoPan (0.5mM) were incubated with stable-isotope labeled PPanSH(D4) (100µM) and levels of both unlabeled CoA and labeled CoA(D4) were measured using mass spectrometry. Cumulative CoA and CoA(D4) levels were used for statistical analysis. e. Stable-isotope labeled PPanSH(D4) (100µM) was added to S2 cells at 4°C and 25°C, incubated for various time intervals and mass spectrometry was used to measure levels of labeled compound in harvested cell extracts. f. Stable-isotope labeled PPanSH(D4) (10, 100, 1000µM) was added to S2 cells for 30min and mass spectrometry was used to measure levels of labeled compound in harvested cell extracts. Data in all the above represent mean ± SD (n = 3), two-tailed unpaired Student’s t-test was used for statistical analysis to compare indicated subsets (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).
we investigated whether the levels of intracellular 4'-phosphopantetheine increased to the same extent as externally added increasing concentrations of 4'-phosphopantetheine under these conditions. We added increasing concentration series (10-100-1000 μM) of labeled 4'-phosphopantetheine to the cells (Figure 5f). The results indicated that the capacity of the cells to accumulate externally provided 4'-phosphopantetheine was not influenced by temperature and was determined by extracellularly concentrations. Finally, we investigated the membrane-permeating efficiency of 4'-phosphopantetheine using a Parallel Artificial Membrane Permeability Assay (PAMPA assay). In this assay, 4'-phosphopantetheine but not CoA, showed membrane permeating properties. Together, these results pointed to a capacity of 4'-phosphopantetheine to permeate membranes via passive diffusion.

**CoA rescues dPANK/fbl and dPPCDC phenotypes**

Our data showed that CoA from external sources could be used to replenish intracellular CoA levels through its hydrolysis product 4'-phosphopantetheine and subsequent conversion back to CoA. The enzyme most likely to be involved in this conversion is the last bifunctional enzyme of the classic CoA biosynthesis pathway, COASY. This hypothesis predicts that CoA, but not vitamin B5, can rescue phenotypes caused by mutations in genes encoding enzymes upstream of 4'-phosphopantetheine in the CoA pathway. As a corollary, CoA would not be predicted to rescue COASY mutant phenotypes.

We aimed to test this hypothesis. In the *Drosophila* genome, we identified single orthologs for all the enzymes involved in CoA biosynthesis, including dPANK/fbl, dPPCDC and dCOASY. We obtained a set of *Drosophila* strains carrying either mutations in genes encoding these enzymes or flies ubiquitously expressing the RNAi construct. Homozygous mutants or flies ubiquitously expressing the RNAi construct showed downregulation of mRNA levels (Supplementary Figure 11) or protein levels (Supplementary Figure 12a) of these enzymes. CoA and 4'-phosphopantetheine levels were also significantly reduced in all conditions (Supplementary Figure 12b-e), with the exception of dCOASY mutants, which showed a significant reduction of CoA, but not of 4'-phosphopantetheine (Supplementary Figure 12f).

It should be stressed that not all mutants with defects in CoA biosynthesis enzymes showed an identical phenotype, which can be explained by the type of fly lines (e.g., RNAi construct-expressing lines, hypomorphic or null mutants) used. This has been reported previously not only for *Drosophila* but also for other organisms. Regardless of the severity of the phenotypes and the developmental stage in which they first arose, the determination of the rescue potential of CoA in the available mutants was a valuable tool for testing our hypothesis. A scheme of the hypothesis, the *Drosophila* life span and the phenotypes of the fly lines used are presented in Supplementary Figure 10.
We first tested two available mutants for dPANK/fbl; the hypomorphic dPANK/fbl<sup>1</sup> and the null mutant dPANK/fbl<sup>null</sup>. Homozygous dPANK/fbl<sup>1</sup> mutants showed reduced levels of dPANK/Fbl protein, and in homozygous dPANK/fbl<sup>null</sup> mutants levels of dPANK/Fbl protein were below detection (Supplementary Figure 12a). Correlating with this, homozygous dPANK/fbl<sup>1</sup> mutants showed a reduced adult lifespan (Figure 6a, Supplementary Figure 13a)<sup>12,19</sup>, whereas homozygous dPANK/fbl<sup>null</sup> mutants developed only until an early L2 larval stage, and pupae were not observed (Figure 6b). Addition of CoA to the food of the homozygous dPANK/fbl<sup>1</sup> mutants increased the life span from 20 d to 40 d (Figure 6a, Supplementary Figure 13a), and the addition of CoA to the food of homozygous dPANK/fbl<sup>null</sup> mutants extended development from the L2 stage to early pupal development (Figure 6b). These results supported our hypothesis. Remarkably, in dPANK/fbl<sup>null</sup> mutants, we detected low levels of CoA and 4’-phosphopantetheine (Supplementary Figure 12c). It is possible that these substances were obtained from a maternal supply or from the flyfood (Supplementary Figure 13b).

To compromise dPPCDC, the enzyme carrying out the third step of the CoA biosynthesis pathway, we used a UAS-RNAi line (dPPCDC RNAi) as well as a dPPCDC mutant and investigated rescue by CoA. Homozygous dPPCDC mutants showed lethality at early second instar larval stage L2 (Figure 6c). dPPCDCRNAi-expressing flies showed a milder phenotype in which adult flies were viable but had a reduced lifespan (Figure 6d). Females also showed sterility associated with small ovaries, and no eggs were produced (Figure 6e, Supplementary Figure 14a-d). The addition of CoA to the food of homozygous dPPCDC mutants extended larval development to late pupal stage (Figure 6c). For the dPPCDC RNAi-expressing flies, supplementation of CoA to the food increased the maximal lifespan from 10 d to 30 d (Figure 6d, Supplementary Figure 14e). In addition, female sterility was rescued, as evidenced by observations of egg production and eclosion of viable offspring (Figure 6e,f and Supplementary Figure 14c,d). These results were also consistent with our hypothesis.

Finally we tested a line with a mutant form of the bifunctional enzyme dCOASY, downstream of 4’-phosphopantetheine. Homozygous dCOASY mutants developed until first-instar larval stage, and addition of CoA to the food did not result in significant rescue (Figure 6g). As a negative control for all rescue experiments, we added vitamin B5 to the food; this did not result in any significant rescue of the phenotypes. A summary of the rescue with CoA in all fly lines is presented in Supplementary Figure 10.

