Comparative Metabolomic Profiling Reveals That Dysregulated Glycolysis Stemming from Lack of Salvage NAD\(^+\) Biosynthesis Impairs Reproductive Development in Caenorhabditis elegans\(*\)**

Received for publication, May 4, 2015, and in revised form, August 22, 2015 Published, JBC Papers in Press, September 8, 2015, DOI 10.1074/jbc.M115.662916

Wenqing Wang\(^*\), Melanie R. McReynolds\(^1\), Jimmy F. Goncalves\(^2\), Muya Shu\(^3\), Ineke Dhondt\(^4\), Bart P. Braeckman\(^5\), Stephanie E. Lange\(^6\), Kelvin Kho\(^6\), Ariana C. Detwiler\(^6\), Marisa J. Pacella\(^5\), and Wendy Hanna-Rose\(^*\)

From the \(^6\)Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802 and \(^4\)Laboratory for Aging Physiology and Molecular Evolution, Department of Biology, Ghent University, Proeftuinstraat 86 N1, 9000 Ghent, Belgium

**Background:** Loss of NAD\(^+\) salvage biosynthesis causes a reproductive delay in *C. elegans.*

**Results:** Loss of salvage biosynthesis causes cytoplasmic, but not mitochondrial, NAD\(^+\) deficiency-related metabolic deficits.

**Conclusion:** Salvage synthesis is required for glycolysis, and in the absence of glycolysis, reproductive progression cannot be sustained.

**Significance:** We elucidate the developmental roles of NAD\(^+\) biosynthetic pathways and the effects of interventions targeting NAD\(^+\) salvage biosynthesis.

Temporal developmental progression is highly coordinated in *Caenorhabditis elegans*. However, loss of nicotinamidease PNC-1 activity slows reproductive development, uncoupling it from its typical progression relative to the soma. Using LC/MS we demonstrate that *pnc-1* mutants do not salvage the nicotinamide released by NAD\(^+\) consumers to resynthesize NAD\(^+\), resulting in a reduction in global NAD\(^+\) bioavailability. We manipulate NAD\(^+\) levels to demonstrate that a minor deficit in NAD\(^+\) availability is incompatible with a normal pace of gonad development. The NAD\(^+\) deficit compromises NAD\(^+\) consumer activity, but we surprisingly found no functional link between consumer activity and reproductive development. As a result we turned to a comparative metabolomics approach to identify the cause of the developmental phenotype. We reveal widespread metabolic perturbations, and using complementary pharmacological and genetic approaches, we demonstrate that a glycolytic block accounts for the slow pace of reproductive development. Interestingly, mitochondria are protected from both the deficiency in NAD\(^+\) biosynthesis and the effects of reduced glycolytic output. We suggest that compensatory metabolic processes that maintain mitochondrial activity in the absence of efficient glycolysis are incompatible with the requirements for reproductive development, which requires high levels of cell division. In addition to demonstrating metabolic requirements for reproductive development, this work also has implications for understanding the mechanisms behind therapeutic interventions that target NAD\(^+\) salvage biosynthesis for the purposes of inhibiting tumor growth.

NAD\(^+\), which is synthesized primarily from vitamin B\(_3\), is a key cellular metabolite. It is well recognized for its role in redox metabolism and has garnered renewed attention as an obligate co-substrate for NAD\(^+\) consumer enzymes that mediate critical cell stress responses and signaling events (for recent reviews, see Refs. 1 and 2). There is widespread interest in pharmacological manipulation of NAD\(^+\) consumer activities because of their roles in health span and disease. As a result, NAD\(^+\) biosynthetic processes, which promote activity of NAD\(^+\) consumers, have been examined as drug targets (3–5). There is interest in both boosting NAD\(^+\) biosynthesis for therapeutic benefit in age-related diseases (6) and inhibiting NAD\(^+\) biosynthesis for therapeutic benefit in cancer (7–9). However, we have not fully elucidated how perturbing the availability of specific NAD\(^+\) biosynthetic precursors or the availability of NAD\(^+\) in various tissues or cellular compartments impacts organism physiology, especially beyond the activity of the NAD\(^+\) consumers. For example, although we have largely eradicated pellagra, the deadly disease resulting from dietary deficiency of vitamin B\(_3\), via supplementation of our food supply, we still lack a basic understanding of the etiology of the hallmark skin, digestive, and nervous system pathologies that are presumably due, at least in part, to a deficiency in NAD\(^+\) biosynthesis (10, 11). Furthermore, the mechanisms leading to toxicity upon treatment with inhibitors of NAD\(^+\) salvage biosynthesis are not well elucidated (9), and effects on development are largely unexamined.

We use the genetically tractable *Caenorhabditis elegans* system as a model to probe the roles of NAD\(^+\) biosynthetic pathways and metabolites in whole organism development and physiology. We have revealed that the reproductive system as well as muscle development and function are particularly sensitive to loss of ability to synthesize NAD\(^+\) via a salvage pathway from nicotinamide and that these phenotypes are caused by the tissue-specific effects of both nicotinamide and nicotinic acid.
levels (12–14). In this study we demonstrate that salvage synthesis has a mild global negative effect on NAD$^+$ levels but that mitochondrial function is preserved when salvage synthesis from nicotinamide (NAM)$^2$ is blocked. Although the overall effect on NAD$^+$ levels upon loss of salvage biosynthesis is mild, we show that efficient glycolysis depends on salvage synthesis from NAM and that the reproductive development phenotype in the NAD$^+$ salvage synthesis mutant results from the block in glycolysis.