To test our hypothesis further, we downregulated COASY with RNAi in mammalian HEK293 cells. Under these conditions, levels of COASY, CoA and histone acetylation were significantly reduced (Supplementary Figure 14g,f). As in dCOASY mutants, levels of 4’-phosphopantetheine remained unaltered in COASY-compromised mammalian cells (Supplementary Figure 14g). The addition of CoA to the medium neither rescued the COASY
Figure 6: External supplementation of CoA rescues dPANK/fbl- and dPPCDC- but not COASY-impaired phenotypes

a. Lifespan analysis of dPANK/fbl1 mutants with (n = 260) or without (n = 207) CoA treatment, compared with control flies with (n = 175) or without (n = 176) CoA treatment. Survival curves were significant with Log-rank (Mantel-Cox) test, between untreated and CoA (9mM) treated dPANK/fbl1 mutants (P value < 0.001).

b. Number of pupae of dPANK/fbl null mutants after treatment of increasing concentrations of CoA or Vitamin B5.

c. Number of pupae of dPPCDC mutants untreated or after treatment with CoA or Vitamin B5.

d. Lifespan analysis of the dPPCDC RNAi line untreated (n = 111), treated with CoA (n = 102) or Vitamin B5 (n = 102). Survival curves were significant with Log-rank (Mantel-Cox) test, between untreated and CoA (18mM) treated dPPCDC RNAi flies (P value < 0.001).

e. Ovarian size of control and dPPCDC RNAi expressing Drosophila females, untreated or treated with CoA or Vitamin B5, imaged with light microscopy. Scale bar = 200µm.

f. Amount of eclosed adult progeny of dPPCDC RNAi females, crossed with control males raised on control food or supplemented with CoA or Vitamin B5.

g. Amount of L1 and L2 dCOASY mutant larvae and controls untreated or treated with CoA or Vitamin B5.

h. Proposed non-canonical CoA supply route starting with extracellular CoA. ENPPs is ecto-nucleotide pyrophosphatases. Data represents mean ± SD (n = 3) in b,c,f,g; Solid thick bars without error bars indicate no pupae or eclosed flies observed.
RNAi-induced decrease in intracellular CoA levels (Supplementary Figure 14g) nor restored histone acetylation levels (Supplementary Figure 14f). These results were also in agreement with our hypothesis.

Taken together, these results demonstrated that impairment of the CoA biosynthetic pathway by genetic manipulation can give rise to highly complex pleiotropic effects affecting lifespan, development and fecundity. These phenotypes can be (partially) rescued by the addition of CoA to the food of affected animals. The added CoA is hydrolyzed to 4'-phosphopantetheine which crosses the plasma membrane via passive diffusion before being converted back to CoA intracellularly in a step requiring COASY (Figure 6h).

**DISCUSSION**

In our study we addressed the basic question of whether cells and organisms have alternative ways to obtain the essential molecule CoA aside from the canonical pathway utilizing vitamin B5. We found that cells and organisms were able to acquire exogenous CoA and convert it into the stable molecule 4'-phosphopantetheine, which entered cells and was converted again into CoA. This newly identified pathway for 4'-phosphopantetheine suggests that this molecule can serve as a transport form of CoA or stable reservoir for rapid access and conversion. The proposed mechanism hypothetically allows a net flow of CoA or 4'-phosphopantetheine between cells and between membrane-bound cellular compartments. Our data further suggest that not all cells or organelles in an organism need to harbor all CoA biosynthetic enzymes to obtain CoA and that the route to CoA does not necessarily need to follow the archetypal path starting with the uptake of vitamin B5. Moreover, these observations hold promise for therapeutic intervention for PKAN, because 4'-phosphopantetheine is stable in serum and passes through cell membranes, thereby allowing for restoration of intracellular CoA levels in cells with defective CoA synthesis. The stability of 4'-phosphopantetheine is in strong contrast to characteristics of the dephosphorylated form, pantetheine, which is degraded rapidly in serum by pantetheinases into vitamin B5 and cysteamine\(^{20,21}\). These results show that the phosphate group protects the molecule from degradation and allows 4'-phosphopantetheine to serve as an effective substrate for CoA biosynthesis from its ready reserve in the circulation.

▶ *size of control and dPPCDC RNAi expressing Drosophila females, untreated or treated with CoA or Vitamin B5, imaged with light microscopy. Scale bar = 200μm.*  
  **f.** Amount of eclosed adult progeny of dPPCDC RNAi females, crossed with control males raised on control food or supplemented with CoA or Vitamin B5.  
  **g.** Amount of L1 and L2 dCOASY mutant larvae and controls untreated or treated with CoA or Vitamin B5.  
  **h.** Proposed non-canonical CoA supply route starting with extracellular CoA. ENPPs is ecto-nucleotide pyrophosphatases. Data represents mean ± SD (n = 3) in b, c, f, g; Solid thick bars without error bars indicate no pupae or eclosed flies observed.*
One intriguing question is whether the proposed route shown here has a physiological function or can be artificially provoked through manipulation of concentrations of extracellular CoA. Compared to CoA concentrations in cytoplasm (0.02-0.14 mM) and mitochondria (2.2-5.0 mM)\cite{38}, the concentrations used in our study (in the micromolar range) were relatively low. Answers may come from previous studies demonstrating that bacteria are able to excrete 4′-phosphopantetheine but not take it up from their environment, which suggests that bacteria-derived 4′-phosphopantetheine may be present in the digestive system of other organisms\cite{39}. The presence of endogenous 4′-phosphopantetheine in mouse serum and in dPANK/fbl\textsuperscript{null} mutants is consistent with a possible source of extracellular 4′-phosphopantetheine. Such a ‘ready’ pool of CoA precursor may function in transport from one organ to another. In addition to being a source for intracellular CoA, extracellular CoA and 4′-phosphopantetheine may have signaling functions, as suggested by reports that CoA has effects on platelet aggregation and vasoconstriction\cite{40,41}. Our results suggest that these effects, which have been attributed to CoA, may in fact be caused by 4′-phosphopantetheine. Future experiments are required to demonstrate the presence and possible effect of a net flow of CoA among organelles, cells and organisms (such as between intestine bacteria to the host).

The ability of 4′-phosphopantetheine to translocate across membranes provides answers long-standing questions regarding the intracellular distribution of CoA and its biosynthetic enzymes. CoA is present in the cytoplasm and in organelles such as mitochondria\cite{38}. All CoA biosynthetic enzymes are present in cytoplasm\cite{42}, but only a subset have been found in mitochondria. It remains unclear how mitochondria obtain CoA, and the localization of COASY (but not the other CoA biosynthetic enzymes) to the mitochondrial matrix, is also unexplained\cite{17,43}. It has been postulated that CoA synthesized in the cytosol can be transported into the mitochondrial matrix by specific CoA transporters localized in the mitochondrial inner membrane\cite{44}. Indeed, evidence for the presence of such CoA transporters has been presented\cite{45}. On the basis of our observations, we hypothesize that 4′-phosphopantetheine is able to pass over the mitochondrial inner membrane and into the matrix and is subsequently converted into CoA by matrix COASY. This might explain the localization of COASY in the mitochondrial matrix\cite{17,43}.

The presence of a mechanism for 4′-phosphopantetheine uptake could have considerable implications for public health. Pathogens and parasites acquire resistance to treatments, and species-specific inhibitors of CoA biosynthetic enzymes are attractive candidates for a new class of antibiotics and anti-malarial drugs\cite{46,47}. Such inhibitors might be more effective antimicrobials when 4′-phosphopantetheine uptake is blocked as well. Alternatively, differences in the capacity for 4′-phosphopantetheine uptake by eukaryotic cells (as described in this manuscript) and bacteria\cite{39} may be further explored as possible targets for antimicrobial strategies.
CoA is essential for coordinating key aspects of cell function. It is therefore not surprising that an extracellular pool exists to facilitate swift replenishment and that it relies on the formation of a stable intermediate. Although these observations raise many new questions about CoA metabolism, they also suggest therapeutic approaches for a range of life-threatening human diseases.