In eukaryotes, NAD$^+$ is synthesized from dietary-derived and salvaged forms of vitamin B$_6$, such as nicotinamide and nicotinic acid and from tryptophan (15, 16) (Fig. 1A). Nicotinic acid is processed by the highly conserved Preiss-Handler (17, 18) pathway to synthesize NAD$^+$. Dietary nicotinamide riboside (NR) or nicotinic acid riboside are used as substrates by nicotinamide riboside kinase to produce intermediates that are processed by Preiss-Handler pathway enzymes to synthesize NAD$^+$ (19, 20). The hydrolysis of NAD$^+$ by NAD$^+$ consumer enzymes produces NAM, which is used to resynthesize NAD$^+$ via the salvage pathway. In vertebrates, salvage biosynthesis requires the enzyme nicotinamide phosphoribosyltransferase (NAMPT), which produces nicotinamide mononucleotide (NMN) from NAM. NMN is then converted directly to NAD$^+$ by nicotinamide mononucleotide adenylyltransferase (NMAT), the penultimate enzyme of the Preiss-Handler pathway (21). However, invertebrates, including C. elegans, use an alternative salvage pathway whereby NAM is converted to nicotinic acid (NA) by a nicotinamidase, encoded by the pnc-1 gene in C. elegans, and fed into the beginning of the Preiss-Handler pathway (Fig. 1A) (21). Although NAMPT and nicotinamidase are distinct enzymes and co-exist only in select organisms (15), they have biologically comparable functions in consuming NAM and producing NAD$^+$. Human NAMPT can partially compensate for the loss of the C. elegans nicotinamidase pnc-1, demonstrating an equivalent biological role for the two enzymes (12). Finally NAD$^+$ is produced from the essential amino acid tryptophan through the kynurenine pathway, which produces quinolinic acid that is converted to nicotinic acid mononucleotide via quinolinic acid phosphoribosyltransferase (21). The C. elegans genome does not encode an apparent homolog of quinolinic acid phosphoribosyltransferase (12, 15). Thus, whether tryptophan can be used to synthesize NAD$^+$ in C. elegans is unclear.

NAD$^+$ is required as an enzyme co-factor in the cytoplasm and the mitochondria. It also has signaling roles in these compartments as well as in the nucleus and the extracellular space. Recognition of these various roles has created new questions about the bioavailability of NAD$^+$ in various compartments and how the molecule is partitioned. Subcellular localization patterns of NAD$^+$ biosynthetic enzymes suggest that different cellular compartments rely on different precursors for NAD$^+$ biosynthesis. It appears that NAM, NA, NR, and nicotinamide riboside can be used for NAD$^+$ biosynthesis in the cytoplasm, whereas NMN may be the sole precursor for mitochondrial NAD$^+$ biosynthesis (22). The presence of distinct biosynthetic pathways combined with the lack of movement of NAD$^+$ between compartments suggests that cells may separately regulate NAD$^+$ bioavailability in a particular compartment and/or respond in a distinct manner to a perturbation in NAD$^+$ bioavailability in one compartment versus another (23). Compartment-specific differences in NAD$^+$ levels have been observed. For example, mitochondria in cardiac and other myocytes are capable of storing a much higher concentration of NAD$^+$ than the cytoplasm or nucleus, and neurons have been shown to have more equal proportions between the mitochondria and the rest of the cell (23–26) Compartmentalization of NAD$^+$ homeostasis also provides a mechanism for cells to link distinct metabolic activities to distinct NAD$^+$ signaling activities (27). In this study, we present evidence that compromised salvage synthesis preferentially affects NAD$^+$ availability outside the mitochondrial compartment.

Mutation of the C. elegans pnc-1 nicotinamidase gene causes a variety of developmental and physiological defects (12, 13, 28). These phenotypes comprise three distinct classes: those that can be mimicked by supplementation of wild-type animals with the PNC-1 substrate NAM, those that are rescued by supplementation of mutants with the PNC-1 product NA, and those where both lack of NA production and accumulation of substrate make a contribution to the phenotypic outcome (12, 13). In particular pnc-1 mutants have a delay in development of the gonad relative to the soma; the gonad develops more slowly than expected and no longer displays the characteristic synchrony with somatic development that is expected in the highly invariant C. elegans developmental program. Providing NA as a supplement to the pnc-1 mutant cultures rescues this delay in development, suggesting that the lack of NAD$^+$ biosynthesis underlies the gonad developmental phenotype. Here we further investigated this hypothesis by measuring metabolite levels in wild-type and mutant animals as well as in animals treated with conditions predicted to alter NAD$^+$ levels. Our data support the hypothesis that a specific lack of NAD$^+$ bioavailability is the underlying cause of the reproductive developmental delay and reveal that this deficit appears to affect the nucleo-cytoplasmic compartment preferentially. We use a metabolomics approach to identify perturbations specifically linked to the change in NAD$^+$ availability and to investigate the mechanism causing the reproductive delay.

**Experimental Procedures**

### C. elegans Culture and Strains

Strains were maintained under standard conditions with *Escherichia coli* OP50 as food at 20 °C (29) unless otherwise specified. N2 is the wild-type strain. UV-irradiated OP50 plates were prepared as described (12), and killing was confirmed by streaking to LB agar.

Strains and alleles used were as follows. *pnc-1(pk9605)* is the *pnc-1* null allele (12). sir-2.1(osh434), sir-2.3(osh444), sir-2.4(n1537), CF1553 (mul184 [pAD76] sod-3p::GFP + rol-6), and NL3909 (unc-119(ed3); pkk1642 [unc-119(+)] + R11A8.5(+))

---

$^2$The abbreviations used are: NAM, nicotinamide; NR, nicotinamide riboside; NAMPT, nicotinamide phosphoribosyltransferase; NMN, nicotinamide mononucleotide; NMAT, nicotinamide mononucleotide adenylyltransferase; NA, nicotinic acid; 3PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PARP, poly(ADP-ribose) polymerase; DHAP, dihydroxyacetone phosphate; 3PGA, 3-phosphoglycerate.
sir-2.1(+)) (30) were provided by the Caenorhabditis Genetic Center. HA2040 (sir-2.1(ok434); sir-2.2(n5136); sir-2.4(n1537)) and HA2041 (sir-2.1(ok434); sir-2.3(ok444); sir-2.4(n1537)) were provided by Anne Hart. sir-2.2(tm2673) and nmat-2(tm2905) were provided by the Mitani laboratory, and nmat-2(tm2905) was outcrossed against N2 three times, is homozygous sterile, and was balanced with hT2. Only non-GFP homozygous nmat-2(tm2905) mutants were used in pheno-
typic analysis.

Metabolite Supplementation
We added filter-sterilized stock solutions to UV-killed OP50 plates to a final concentration of 25 mM NA (Alfa Aesar), 2.5 mM Metabolite Supplementation.

Phenotypic Analysis
We performed targeted LC-MS and GC-MS analysis with the Metabolomics Core Facility at Penn State to measure NAD⁺ and glycolytic intermediates. ~50 μl of worms were collected in double distilled H₂O, flash-frozen in liquid nitrogen, and stored at ~80 °C. 15-μl samples were extracted in 1 ml of 3:3:2 acetonitrile:isopropyl alcohol:H₂O with 1 μM chlorpro-
pamide as the internal standard. Samples were homogenized using a Precellys™ 24 homogenizer. Extracts from samples were dried under a vacuum, resuspended in HPLC Optima Water (Thermo Scientific), and divided into two fractions, one for LC-MS and one for BCA protein analysis. Samples were ana-
yzed by LC-MS using a modified version of an ion-pairing reversed phase negative ion electrospray ionization method (31). Samples were separated on a Supelco (Bellefonte, PA) Titan C18 column (100 × 2.1-mm 1.9-μm particle size) using a water-methanol gradient with tributylamine added to the aque-
ous mobile phase. The LC-MS platform consisted of Dionex Ultimate 3000 quaternary HPLC pump, 3000 column compart-
ment, 3000 autosampler, and an Exactive plus orbitrap mass spectrometer controlled by Xcalibur 2.2 software (all from ThermoFisher Scientific, San Jose, CA). The HPLC column was maintained at 30 °C and a flow rate of 200 μl/min. Solvent A was 3% aqueous methanol with 10 mM tributylamine and 15 mM acetic acid; solvent B was methanol. The gradient was 0 min, 0% B; 5 min, 20% B; 7.5 min, 20% B; 13 min, 55% B; 15.5 min, 95% B, 18.5 min, 95% B; 19 min, 0% B; 25 min 0% B. The orbitrap was operated in negative ion mode at a maximum resolution (140,000) and scanned from m/z 85 to m/z 1000. Metabolite levels were corrected to protein concentrations determined by BCA assay (Thermo Fisher).

NAD/NADH Ratio
300 μl of mixed stage N2 or pnc-1 animals (cultured on live or UV-killed OP50) were collected in M9, snap-frozen in liquid nitrogen, and stored at ~80 °C. 50 μl of thawed sample was added to duplicate wells of a black 96-well plate. NAD and NADH measurements were performed using the Elite Fluorimetric NAD/NADH Ratio Assay kit (eENZYME, LLC, Gai-
thersburg, MD) according to the manufacturers’ instructions.

RNAi
We generated an nprt-1 RNAi construct by PCR amplifica-
tion of nprt-1 cDNA and insertion into vector pPD129.36 (32). Primer sequences were nprtRNAiSacII (5′-ccgctggatcttgc-
cattgtgc-3′) and nprtRNAiXhoI (5′-ctcgagcccaaatcctc-
ccgaaatc). qns-1, pfk-1.1, pfk-1.2, tpi-1, cyc-1, nuo-1, atp-2, atp-5, sir-2.2, and sir-2.3 RNAi clones were from the C. elegans RNAi Library (Source BioScience, Nottingham, UK). Constructs were confirmed by sequencing. RNAi feeding assays were carried out as described (33). We transferred L4 animals to RNAi plates and scored their progeny for gonad developmental delay. E. coli strain HT115 carrying the empty RNAi vector pPD129.36 was used as a control.

Phenotypic Analysis
Gonad Development—Mid-L4 stage animals with an open lumen in the vulva and the uterus are normal. “Delayed” ani-
mals are those that do not yet have an open uterine lumen when the vulval lumen achieves its characteristic mid-L4 morphology (12).

Mitochondrial Sensitivity—For the paralyzing assay we treated L4 animals that were aged 24 h with 20 mM sodium azide (Sigma) in a depression slide at time 0. The number of animals that were moving was recorded every 5 s until all ani-
mals were paralyzed. For the recovery assay we treated the ani-
mals with 5 mM sodium azide for 15 min (all animals were paralyzed). We replaced the sodium azide with M9 buffer at
time 0 and recorded the number of animals that had started moving again every 5 min until all worms recovered. All assays were repeated at least three times. Thrashing assays were performed as previously described (13).

**O₂ Consumption and Heat Production Methods**—Synchronized day 2 adults were collected and washed in S-basal medium. 400–600 µl of worm suspension (dependent on worm number) was used for live oxygen consumption (928 six-channel oxygen system, Strathkelvin Instruments) and heat production measurements (2277 Thermal Activity Monitor, Thermometric). To prevent death of the pnc-1 mutants from internal rupture due to inability to lay eggs, 300 µM fluorodeoxyuridine (FUdR) was supplemented to pnc-1 mutants and N2 controls beginning at the L4 stage. Heat production and oxygen consumption data were normalized to total protein determined by BCA assay of a parallel aliquot of worm suspension. All essays were repeated three times independently.