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AUTHOR CONTRIBUTIONS

COMPETING FINANCIAL INTEREST
REFERENCES


4'-PHOSPHOPANTETHEINE IS A SOURCE FOR COENZYME A SYNTHESIS


4′-Phosphopantetheine is a Source for Coenzyme A Synthesis


ONLINE METHODS

Drosophila S2 Cell Culture, RNA Interference, and CoA and 4’-phosphopantetheine treatment

Drosophila Schneider’s S2 cells were maintained at 25°C in Schneider’s Drosophila medium (Invitrogen) supplemented with 10% fetal calf serum (Gibco) and antibiotics (penicillin/streptomycin, Invitrogen) under laboratory conditions. Synthesis of RNAi constructs and RNA interference (dsRNA) treatment was carried out as described previously. Non-relevant (human gene; hMAZ) dsRNA was used as control. The cells were incubated for 4 days to induce an efficient knock-down. Cells were then subcultured, with or without CoA (Sigma-Aldrich, Cat. No: C4780, 95% – which is used for all the experiments wherever stated below) or 4’-phosphopantetheine (PPanSH) (Acies Bio, >92%) at different concentrations and were maintained for additional 3 days until analysis for rescue efficiency of the compounds was performed. The stock solutions of compounds were made in sterile water and stored in -20°C until use.

HoPan treatment of Drosophila S2 Cell in combination with CoA or 4’-phosphopantetheine treatment

Drosophila Schneider’s S2 cells were maintained at standard conditions as explained above. Cells in the exponential phase of growth were used for all the experiments. Different indicated concentrations of CoA or 4’-phosphopantetheine (deuterium labelled PPanSH(D4) or unlabelled PPanSH) were added to S2 cells either in the presence or absence of 0.5mM HoPan (Zhou Fang Pharm Chemical,; 99%) for 48hrs. Similarly, Drosophila S2 cells were treated with different concentrations of PPanSH(D4) at either 25°C or 4°C and cells were then harvested at various time points to access transport of PPanSH(D4). Stable isotope labelled PPanSH containing 4 deuterium atoms was purchased as a sodium salt (from Syncom; synthesized as previously described, 99.7%). As a read out, cell count, intracellular total CoA and PPanSH levels (both labelled and unlabeled levels wherever appropriate) and histone acetylation levels were analyzed as explained below.

Drosophila S2 Cell Immunofluorescence Staining

For immunofluorescence Drosophila S2 cells were seeded on Poly-L-Lysine coated (Sigma-Aldrich) glass microscope slides and allowed to settle for 45min. Cells were fixed with 3.7% formaldehyde (Sigma Aldrich) for 20min, washed briefly with phosphate-buffered saline (PBS) + 0.1% Triton-X-100 (Sigma Aldrich) and permeabilized with PBS + 0.2% Triton-X-100 for 20min. The slides were incubated in primary antibody (rabbit anti-AcLys, Cell Signaling Cat No: 9441, 1:500) to visualize histone acetylation levels in PBS + 0.1% Triton-X-100 overnight and after an additional washing step in PBS + 0.1% Triton-X-100 they were incubated in secondary goat anti-rabbit-Alexa488 antibody (Molecular Probes) for two hours at room temperature (RT).
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F-actin was detected with Rhodamin-Phalloidin (20U/ml) (Invitrogen) and DNA by staining with DAPI (0.2μg/ml) (Thermo Scientific). Finally the samples were mounted in 80% glycerol and analyzed using a Leica fluorescence microscope with Leica software. Adobe Photoshop and Illustrator (Adobe Systems Incorporated, San Jose, California, USA) were used for image assembly.

**HoPan treatment of mammalian HEK293 Cells in combination with CoA and 4’-phosphopantetheine treatment**

HEK293 cells were maintained in dMEM (Invitrogen) supplemented with 10% fetal calf serum (Gibco) and antibiotics (penicillin/streptomycin, Invitrogen). For HoPan treatment, cells were cultured in custom made dMEM without Vitamin B5 (Thermo Scientific) supplemented with dialyzed FCS (Thermo Scientific). CoA or PPanSH was added to HEK293 cells for the final concentration of 25μM, either in the presence or absence of HoPan (0.5mM) for 4 days, followed by analysis for phenotype and rescue efficiency of CoA and PPanSH.

**Knockdown of COASY by siRNA in mammalian HEK293 cells**

HEK293 cells were maintained as described above. HEK293 cells were transfected with 200nM COASY siRNA (GE Healthcare human COASY 80347 smartpool Cat no: M-006751-00-0010) or non-targeting siRNA (GE Healthcare Cat no: D-001206-13-20) using lipofectamine 2000 (Invitrogen). 4hrs after transfection CoA was added in a final concentration of 25μM. Cells were cultured for 3 days and then harvested for HPLC analysis of total CoA and PPanSH levels and Western blot (histone acetylation) as described below.

**Western blot analysis and Antibodies**

For Western blot analysis, cells were collected and washed with PBS, followed by centrifugation. The cells were lysed and sonicated in 1X Laemmli Sample Buffer and boiled for 5min with 5% β-mercaptoethanol (Sigma). Protein content was determined using DC protein assay (BioRad). Equal amounts of protein were loaded on a 10 or 12.5% SDS-PAGE gel, transferred onto PVDF membranes and blocked with 5% milk in PBS/0.1% Tween, followed by overnight incubation with primary antibodies. The primary antibodies used were: rabbit-anti dPANK/fbl, 1:4000 Eurogentec custom made as described previously, mouse anti-tubulin (Sigma Aldrich Cat no: TS168, 1:5000), anti-acetyl-Histone3 (Active Motif Cat no: 39139, 1:2000), anti GAPDH (Fitzgerald Cat no: 10R-G109a, 1:10000), rabbit anti COASY (Abcam Cat no: AB129012, 1:1000). Appropriate HRP-conjugated secondary antibodies (Amersham) were used and detection was performed using enhanced chemi-luminescence (Pierce cat nog: 32106) and Amersham hyperfilm (GE healthcare). Band intensities were quantified with Image-studio software. Full uncut gel images for all Westerns displayed in this paper are shown in Supplementary Figures 15 and 16.
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**C. elegans Media and Strains**
Standard culturing conditions were used for *C. elegans* maintenance at 20°C. N2 strain was used as a wild-type control. VC927, the PANK deletion mutant pnk-1 (ok1435)/ht2[bli-4(e937) let-2 (q782)qIs48](I;III), was obtained from the *Caenorhabditis* Genetics Center. To obtain synchronous cultures, worms were bleached with hypochlorite, and allowed to hatch in M9 buffer (3g KH₂PO₄, 6g Na₂HPO₄, 5g NaCl, 1ml 1M MgSO₄, H₂O to 1 liter) overnight and cultured on standard Nematode Growth Medium (NGM) plates seeded with OP50 strain of *Escherichia coli*.

**C. elegans Lifespan Assay**
After synchronization, *C. elegans* L1 animals were grown on control NMG plates or NGM plates supplemented with 400μM CoA. The life span experiments were started by transferring 100 one-day old adults per condition on NGM plates, which contained 5-fluoro-2′deoxy-uridine (FUDR) to inhibit growth of offspring. Once a day surviving animals were counted, the worms that disappeared or crawled out of the plate were excluded from the analysis.

**C. elegans Motility Assay**
After synchronization, L1 *C. elegans* were grown on control NMG plates or NGM plates containing various concentrations of CoA. One-day old adults were placed in a drop of M9 buffer and allowed to recover for 30sec. During the following 30sec, the number of body bends were counted. A movement was scored as a bend when both the anterior and posterior ends of the animal turned to the same side. At least 15 worms were scored per condition and each experiment was repeated thrice. The sequential light microscopy images demonstrating movements of *C. elegans* in M9 buffer were captured using Leica MZ16 FA microscope at 32x magnification within the time frame of 1sec and processed using ImageJ (National Institutes of Health, Maryland, USA) and Adobe Photoshop (Adobe Systems Incorporated, San Jose, California, USA).

**Drosophila Maintenance and Crosses**
*Drosophila melanogaster* stocks/crosses were raised on standard cornmeal agar fly food (containing water, agar 17 g/L, sugar 54 g/L, yeast extract 26 g/L and nipagin 1.3 g/L) at 25°C. The stocks were either obtained from the Bloomington Stock Centre (Indiana University, USA), VDRC (Vienna *Drosophila* RNAi Collection, Vienna, Austria) or from the Exelixis Collection (Harvard Medical School) and rebalanced over eGFP-positive balancers to identify homozygous (eGFP negative) progeny. The stocks used were: *w[1118]; dPANK/fbl*, hypomorphh; *dPANK/fbl* RNAi; *Mi[+]mDint2]=MIC/fbl[MI04001]/TM3, Sb[1] Ser[1], Bloomington 36941); *dPPCDC* mutant (*w[1118]; PBac[+mC]=WH]Ppcdc[f00839]/CyO, Bloomington 18377); UAS-*dPPCDC* RNAi line (VDRC 104495); *dCOASY* mutant (PBac{RB})
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Ppat-Dpck[e00492], Exelixis). The UAS-RNAi constructs were expressed ubiquitously using the Actin-Gal4 drivers (y[1] w[+] P[w[+mC]=Act5C-GAL4]25FO1/CyO, y[+], Bloomington 4414). As controls we used the heterozygous flies/larvae for the mutants and the Actin-Gal4 driver crossed to isogenic w1118 flies (Actin-Gal4/+) for the RNAi-constructs expressing flies.

Drosophila Larval Collection and Larval Count Experiment
One week old flies (in the ratio 10 females and 5 males) were kept on 5 ml of standard fly food in a vial at 25°C with or without various concentrations of CoA or Vitamin B5 (Sigma, Cat. No. P5155). The flies were allowed to lay eggs for 2 days and parent flies were then discarded. The L1, L2 and L3 larvae were collected from the food with 20% sucrose at appropriate time (day 4, 6 and 8 respectively) for larval counting and stored in -80°C until analysis. The pupal count was performed between days 10 and 12.

Drosophila HoPan Toxicity and CoA Rescue Experiment
One week old w1118 flies (in the ratio 10 females and 5 males) were kept in vials containing standard fly food with or without HoPan and CoA at indicated concentrations. The flies were allowed to lay eggs for 2 days, after which the adults were discarded. The resulting offspring were allowed to develop. The numbers of flies which eclosed were counted to evaluate HoPan toxicity and CoA rescue efficiency.

Drosophila Life Span
One-day old female adult flies of Drosophila homozygous mutants or RNAi-constructs expressing lines, were collected with appropriate controls and were kept on standard fly food at 25°C with or without CoA or Vitamin B5 (Sigma) at necessary concentration (in 50 μl added on top of the fly food and dried before flies were added). The flies were counted every 12-24 hrs and flipped to new fly food vials with or without CoA or Vitamin B5.

Drosophila Ovary Rescue Experiment
UAS-dPPCDC RNAi constructs were ubiquitously expressed under the control of Actin-Gal4. The crosses were raised at 25°C. F1 RNAi-construct expressing females and control females were collected shortly after eclosion and transferred to standard fly food or food containing Vitamin B5 or CoA (18 mM). Flies were maintained for 2 days on this food at 25°C. After this period extra yeast and w1118 control males were added and the crosses were kept at 25°C for another 2 days. After this 4 day period ovaries were dissected and stained for further analysis. The vials (or plates) from the crosses (with eggs that were being laid during the 4 day period of CoA treatment) were kept for another 10 days and offspring numbers were counted after eclosion.
RNA Isolation, Quantitative Real-Time PCR, and Primers

*Drosophila* larvae and samples of 1-day old adult flies or larvae were collected for homozygous *dPPCDC* mutants, *dPPCDC* RNAi-construct expressing lines and for homozygous *dCOASY* mutants, followed by brief washing with PBS. The samples were lysed in TRIZOL (Invitrogen) for RNA extraction and reverse transcribed using M-MLV (Invitrogen) and oligo(dt) 12-18 (Invitrogen). SYBR green (Bio-Rad) and Bio-Rad Real-Time PCR with specific primers were used for gene expression level analysis. The expression levels were normalized for rp49 (house-keeping gene). The Primer sequences used were *dPPCDC* (TGCACCTGCGATGAATACCC; TCGGCTGAAAGCGGATAAC), *dCOASY* (GGCTGTGCGGCGGATTATTG; CGGGTTAAAGGCTGCTCTGG) and *rp49* (GCACCAAGCACTTCATCC; CGATCTCGCCGCAGTAAA) (Biolegio).

*Drosophila* Ovary dissection and staining

*Drosophila* ovaries were collected in cold PBS and fixed in 4% formaldehyde (from methanol-free 16% Formaldehyde Solution, Thermo Scientific) for 45min at RT. The fixed tissue was washed in PBS + 0.1% Triton-X-100 for 1hr at RT and afterwards permeabilized in PBS + 0.2% Triton-X-100 for 1hr. Finally the ovaries were stained with Rhodamin-Phalloidin (20U/ml) to detect F-actin and DAPI (0.2 µg/ml) for DNA. Finally the samples were mounted in 80% glycerol and analyzed on a Zeiss-LSM780 NLO confocal microscope with Zeiss Zen software. Adobe Photoshop and Illustrator (Adobe Systems Incorporated, San Jose, California, USA) were used for image assembly.

PAMPA assay procedure

Parallel Artificial Membrane Permeability Assay (PAMPA) was performed and processed according to manufacturer’s instructions (BD Gentest Pre-coated PAMPA plates). Briefly, two superimposed wells are separated by an artificial lipid-oil-lipid membrane. The compound of interest (PPanSH, CoA, caffeine, amiloride) was added to the bottom well in phosphate-buffered saline, whereas the top well was filled with phosphate-buffered saline alone. After 5hrs of incubation at RT, concentrations of the different compounds were measured using UV-VIS absorption spectroscopy (BMG Labtech SPECTROstar Omega) along with calibration curves for all compounds. The permeability efficiency was further calculated according to manufacturer’s instructions (Supplementary figure 9e). For caffeine and amiloride, four replicates were performed; for PPanSH and CoA twelve replicates were performed. Caffeine and amiloride were obtained from Sigma.