**sod-3::GFP Expression**—L4 animals were placed on fresh plates and imaged on a Nikon SMZ1500 stereoscope. Images were collected and analyzed using NIS-Elements software from Nikon. The pharynx of each animal was selected, and the mean GFP signal for each animal was calculated.

**Statistical Analysis**

Fisher’s exact test was carried out to determine p values in gonad development, thrashing, and sodium azide treatment assays. For quantification of sod-3::GFP intensities, oxygen consumption, and heat production assays, p values were calculated using Student’s t test. In LC-MS and GC-MS analysis we used Welch’s two-sample t test to calculate p values. In all figures: §, \(0.05 < p < 0.1\); *, \(0.01 < p < 0.05\); **, \(0.001 < p < 0.01\); ***, \(p < 0.001\).

**Results**

**Loss of Salvage NAD⁺ Biosynthesis Resulted in Minor Global Depletion of NAD⁺**

We used LC-MS to investigate if salvage biosynthesis of NAD⁺ from NAM is affected by loss of pnc-1 function using the pnc-1(pk9605) allele (12). pnc-1(pk9605) mutants showed a 19-fold increase in substrate NAM levels and an 11-fold decrease in NA product levels relative to wild-type animals (Fig. 4A). The C. elegans genome encodes a second nicotinamidase called PNC-2. Kinetically, PNC-2 is a 0.05 fold increase in substrate NAM levels and an 11-fold decrease in NA product levels relative to wild-type animals (Fig. 1). We next tested the consequences of decreasing NAD⁺ levels in the pnc-1 mutant using two strategies. First, we supplemented cultures with a precursor for NAD⁺ biosynthesis that is processed via a pathway other than NAM salvage. Supplementation experiments were conducted on UV-killed E. coli OP50 strain as a food source to prevent metabolism of the supplement by the E. coli. Supplementation of cultures with 1.25 mM NR effectively rescued the reproductive delay in pnc-1 mutants (Fig. 2A), and we confirmed that NR-supplementation elevates NAD⁺ levels (Fig. 2B) as previously reported (35, 36). We also measured NAD⁺ levels in pnc-1 mutants that were supplemented with NA, which rescues the gonad delay (12). We found a trend toward an increase in NAD⁺ levels with an average 70% increase; however, there was no significant difference upon statistical analysis (\(p = 0.147\)) (Fig. 2B). Second, we used a genetic strategy to reduce consumption of NAD⁺ in vivo. PARPs are major consumers of NAD⁺, and an increase in NAD⁺ has been observed in pme-1/PARP loss-of-function mutants (36). The reproductive delay associated with pnc-1(pk9605) is alleviated by the presence of a pme-1 mutation (Fig. 2C), and we confirmed higher NAD⁺ levels in the pme-1; pnc-1 double mutants (Fig. 2D).

We next tested the consequences of decreasing NAD⁺ availability. We predicted that knockdown of other enzymes in the NAD⁺ salvage/Preiss-Handler pathway should both reduce NAD⁺ availability and mimic the pnc-1 gonad development phenotype. RNAi of nprt-1 or qns-1 each recapitulate a pnc-1-like gonad developmental delay in a portion of the population (Figs. 3A and 4). Although the NAD⁺ levels trend toward lower average levels (Fig. 3B), the differences were not statistically significant.

We also tested the effects of reducing nicotinamide mononucleotide adenyltransferase activity. A predicted null allele of nmat-2, tm2905, has a severe gonad development phenotype...
We could not measure NAD$^+$ levels in the nmat-2(tm2905) mutant because the homozygous animals are sterile (data not shown), precluding our ability to collect the thousands of animals required for LC-MS analysis. However, we used this allele to demonstrate that the NR-mediated rescue of gonad developmental delay is because of activity as an NAD$^+$ biosynthetic precursor. We repeated the NR supplementation experiment in the nmat-2(tm2905) mutant, which should be compromised in ability to use NR to synthesize NAD$^+$. As expected, the nmat-2 mutants are not rescued upon supplementation with NR (Fig. 3C).

The correlation between a mild deficit in NAD$^+$ bioavailability and a gonad developmental delay is further supported by the identification of culture conditions that simultaneously increase the penetrance of the gonad delay phenotype and exacerbate the NAD$^+$ deficiency in the pnc-1 mutant. We previ-
viously reported that culturing animals on UV-killed *E. coli* exacerbates the *pnc-1* gonad developmental defects relative to growth on live food (e.g. compare phenotype penetrance in Fig. 2, A and C) (12), and we show here that the UV-killed conditions also further depress NAD^+^ levels (Fig. 1D), still without affecting the NAD/NADH ratio (Fig. 1E). Together our experiments are consistent with the conclusion that lack of NAD^+^ salvage biosynthesis from NAM results in a mild deficit in NAD^+^ bioavailability, and this deficit in the availability of NAD^+^ itself and not, for example, other intermediate metabolites in the biosynthetic pathway, is causative of a developmental delay in the reproductive system.

**FIGURE 2. Increasing NAD^+^ levels in *pnc-1* mutants rescues gonad developmental delay.** Supplementation of 1.25 mM NR to *pnc-1*(pk9605) mutants effectively rescues the gonad developmental defects (A) and raises NAD^+^ levels (B); *pme-1* (ok988) loss-of-function also rescues the gonad developmental defects of a significant percentage of *pnc-1* mutants (C) and moderately increases NAD^+^ levels (D). NAD^+^ levels were measured using LC-MS. In histograms, error bars are S.E. ***, p < 0.001**, calculated using Fisher’s exact test. Box plots are as described in Fig. 1. *, 0.01 < p < 0.05; ***, p < 0.001, calculated with Welch’s two sample t test. Animals in A and B were cultured on UV-killed OP50 plates. Animals in C and D were cultured on standard live OP50 plates.

**FIGURE 3. Inhibiting NAD^+^ biosynthesis causes a gonad developmental delay.** A, RNAi of *nprt-1* or *qns-1* causes gonad developmental delay. B, NAD^+^ levels in *nprt-1* or *qns-1* RNAi animals showed a mild decreasing trend compared with animals on control RNAi; however, the difference is not statistically significant. C, *nmat-2* mutants have a delay in gonad development, which cannot be rescued by 1.25 mM NR supplementation. In histograms, error bars are S.E. ***, p < 0.001**, calculated using Fisher’s exact test. Box plots are as described in Fig. 1. Animals in A and B were cultured on *E. coli* strain HT115 for RNAi. Animals in C were cultured on UV-killed OP50 plates.
PNC-1 Loss-of-function Inhibited Sirtuin Activity but This Effect Did Not Cause the Developmental Phenotype

We next considered the question of how a decrease in biosynthesis of NAD+ might result in a reproductive delay. We investigated several hypotheses to relate NAD+ bioavailability to the developmental phenotype. First, we predicted that lack of NAD+ bioavailability would compromise NAD+ consumer activity (37) and that reduced consumer activity might underlie the phenotype. We specifically examined sirtuin NAD+ consumer activity by using a sod-3::GFP reporter as a readout of SIR-2.1 activity. Expression of sod-3::GFP was elevated by overexpression of SIR-2.1 (Fig. 5, A and B, and Berdichevsky et al. (30)). As predicted, SIR-2.1-mediated up-regulation of sod-3::GFP required pnc-1 (Fig. 5, A and B), supporting the conclusion that SIR-2.1 activity is reduced in the absence of PNC-1.

To look for a functional relationship between reduced sirtuin activity and reproductive development, we examined animals carrying a sir-2.1 deletion allele but found no gonad developmental delay (data not shown). We also functionally examined sir-2.2, sir-2.3, and sir-2.4 mutants as well as triple mutant strains containing alleles of sir-2.1, sir-2.2, and sir-2.4 and of sir-2.1, sir-2.3, and sir-2.4. We also did RNAi of sir-2.2 in the sir-2.3 mutant background and RNAi of sir-2.3 in a sir-2.2 mutant background to check for redundancy of these two mitochondrial sirtuins. No gonad delay phenotype was associated with any sirtuin single mutant or mutant combination (data not shown). We have already shown that a reduction in PME-1/PAKP1 activity can ameliorate the gonad delay instead of mimicking the phenotype (Fig. 2C), suggesting that a lack of PARP activity is not causing the phenotype. Thus, although our experimental results are consistent with a reduction of SIR-2.1 activity, which could reflect impaired NAD+ bioavailability in the nucleo-cytoplasmic compartment or increased levels of sirtuin inhibitor NAM (38–40), we found no evidence that reduced NAD+ consumer activity underlies the reproductive phenotype of pnc-1 mutants.

Mitochondrial Activity in pnc-1 Mutants Is Not Impaired

Mitochondrial NAD+ represents a substantial portion of the cellular NAD+ pool (23, 24) where it is used for important energetic processes such as the TCA cycle and oxidative phosphorylation. We next sought to examine mitochondrial NAD+–mediated processes for evidence of limited NAD+ bioavailability in the pnc-1 mutants. Simultaneously, we aimed to test our second hypothesis to explain the reproductive delay of pnc-1 mutants, which is that gonad development is an energetically demanding process, and a lower rate of energy production, perhaps due to a lack of mitochondrial NAD+ availability, could negatively influence gonad development. We directly tested oxygen consumption as a measure of oxidative phosphorylation and found no difference between pnc-1 mutants and wild-type animals (Fig. 6A). Heat production is also unchanged (Fig. 6B), suggesting that the overall metabolic rate is unchanged. Thus, a simple lack of energy provided by the mitochondria to sustain gonad development is not an adequate explanation of the reproductive developmental delay.

Metabolomics Analysis of pnc-1 Mutants

NAD+ is a major hub in the metabolic network (15), and our data are consistent with a mild, but functionally relevant, deficit of NAD+ bioavailability in the nucleo-cytoplasmic compartment. We hypothesized that multiple processes in addition to NAD+ consumer activity would be affected by a deficit in NAD+ availability. To seek further evidence of perturbation

---

**FIGURE 4.** A, a wild-type N2 animal showing an open uterine lumen (arrow) at the mid L4 stage, determined by the “Christmas tree” shaped vulva (asterisk). B, a representative pnc-1 mutant animal has an under-developed uterine lumen (open arrow) at the same mid L4 stage. C and D, representative N2 animals fed with nprt-1 or qns-1 RNAi showing underdeveloped uterine lumens (open arrow) at the mid L4 stage, a phenotype similar to pnc-1 mutants.

**FIGURE 5.** Loss of pnc-1 function inhibits sir-2.1 activity. A, representative pictures showing intensity of a sod-3::GFP reporter (muIs84). Expression level in a sir-2.1 overexpression background (pkIs1642) is reduced upon loss of pnc-1 function. B, quantification of GFP intensity in strains carrying muIs84 as indicated. Error bars are S.D. **, 0.001 < p < 0.01, ***, p < 0.001, calculated using Student’s t test. C, LC-MS measurement of acetyllysine in wild-type N2 and pnc-1(pk9605) mutants. Box plots are as described in legend to Fig. 1. **, 0.001 < p < 0.01, calculated with Welch’s two sample t test.
of NAD\textsuperscript{+} biosynthesis and to reveal specific changes that might influence gonad development, we used a metabolomics approach to measure steady-state levels of hundreds of metabolites in wild type and pnc-1 mutants. The loss of pnc-1 activity is expected to have metabolic changes beyond those directly affected by compromised NAD\textsuperscript{+} production. For example, we have demonstrated phenotypic consequences to the increase in NAM (12, 13). To allow us to identify those perturbations specifically relevant to compromised NAD\textsuperscript{+} biosynthesis and to the delay in gonad development, we also profiled the mutant under two other conditions. We profiled pnc-1 mutants supplemented with NA; we would expect to see rescue of relevant perturbations in this condition. We also profiled pnc-1 mutants on UV-killed E. coli where we would expect to see exacerbation of relevant perturbations.