Serum collection from PKAN patients

Serum was collected from the blood of PKAN patients and respective healthy family members (control) using standard protocols. Briefly, venous blood was collected in commercially available red topped Vacutainer tubes (Becton Dickinson) and allowed to remain at RT for 15-30min.
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undisturbed for the blood clotting. The tubes were then centrifuged at 2,000 g for 10 min at 4°C. The resulting supernatant serum was immediately transferred to 2 ml cryovials and maintained at -80°C until CoA stability assessments were performed. Blood samples and clinical data were obtained under OHSU’s IRB-approved repository protocol #7232 following informed consent.

CoA and pantethine serum stability measurements

CoA serum stability studies were conducted in commercially obtained serum and in serum collected from PKAN patient and healthy family members as controls. Human and Mouse sera were purchased from Sigma and Fetal calf serum was purchased from Gibco. Additionally, dMEM medium with or without 10% fetal calf serum was used for evaluating CoA stability. Briefly, all sera and samples were incubated for 30 min at 37°C with or without pre-conditioning compounds at final concentration 10 mM [Adenosine 5′-triphosphate (ATP), Adenosine 5′-diphosphate (ADP), Ethylenediaminetetraacetic acid (EDTA), Levamisole, Suramin, 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium (DIDS) and sodium fluoride (NaF), all purchased from Sigma] followed by addition of CoA 20 μM in the ratio of 1:1 and incubated at 37°C after brief vortex for indicated time intervals. For heat inactivation, all sera were incubated for 30 min at 56°C after which CoA was added. Serum samples at different time points were collected, deproteinized and analyzed by HPLC as described below. For pantethine serum stability, pantethine (Sigma) was incubated in fetal calf serum, mouse serum and human serum for 15 min in 37°C and total levels of pantetheine and cysteamine was measured using HPLC.

Mice and CoA intravenous injection study

Adult male mice of C57BL/6J 129/SvJ mixed genetic background were used for this study. Two mice (approximately 25-30g wt) were used for each condition. 0.1 mg or 0.5 mg CoA in 0.25 ml saline solution was injected intravenously (i.v) into the tail vein. Saline solution (0.25 ml) was injected to control groups. After 30 min and 6 hrs blood samples were collected and further processed to obtain plasma followed by sample preparation for HPLC or LC-MS analysis as indicated below. All animal studies were approved by the Ethics Committee of the Foundation IRCCS Neurological Institute C. Besta, in accordance with guidelines of the Italian Ministry of Health: Project no. BT4/2014. The use and care of animals followed the Italian Law D.L. 116/1992 and the EU directive 2010/63/EU.

HPLC sample preparation protocol for total CoA and 4′-phosphopantetheine measurement

Samples were briefly washed with ice-cold PBS solution. Samples were sonicated thoroughly in 100 μl ice-cold PBS and centrifuged for 10-15 min at 4°C to collect supernatant. Tris(2-carboxyethyl)phosphine hydrochloride (Sigma) (50 mM; 10 μl) was added to 50 μl sample
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Supernatant and were incubated at RT for 15min after vortex-mixing. Saturated ammonium sulfate solution or Millipore 3KD centrifugal filter units were used to remove proteins. The samples were centrifuged at 14,000 rpm for 15min at 4°C. The clear supernatant (50μl) or the filtrate was derivatized with 45μl of ammonium 7-flurobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F, Sigma) (1mg/ml in borax buffer - 0.1M containing 1mM EDTA disodium, pH 9.5), and 5μl ammonia solution (12.5% v/v, Merck Millipore) at 60°C for 1hr. The derivatized samples were placed in a refrigerated autosampler (10°C) in the Shimadzu HPLC system, and injected for total CoA and PPanSH analysis using optimized chromatographic separation conditions combined with fluorescence detection (described below).

**Chromatography separation condition**

Chromatographic analysis was performed with a Shimadzu LC-10AC liquid chromatograph, SCL-10A system controller, SIL-10AC automatic sample injector and LC-10AT solvent delivery system. Shimadzu RF-20Axs fluorescence detector was used for derivatized sample extract analysis. The fluorescence detector was set at excitation and emission wavelengths of 385nm and 515nm, respectively. Signal output was collected digitally with Shimadzu Labsolution software and postrun analysis were performed. Chromatographic separation of the analytes was achieved with a Phenomenex Gemini C18 guard column (4 x 3mm) connected to a Phenomenex Gemini NX-C18 analytical column (4.6 x 150mm; 3μm particles) at 45°C. The two mobile phases consisted of A: 100mM ammonium acetate buffer (pH 4.5) and B: acetonitrile. Flow rate was maintained at 0.8ml/min with a slow gradient elution: 0% B till 7min, 20% B at 20min, 20% B at 22min, 50% B at 23min, maintained at 50% B till 27min, 0% B at 28min and 7-10min for column re-equilibration.

**Sample preparation for mass spectrometry and instrumental parameters**

Samples were briefly washed with ice-cold PBS solution. Samples were then sonicated thoroughly in 100μl ice-cold milliQ (MQ) water containing 50mM Tris(2-carboxyethyl)phosphine hydrochloride. Subsequently 100μl saturated ammonium sulfate was added to each sample and centrifuged for 20 min at 10°C, 16,100g to collect supernatant. To 150μl of supernatant, 15μl of ammonium hydroxide (12.5%) was added and 20μl was injected for LC-MS (liquid chromatography-mass spectrometry) analysis. For mouse plasma analysis, 50μl of MQ water containing 50mM Tris(2-carboxyethyl)phosphine hydrochloride was added to 50μl of plasma and processed further as mentioned above. Appropriate dilution series of standard CoA, PPanSH and PPanSH(D4) was processed similarly before analysis. The LC separation of metabolites were obtained using Phenomenex Gemini NX-C18 analytical column (4.6 x 150mm; 3μm particles) at 45°C. The flow was maintained at 1ml/min with optimized mobile phase gradient of MQ water (A), 200mM ammonium acetate (NH₄Ac) in 95/5 MQ water/acetonitrile adjusted to pH 4.5 with acetic acid (B), and acetonitrile (C). The separated analytes
were detected with positive mode mass spectrometry (Sciex API5000 Q-trap) under unit resolution. The targeted Q1/Q3 mass/charge ions of PPanSH, PPanSH(D4), CoA and CoA(D4) were 359.1/261.1, 363.1/265.1, 768/261.1, and 772/265.1 respectively. The absolute concentration was finally calculated using linear regression analysis of respective standard compounds, except CoA(D4) which was estimated indirectly using CoA standards.