We profiled five biological replicates for each condition. 361 named metabolites were identified in our analysis (supplemental Table S1). Of these, 85 ($p < 0.05$) were altered by mutation of pnc-1 and another 38 trended toward a change ($0.05 < p < 0.10$) (Table 1, Fig. 7). These 123 metabolites ranged across all subtypes of metabolites examined and are involved in a wide variety of metabolic pathways (Table 1). Supplementation of mutant animals with NA reversed the pnc-1-induced change of 62 of these metabolites (Table 1, Fig. 7).

**Metabolomics Results Are Consistent with Functional Experiments Involving Mitochondria and Sirtuins**

In support of our conclusion that mitochondrial respiration is not compensated in the pnc-1 mutant, TCA cycle metabolites are not perturbed (supplemental Table S1). The only change in TCA cycle metabolites upon loss of pnc-1 was a trend toward more α-ketoglutarate ($0.05 < p < 0.1$), but this phenotype did not correlate with a lack of NAD\textsuperscript{+} biosynthesis or the reproductive development phenotype because it was neither rescued by supplementation with NA nor exacerbated by UV-killed E. coli (Table 1).

Consistent with the detected reduction in sirtuin activity, acetyllysine levels are elevated in pnc-1 mutants (Table 1 and Fig. 5C). Interestingly, just as reduced sirtuin activity does not functionally correlate with gonad development, the increase in acetyllysine does not correlate with lack of NAD\textsuperscript{+} biosynthesis or the gonad development phenotype because it was neither rescued by supplementation with NA nor exacerbated by UV-killed food (supplemental Table S1).

**A Minority of the Metabolic Changes Associated with pnc-1 Correlate Well with the Reproductive Development Phenotype**

**Amino acid and Dipeptide Metabolites**—Loss of pnc-1 function altered approximately one-third of the detected amino acid-related metabolites (supplemental Table S1). The changes reversed by supplementation with NA included positive and negative changes in various amino acid metabolic pathways (Table 1). Four of these changes (saccharopine, α-hydroxyisocaproate, 2-hydroxybutyrate, and N-acetyltornithine) were exacerbated by UV-killed E. coli, but no particular pathway was prominent (Table 1). Thus, amino acid metabolites did not become a primary focus for investigating the reproductive delay. Twenty dipeptides out of 101 examined were altered by loss of pnc-1 (supplemental Table S1), and NA rescued 15 of these, which were all altered in the same direction, displaying an increase upon loss of pnc-1 (Table 1). However, none of these changes were exacerbated by UV-killed E. coli (supplemental Table S1).

**Fatty Acid Metabolism**—Notably, inactivation of pnc-1 caused increased levels of 8 of 10 detected acylglycerophosphocholines as well as choline phosphate (supplemental Table S1). The changes in choline phosphate as well as three of eight acylglycerophosphocholines were rescued by supplementation with NA (Table 1), suggesting a possible link to NAD\textsuperscript{+} biosynthesis. However, these effects were not significantly exacerbated by UV-killed E. coli (supplemental Table S1) and thus do not meet our criteria for candidates with the most direct functional association to the reproductive delay phenotype.

Of the 33 changes in fatty-acid-related metabolites associated with loss of pnc-1, NA reversed 10 (Tables 1 and supplemental Table S1). And only three (the long chain fatty acid oleate, 2-hydroxyglutarate, and myo-inositol) had changes that were exacerbated by UV-killed food (supplemental Table S1). Thus, no specific aspect of fatty acid metabolism stood out as a strong candidate for association with the gonad developmental delay.

**Nucleotide Metabolism**—Numerous nucleotide metabolites were both increased and decreased by loss of pnc-1, and strikingly, most of these changes were reversed by supplementation of cultures with NA, suggesting an association with NAD\textsuperscript{+} biosynthesis (Tables 1 and supplemental Table S1). However, only one metabolite, 3-aminoisobutyrate, had a pnc-1-mediated change that was exacerbated on UV-killed E. coli. Thus, we did not focus on nucleotide-related metabolites in our search for a cause for the developmental delay.

**Carbohydrate Metabolism**—Carbohydrate metabolism is the category in which the largest percentage of measured metabolites were altered by pnc-1, and more than half of these changes were restored by NA supplementation (Tables 1 and supplemental Table S1). pnc-1 mutants have accumulation of a number of mono-, di-, and tri-saccharides (Tables 1 and supplemental Table S1), which are glycogen breakdown intermediates used for energy storage. Glycolysis was the only specific pathway where more than one of the changes induced by loss of pnc-1 was both rescued by NA and exacerbated by UV-killed E. coli (supplemental Table S1).
### TABLE 1
List of metabolites with altered levels in *pnc-1* mutants relative to wild type

Metabolites highlighted in green are those whose levels are rescued in *pnc-1* mutants with NA supplementation. Metabolites highlighted in blue are those whose levels are both rescued in NA-supplemented *pnc-1* mutants and exacerbated in *pnc-1* mutants cultured on UV-killed OP50 plates. Blue metabolites met all criteria to be considered candidates for investigating a relationship to the reproductive phenotype. *, *p* < 0.05; §, 0.05 < *p* < 0.1; calculated with Welch’s two sample t test.