Statistical Analysis
All experimental results are presented as mean of at least 3 independent experiments ± SD, unless otherwise stated. Statistical significance was determined by a two-tailed unpaired Student’s t test between appropriate groups wherever applicable. For life span survival curve, more than 80 flies or C.elegans were used in each group and statistical significance was determined using Log-rank (Mantel-Cox) test (See figure legends for exact number or flies/C.elegans used in survival analysis). Statistical P values ≤ 0.05 were considered significant (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).
Supplementary Figure 1: CoA supplementation rescues HoPan induced phenotypes in Drosophila S2 cultured cells

a. Relative cell counts of control (100%) and HoPan treated cells in the presence of increasing concentrations of CoA. Data points represent mean ± SD (n = 3).

b-d. Protein acetylation levels were visualized in control (b) and HoPan treated cells without (c) and with (d) CoA. An antibody against acetylated Lysine (green), Rhodamin-Phalloidin (red), marking F-actin, and DAPI (blue, DNA) were used. Scale bars indicate 20 μm.
Supplementary Figure 2: Phenotypes induced by impaired pantothenate kinase in C. elegans are rescued by external supplementation of CoA

a. Quantification of motility in C. elegans pantothenate kinase (pnk-1) mutants with and without addition of different CoA concentrations (0, 50, 100, 200, 400 and 800 μM) to the food. Error bars indicate ± SD (n = 15, except for 0 μM where n = 45) and two-tailed unpaired Student’s t-test was used to assess statistical significance (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).

b. Lifespan analysis of C. elegans pnk-1 mutants untreated (n = 96) and with CoA treatment (100 μM; n = 101 and 400 μM; n = 90). Survival curves were found to be significant with P value < 0.001, analyzed with Log-rank (Mantel-Cox) test, between untreated and CoA treated pnk-1 mutants.

c-e. Representative serial images demonstrating movements of C. elegans wild-type (c) and pnk-1 mutants without (d) and with (e) CoA treatment (400 μM); still images are given; ‘merged’ indicates superimposed images. Scale bar represents 200 μm.
Supplementary Figure 3: CoA degradation profile in medium and fetal calf serum

Relative CoA levels determined by HPLC analysis in PBS, medium, medium containing fetal calf serum and in fetal calf serum (FCS) after 3 hrs incubation. Each bar represents mean data value (n = 3) ± SD and two-tailed unpaired Student’s t-test was used to analyse significance (***P ≤ 0.001).
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Supplementary Figure 4: Chromatogram profile of CoA and its thiol containing conversion product in fetal calf serum

a-e. HPLC chromatogram profile of CoA stability in PBS (a) and FCS (b) compared with standard 4’ phosphopantetheine (PPanSH, c), Pantetheine (d) and Dephospho-CoA (e). CoA is migrating at 17.65 min; PPanSH at 18.27 min; Pantetheine at 21.61 min and Dephospho-CoA at 18.85. CoA is stable in PBS and converted in serum into a thiol-containing compound exactly migrating as PPanSH standard at 18.27 min.
Supplementary Figure 5: Pantetheine stability, CoA conversion in patient’s serum and CoA and 4'-phosphopantetheine stability in human serum

**a.** Pantetheine is rapidly degraded in serum. Pantetheine was incubated for 15 min at 37°C in fetal calf serum, mouse serum and human serum and levels of total pantetheine and cysteamine were measured using HPLC. **b.** CoA hydrolysis to PPanSH in serum derived from PKAN patients and their healthy family members as a control. CoA (20µM) was incubated for 3 hrs at 37°C in serum derived from PKAN patient and in serum derived from their healthy family members as a control. Levels of CoA and PPanSH were measured using HPLC analysis. In patient’s serum CoA was as efficiently hydrolysed into PPanSH as in serum derived from healthy family members. Error bars represent ± SD where applicable for mean values (n = 3). Genders were indicated as female=F and male=M. **c.** CoA was added to human serum and concentrations of CoA and PPanSH in human serum over 6 hrs were determined by HPLC analysis. Data points indicate mean value ± SD (n = 3).
Supplementary Figure 6: HPLC chromatogram profile and confirmation by mass spectrometry of endogenous 4'-phosphopantetheine in mouse serum

a. HPLC chromatogram profile in untreated fresh mouse serum (solid line), shows a peak which co-migrates exactly with PPanSH as visible when the sample was spiked with standard PPanSH (dotted line). These results indicate the presence of endogenous PPanSH. b-c. Confirmation of endogenous PPanSH by mass spectrometry in mouse plasma (c) compared to standard PPanSH (b). d. Mass spectrometry was used to confirm the presence of elevated levels of PPanSH in plasma, 6 hrs after CoA injection (0.5mg) in mice.
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Supplementary Figure 7: CoA degradation correlates with the appearance of 4’-phosphopantetheine

a-c. 4’-phosphopantetheine measurements of the experiment shown in main Figure 4. Fetal calf serum (a), mouse serum (b) and human serum (c) were heat-inactivated or pre-treated with 10mM of EDTA, or ATP or ADP, or with the inhibitors Sodium fluoride (NaF) or Suramin and levels of PPanSH were determined as described for main Figure 4. Data represents mean value ± SD (n = 3) and solid black bars without error bars indicate no PPanSH was detected, wherever applicable.
**Supplementary Figure 8: External supplementation of 4’-phosphopantetheine rescues CoA-deprived phenotypes**

a-f. Immunofluorescence was used to visualize protein acetylation levels in control (a,d), dPANK/fbl RNAi treated (b) and HoPan treated (e) S2 cells with and without PPanSH (c,f). An antibody against acetylated Lysine (green), Rhodamin-Phalloidin (red), marking F-actin, and DAPI (blue, DNA) were used. Addition of PPanSH rescues acetylation defects of dPANK/fbl RNAi and HoPan treated S2 cells. Scale bars indicate 20µm.

g. Cell count of mammalian HEK293 control cells (100%), cells treated with HoPan with and without CoA or PPanSH added to the medium. Data indicate mean values ± SD (n = 3) and two-tailed unpaired Students t-test was used for statistical analysis.

h. Relative CoA levels were determined by HPLC of control (100%) and HoPan treated HEK293 cells with and without CoA or PPanSH added to the medium. Data indicate mean values ± SD (n = 3) and two-tailed unpaired Students t-test was used for statistical analysis.

i. Western blot analysis and quantification to determine histone acetylation levels of control HEK293 cells, cells treated with HoPan with and without CoA or PPanSH. Data represents mean values ± SD (n = 3) and two-tailed unpaired Students t-test was used for statistical analysis. In all the above data representation, statistical significance was indicated as applicable (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).
Supplementary Figure 9: 4'-phosphopantetheine is taken up by Drosophila S2 cells and converted in CoA

a-d. Mass spectrometry was used to detect the levels of 4’phosphopantetheine labelled with stable isotope (deuterium), PPanSH(D4) and CoA labelled with stable isotope (deuterium) (CoA(D4)). S2
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Cells were left untreated (a, b) or treated with HoPan (c, d) and PPanSH(D4) was added to the medium of untreated and HoPan treated cells. Levels of PPanSH(D4) (a, c) and levels of CoA(D4) (b, d) were measured. Chemical structures of PPanSH(D4) and CoA(D4) are given. e-f. Schematic overview of a Parallel Artificial Membrane Permeability Assay (PAMPA assay). Experiments were performed and the permeability parameter was calculated according to the manufacturer’s instructions. (formulas are depicted in the figure and the parameters are explained below). (f) Compounds that are below the assay threshold are predicted to be unable to pass membranes passively, whereas compounds above the threshold are able to pass membranes passively. In contrast to CoA, PPanSH shows a permeating property (although the permeability parameter of PPanSH is lower compared to the positive control). Caffeine and amiloride were used as a positive and negative control respectively. Data values represents mean ± S.E.M with replicated for PPanSH and CoA; n = 12 and caffeine and amiloride; n = 4). Abbreviations are as follows:

Ceq = Equilibrium Concentration
CD = Concentration in donor well
VD = Volume of donor well (0.3 ml)
CA = Concentration in acceptor well
VA = Volume of acceptor well (0.2 ml)
P = Permeability
S = Membrane area (0.3 cm²)
t = Incubation time (18000 s)