| Metabolite type | Metabolism Pathway | Metabolite Name | Fold change in *pnc-1* | Rescue in presence of NA | Exacerbated on UV-killed food |
|-----------------|-------------------|----------------|------------------------|--------------------------|-------------------------------|
| Amino acids     | Glycine, serine and threonine metabolism | O-acetylserine | 0.45 * | | |
|                 |                    | N-acetylserine | 0.69 * | | |
|                 |                    | homoserine     | 1.62 § | | |
|                 |                    | betaine        | 1.28 * | | |
|                 | Glutamate metabolism | glutamate      | 1.22 * | | |
|                 |                    | glutamine      | 1.57 * | | |
|                 | Lysine metabolism  | 2-aminoadipate | 1.18 * | | |
|                 |                    | pipicolate     | 2.10 * | | |
|                 |                    | saccharopine   | 3.03 * | § | |
|                 |                    | N6-acetyllysine| 1.29 * | | |
|                 | Phenylalanine & tyrosine metabolism | tyrosine       | 1.17 * | | |
|                 |                    | 3-(4-hydroxyphenyl)lactate | 0.61 § | | |
|                 | Tryptophan metabolism | tryptophan   | 1.14 * | | |
|                 |                    | antranilate    | 1.50 * | | |
|                 | Valine, leucine and isoleucine metabolism | alpha-hydroxyisocaproate | 0.57 * | | |
|                 |                    | leucine        | 1.12 § | | |
|                 |                    | isovalerylcamitine | 1.38 * | | |
|                 | Cysteine, methionine, SAM, taurine metabolism | N-acetylhomocysteine | 0.34 * | | |
|                 |                    | 2-hydroxybutyrate (AHB) | 0.57 * | | |
|                 | Urea cycle; arginine-, proline-, metabolism | arginine | 1.19 § | | |
|                 | Polyamine metabolism | N-acetyltorine | 1.55 * | | |
| Peptide         | Dipeptide          | 5-methylthioadenosine (MTA) | 1.14 § | | |
|                 |                    | N-acetylputrescine | 1.84 § | | |
### TABLE 1—continued

| Carbohydrate | Aminosugars metabolism | Fructose, mannose, galactose, starch, and sucrose metabolism | Glycolysis, gluconeogenesis, pyruvate metabolism | Nucleotide sugars, pentose metabolism | Oligosaccharide | Energy | Lipid |
|--------------|------------------------|-------------------------------------------------------------|--------------------------------------------------|-------------------------------------|----------------|--------|-------|
| lysylleucine | 1.14 §                 | *                                                           | *                                                | *                                   |                |        |       |
| lysylvaline  | 1.15 *                 | *                                                           | *                                                | *                                   |                |        |       |
| phenylalanyleucine | 1.46 § | *                                                      | *                                                | *                                   |                |        |       |
| alpha-glutamyltyrosine | 1.66 * | §                                                       | *                                                | *                                   |                |        |       |
| gamma-glutamyl |                                      |                                                            | *                                                | *                                   |                |        |       |
| gamma-glutamylleucine | 1.41 § | *                                                      | *                                                | *                                   |                |        |       |
| gamma-glutamyl/phenylalanine | 1.68 * | *                                                      | *                                                | *                                   |                |        |       |
| Polypeptide | val-val-val | 1.58 * | *                                                               | *                                                | *                                   |                |        |       |
| leu-leu-leu | 1.92 * | *                                                      | *                                                | *                                   |                |        |       |
| glucosamine  | 2.77 *                 |                                                            | *                                                | *                                   |                |        |       |
| N-acetylglucosamine | 2.19 * | *                                                      | *                                                | *                                   |                |        |       |
| galactose    | 2.59 *                 |                                                            | *                                                | *                                   |                |        |       |
| mannose      | 2.02 *                 |                                                            | *                                                | *                                   |                |        |       |
| mannose-6-phosphate | 1.49 * | §                                                | *                                                | *                                   |                |        |       |
| sorbitol     | 2.63 *                 |                                                            | *                                                | *                                   |                |        |       |
| trehalose    | 5.44 *                 |                                                            | *                                                | *                                   |                |        |       |
| maltotriose  | 2.17 §                 |                                                            | *                                                | *                                   |                |        |       |
| glycerate    | 0.63 *                 |                                                            | *                                                | *                                   |                |        |       |
| glucose-6-phosphate (G6P) | 1.61 * | *                                       | *                                                | *                                   |                |        |       |
| glucose      | 1.46 *                 |                                                            | *                                                | *                                   |                |        |       |
| 3-phosphoglycerate | 0.65 § | §                         | *                                                | *                                   |                |        |       |
| dihydroxyacetone phosphate (DHAP) | 3.20 * | §                                 | *                                                | *                                   |                |        |       |
| pyruvate     | 1.34 §                 |                                                            | *                                                | *                                   |                |        |       |
| lactate      | 0.58 *                 |                                                            | *                                                | *                                   |                |        |       |
| 6-phosphogluconate | 1.74 § | §                           | *                                                | *                                   |                |        |       |
| gluconate    | 2.79 *                 |                                                            | *                                                | *                                   |                |        |       |
| ribose       | 0.52 *                 |                                                            | *                                                | *                                   |                |        |       |
| ribulose     | 0.68 *                 |                                                            | *                                                | *                                   |                |        |       |
| maltotetraose | 2.33 * | *                                                      | *                                                | *                                   |                |        |       |
| Krebs cycle  | alpha-ketoglutarate     | 1.58 §                                                      | *                                                | *                                   |                |        |       |
| Medium chain fatty acid | pelargonate (9:0) | 1.30 *                                              | *                                                | *                                   |                |        |       |
| Long chain fatty acid | myristate (14:0) | 1.17 §                                              | *                                                | *                                   |                |        |       |
| margarate (17:0) | 0.64 §                                   |                                                            | *                                                | *                                   |                |        |       |
| oleate (18:1n9) | 1.47 *                                   |                                                            | *                                                | *                                   |                |        |       |
| Fatty acid, dicarboxylate | 2-hydroxyglutarate | 0.53 §                                              | *                                                | *                                   |                |        |       |
| Fatty acid, amide | oleamide | 1.61 *                                             | *                                                | *                                   |                |        |       |
| Fatty acid metabolism (also BCAA metabolism) | propionylcarnitine | 1.67 *                                             | §                                                | *                                   |                |        |       |
| Bile acid metabolism | glycocholate | 1.84 *                                         | *                                                | *                                   |                |        |       |
| taurocholate | 2.08 *                 |                                                            | *                                                | *                                   |                |        |       |
| taurodeoxycholate | 1.76 *                     |                                                            | *                                                | *                                   |                |        |       |
| glycodeoxycholate | 4.58 *                                |                                                            | *                                                | *                                   |                |        |       |
| Glycerolipid  | choline phosphate       | 1.95 *                                                      | *                                                | *                                   |                |        |       |
| Metabolism                                      | Compound                                      | Value  |
|------------------------------------------------|-----------------------------------------------|--------|
| **Ethanolamine**                               |                                               |        |
| C. elegans NAD⁺ Biosynthesis                   |                                               |        |
| **Inositol metabolism**                        |                                               |        |
| **Lysolipid**                                  |                                               |        |
| 1-arachidonoylglycerophosphoethanolamine        | 0.50 §                                        |        |
| 2-arachidonoylglycerophosphoethanolamine        | 0.54 §                                        |        |
| 1-stearylglycerophosphoglycerol                | 0.57 §                                        |        |
| 2-stearylglycerophosphoethanolamine            | 0.55 §                                        |        |
| 2-palmitoleoylglycerophosphocholine            | 2.17 §                                        |        |
| 2-stearylglycerophosphocholine                 | 3.89 *                                        |        |
| 1-oleoylglycerophosphocholine                  | 3.62 §                                        |        |
| 2-oleoylglycerophosphocholine                  | 5.90 *                                        |        |
| 1-linoleoylglycerophosphocholine               | 4.39 *                                        |        |
| 2-linoleoylglycerophosphocholine               | 5.91 *                                        |        |
| 2-eicosatrienoylglycerophosphocholine          | 4.56 *                                        |        |
| 2-arachidonoylglycerophosphocholine            | 3.03 *                                        |        |
| 1-oleoylglycerophosphoinositol                 | 0.65 §                                        |        |
| 1-linoleoylglycerophosphoinositol              | 0.51 §                                        |        |
| 1-arachidonoylglycerophosphoinositol           | 0.52 *                                        |        |
| 2-oleoylglycerophosphoinositol                 | 0.56 §                                        |        |
| **Monoacylglycerol**                           |                                               |        |
| 1-dihomo-linolenyl-glycerol (1-monoeicosatrienoin) | 6.37 *                              |        |
| **Sterol/Steroid**                             | 7-dehydrocholesterol                          | 2.27 * §|