Supplementary Figure 10: External supplementation of CoA rescues dPANK/fbl- and dPPCDC- but not COASY impaired phenotypes

From left to right; Overview of the well-known CoA biosynthesis route in which the enzymatic conversion steps 1, 2 and 3, upstream of PPanSH and the combined enzymatic step 4-5 downstream of PPanSH are indicated. Mutant lines and/or RNAi lines used for manipulating conversion steps upstream and downstream of PPanSH are indicated. The upper image represents time scale and images of normal Drosophila developmental and adult stages. Fly line and mutant-specific developmental arrest is indicated under control conditions (dotted line) and after CoA supplementation to the food (solid line).
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Supplementary Figure 11: Quantitative real-time PCR data demonstrates reduced mRNA expression of dPPCDC and dCOASY in Drosophila RNAi lines and mutant flies

a. mRNA expression levels of dPPCDC normalized with house-keeping gene (rp49) expression levels in 1 day old adult dPPCDC RNAi Drosophila female flies and in age-matched control flies.

b. mRNA expression levels of dPPCDC normalized with house-keeping gene (rp49) expression levels in L2 stage control larvae and in L2 stage dPPCDC mutant larvae.

c. mRNA expression levels of dCOASY normalized with house-keeping gene (rp49) expression levels in L1 stage control larvae and in L1 stage dCOASY mutant larvae. All data presented above indicate the mean ± SD (n = 3) and two-tailed unpaired Student’s t-test was used for statistical analysis (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).
Supplementary Figure 12: External supplementation of CoA rescues dPANK/fbl- and dPPCDC- but not COASY impaired phenotypes

a. Western blot analysis of dPANK/Fbl protein expression levels of control animals, homozygous hypomorphic (dPANK/fbl^1) mutants and homozygous null (dPANK/fbl^null) mutants. Tubulin as loading control.

b. CoA and PPanSH levels measured by HPLC analysis in 1-day old hypomorphic homozygous (dPANK/fbl^1) mutant and control adult flies. CoA and PPanSH levels in mutant larvae are presented as percentages of CoA levels in control larvae.

c. CoA and PPanSH levels measured by HPLC in early L2 stage null homozygous (dPANK/fbl^null) mutant and control larvae. CoA levels in mutant larvae are presented as percentages of CoA levels in control larvae.

d. Relative CoA and PPanSH levels measured by HPLC in 1-day old females of the dPPCDC RNAi fly line compared to control flies.

e. Relative CoA and PPanSH levels measured by HPLC of the L2 larval stage of control and homozygous dPPCDC mutant larvae.

f. Relative CoA and PPanSH levels measured by HPLC of 1-day old homozygous dCOASY mutant larvae, compared to control. All the data sets in b-f indicate mean ± SD (n = 3) in the above representations and two-tailed unpaired Student’s t-test was used for statistical analysis (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).
Supplementary Figure 13: Life span survival of Drosophila dPANK mutant flies and endogenous 4’ phosphopantetheine levels in food sources.

a. Lifespan analysis of hypomorphic (dPANK/fbl1) homozygous female mutants untreated (n = 207) and treated with various concentrations of CoA (6mM, n = 105; 9mM, n = 115; and 12mM, n = 88) added to the food. Survival curves were found to be significant with P value < 0.001, analyzed with Log-rank (Mantel-Cox) test, between untreated and all CoA treated dPANK/fbl1 mutants.

b. In various animal food sources (yeast, E.coli and mouse liver) levels of CoA and PPanSH were measured and found to be present (n = 2).
**Supplementary Figure 14: External supplementation of CoA rescues phenotypes of dPPCDC RNAi lines and COASY is required for CoA rescue in mammalian cells.**

a-c. Ovaries of 4-day old control and dPPCDC RNAi expressing flies, stained with Rhodamin-Phalloidin (magenta, marking F-actin) and the nuclear marker DAPI (green) and imaged with confocal microscopy. (a) In wild-type ovarioles strings of developing egg-chambers, from the germarium up to stage 9 were visible. Mature eggs were also found (marked by asterisks), identifiable by the presence of yolk. (b) In ovaries of the dPPCDC RNAi expressing flies, egg-chambers developed normally until...
stage 7. From stage 8 on, fragmented and condensed DNA was visible, indicating apoptosis (marked by white arrowheads). No egg-chambers older than stage 8/9 or mature eggs were found in these ovaries. (c) CoA treatment of the dPPCDC RNAi expressing flies improved egg-production significantly and eggs developed to maturity (marked by asterisks). Scale bars =100μM. d. Increased fertility of dPPCDC RNAi expressing females. Untreated, Vitamin B5 treated and CoA treated dPPCDC RNAi expressing females were mated with control males and put onto apple juice plates to allow egg laying for 4 days. For untreated and Vitamin B5 treated females, no or only very few eggs were observed on the plates (compare Figure 6e). CoA treated females produced a significant number of eggs that developed into pupae which eclosed resulting in viable offspring (compare Figure 6f). Scale bar = 1 cm. e. Lifespan analysis of adult female dPPCDC RNAi flies untreated (n = 111) and treated with various concentrations of CoA (9mM, n = 106; 18mM, n = 102; 21mM, n = 104) added to the food. Survival curves were found to be significant with P value < 0.01 for CoA 9mM treatment and P value < 0.001 for CoA (18 and 21mM) treatment compared to control untreated dPPCDC RNAi mutants, analyzed with Log-rank (Mantel-Cox) test. f. RNAi was used to down-regulate COASY in HEK293 cells treated or non-treated with CoA and acetyl histone3 levels were quantified (n = 5). Insert: Western blot analysis showing successful down-regulation of human COASY by RNAi and decreased histone acetylation (and quantification). GAPDH was used as loading control. g. Relative levels of CoA and PPAnSH were measured in control HEK293 and COASY down-regulated cells treated with medium with and without addition of CoA (n = 4). The data (f,g) indicate the mean ± SD and two-tailed unpaired Student’s t-test was used for statistical analysis ( **P ≤ 0.01, ***P ≤ 0.001).
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Supplementary Figure 15: Full uncut gel images for Western blots presented in Figures 1b and 1h and Supplementary Figure 8i

a-b. Full uncut gel images for the anti-dPANK/Fbl and anti-Tubulin Western blots presented in Figure 1b. The part of the Western blot used for the figure is outlined by a dashed line. c-d. Full uncut gel images for the anti-acH3 and anti-GAPDH Western blots presented in Figure 1h. The part of the Western blot used for the figure is outlined by a dashed line. e-f. Full uncut gel images for the anti-acH3 and anti-GAPDH Western blots presented in Supplementary Figure 8i. The part of the Western blot used for the figure is outlined by a dashed line.
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Supplementary Figure 16: Full uncut gel images for Western blots presented in Supplementary Figures 12a and 14f

a-b. Full uncut gel images for the anti-dPANK/Fbl and anti-Tubulin Western blots presented in Supplementary Figure 12a. The part of the Western blot used for the figure is outlined by a dashed line.

c-e. Full uncut gel images for the anti-COASY, anti-acH3 and anti-GAPDH Western blots presented in Supplementary Figure 14f. The part of the Western blot used for the figure is outlined by a dashed line.
4′-Phosphopantetheine (PPanSH) was synthesized in a three-step procedure as described below (a/b/c) (Supplementary Note). In the first step, commercially available pantothenic acid was coupled with synthesized S-tritylcysteamine. The obtained S-tritylpantetheine was then phosphorylated with freshly prepared dibenzylchlorophosphate. Finally, removal of benzyl groups provided 4′-phosphopantetheine.
D-Pantothenic acid was prepared from its hemicalcium salt (Aldrich, ≥ 99.0 %) by reacting with oxalic acid in distilled water. The precipitated calcium oxalate was filtered off, while the protonated form of D-pantothenic acid was obtained by evaporation of water. S-tritylcysteamine was synthesized from cysteamine hydrochloride and trityl chloride. Dibenzylchlorophosphate was prepared by reacting dibenzylphosphite with N-chlorosuccinimide in toluene as a solvent. All other chemicals were obtained from commercial sources and used without further purification; cysteamine hydrochloride (Aldrich, ≥ 98.0 %), trityl chloride (Aldrich, 97.0 %), N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide (EDC) (Aldrich, ≥ 97.0 %), 1-hydroxybenzotriazole hydrate (HOBT) (Aldrich, ≥ 97.0 %), dibenzylphosphate (Aldrich, technical grade), N-chlorosuccinimide (Aldrich, 98 %). Column chromatography was carried out using Silica gel 60 Å, 60-100 mesh (Aldrich). Cation exchange chromatography was performed on DOWEX 50WX2, hydrogen form, 100-200 mesh (Aldrich). 1H and 13C NMR were recorded at 25°C with Varian Unity Inova 300 MHz spectrometer (300 MHz/75 MHz). The chemical shifts (δ) are reported in ppm units relative to TMS as an internal standard where spectra recorded in CDCl3 or relative to residual solvent signal when D2O was used. High-resolution mass spectra were obtained on AutospecQ mass spectrometer with negative electrospray ionization.

a) Coupling reaction – synthesis of S-tritylpantetheine
In dried acetonitrile (100ml) the following were prepared separately: (A) D-pantothenic acid (2.19g, 10.0mmol), (B) S-tritylcysteamine (3.19g, 10.0mmol) and (C) N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide (EDC) (1.55g, 10.0mmol) together with 1-hydroxybenzotriazole hydrate (HOBT) (1.35g, 10.0mmol). After A, B and C were mixed together, triethylamine (10.4ml, 75mmol) was added. The mixture was stirred at room temperature for 24 h and quenched with addition of water (400ml). The product was extracted with diethyl ether (3x250ml). The combined organic phases were washed with 1 M hydrochloric acid, saturated aqueous solution of NaHCO3 (500ml), and brine (500ml). The organic layer was dried over sodium sulfate and concentrated in vacuum S-tritylpantetheine (3.53g, 68%) was synthesized as pale-yellow crystals. 1H NMR (300 MHz, CDCl3) δ 0.85 (s, 3H), 0.92 (s, 3H), 2.29 (app t, J = 6.2 Hz, 2H), 2.38 (td, J = 2.3, 6.6, 6.8 Hz, 2H), 3.03 (m, 2H), 3.38-3.49 (m, 4H), 3.92 (s, 1H), 6.20 (t, J = 5.7 Hz, 1H, NH), 7.17-7.29 (m, 10 H), 7.36-7.45 (m, 5H).

b) Phosphorylation – synthesis of S-trityl-4‘-dibenzylphosphopantetheine
Dibenzylchlorophosphate was freshly prepared by allowing a reaction of dibenzylphosphite (2.16g, 8.24mmol) with N-chlorosuccinimide (1.21g, 9.06mmol) in toluene (40ml) at room temperature for 2 h. The mixture was filtered and the filtrate was evaporated under vacuum and added to a solution of S-tritylpantetheine (2.86g, 5.49mmol), diisopropylethylamine (3.06ml), 4-dimethylaminopyridine (0.067g, 0.55mmol) in dry acetonitrile (50ml). The mixture was stirred for 2 h at room temperature. Acetonitrile was removed under vacuum. Products were extracted into organic phase in dichloromethane (3x100ml) – aqueous NaHCO3...
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(100ml) system. The organic extracts were washed with water (100ml), and dried over Na₂SO₄. Evaporation of solvent gave a crude S-trityl-4’-dibenzylphosphopantetheine as a dark brown oil (4.69g), which was further purified by flash chromatography (SiO₂, EtOAc, MeOH) to give a semicrystalline pale yellow product (0.640g, 0.82mmol). The yield of the synthesis and purification of S-trityl-4’-dibenzylphosphopantetheine is 15 %. ¹H NMR (300 MHz, CDCl₃) δ 0.75 (s, 3H), 1.03 (s, 3H), 2.32 (app t, J = 6.1 Hz, 2H), 2.4 (app t, J = 6.5 Hz, 2H), 3.06 (app q, J = 6.3 Hz, 2H), 3.47 (app q, 6.0 Hz, 2H), 3.60 (dd, J = 9.9, 7.3 Hz 1H), 3.85 (s, 1H), 4.00 (dd, J = 9.9, 7.0 Hz, 1H), 4.99-5.04 (m, 4H), 5.80 (t, J= 5.5 Hz, 1H, NH), 7.16-7.32 (m, 20H), 7.38-7.40 (m, 5H).

c) Deprotection – synthesis of 4’-phosphopantetheine

Naphthalene (12.9g, 100.6mmol) dissolved in tetrahydrofuran (70ml) was added to sodium metal (Na) (2.21g, 96.1mmol) in tetrahydrofuran (50mL). After 2 h the solution was cooled to −(35±5)°C and S-trityl-4’-dibenzylphosphopantetheine (1.85g, 2.37mmol) dissolved in tetrahydrofuran (70ml) was slowly added. The mixture was stirred for 2 h while maintaining the temperature below −30°C. The reaction was quenched by addition of water (100ml) and then dichloromethane (200ml) was added. Phases were separated and the aqueous phase (together 500ml) was washed with dichloromethane (200ml) and diethylether (3x200ml), concentrated under vacuum and passed through the cation exchange column (DOWEX 50WX2, 200g). Fractions were analyzed by LCMS and those containing the product were pooled and concentrated under vacuum. 4’-phosphopantetheine was precipitated with addition of Ca(OH)₂ as a calcium salt (332mg, 0.838mmol, 35%). The structure of the product was confirmed by comparison of NMR data with the literature¹³ and by HRMS. ¹H NMR (300 MHz, D₂O) δ 0.86 (s, 3H), 1.08 (s, 3H), 2.54 (app t, J = 6.3 Hz, 2H), 2.87 (app t, J = 6.3 Hz, 2H), 3.43 (dd, J = 10.3, 5.0 Hz, 1H), 3.54 (m, 4H), 3.76 (dd, J = 10.3, 6.5 Hz, 1H), 4.14 (s, 1H). The HRMS mass for C₁₁H₂₂N₂O₇SP [M-H]⁻ was found to be 357.0880, which corresponds to the expected mass of 357.0885. The purity of the compound was determined to be >92%, using HPLC coupled with UV detection at 205nm.
SUPPLEMENTAL REFERENCES