**Nucleotide**

| Metabolism                                      | Compound                                      | Value  |
|------------------------------------------------|-----------------------------------------------|--------|
| Purine metabolism, (hypo)xanthine/inosine containing | xanthine                                     | 1.26 * §|
| Purine metabolism, adenine containing           | inosine 5'-monophosphate (IMP)                | 1.61 * §|
| Purine metabolism, guanine containing           | adenosine 3'-monophosphate (3'-AMP)           | 0.38 * §|
| Purine metabolism, guanine containing           | adenosine-2',3'-cyclic monophosphate          | 0.49 * §|
| Purine metabolism, guanine containing           | guanosine 5'-monophosphate (5'-GMP)           | 1.17 * §|
| Purine metabolism, guanine containing           | guanosine-2',3'-cyclic monophosphate          | 0.52 § |
| Purine metabolism, guanine containing           | guanosine 3'-monophosphate (3'-GMP)           | 0.39 * §|
| Purine metabolism, urate metabolism             | urate                                         | 1.94 * §|
| Purine metabolism, urate metabolism             | allantoin                                      | 1.60 * §|
| Pyrimidine metabolism, cytidine containing      | cytidine-3'-monophosphate (3'-CMP)            | 0.28 * §|
| Pyrimidine metabolism, cytidine containing      | cytosine-2',3'-cyclic monophosphate           | 0.53 * §|
| Pyrimidine metabolism, thymine containing       | 3-aminoisobutyrate                             | 0.71 * §|
| Pyrimidine metabolism, thymine containing       | uridine-2',3'-cyclic monophosphate            | 0.21 * §|
| Cofactors and vitamins                          |                                               |        |
| Folate metabolism                               | dihydrobiopterin                             | 2.22 * §|
| Nicotinate and                                  | nicotinamide                                  | 19.09 * §|
C. elegans NAD⁺ Biosynthesis

TABLE 1—continued

| Metabolite Type       | Table 1—Continued | NAD⁺ | Nicotinate | Pantothenate and CoA Metabolism | Flavin Adenine Dinucleotide (FAD) | Flavin Mononucleotide (FMN) | Benzoate Metabolism | Chemical Metabolism |
|-----------------------|-------------------|------|------------|--------------------------------|---------------------------------|------------------------|-------------------|--------------------|
| pnc-1/N2              |                   |      |            |                                |                                 |                        |                   |                    |
| 123                   |                   |      |            |                                |                                 |                        |                   |                    |
| pnc-1 UV/pnc-1        |                   |      |            |                                |                                 |                        |                   |                    |
| 14                    |                   |      |            |                                |                                 |                        |                   |                    |
| 145                   |                   |      |            |                                |                                 |                        |                   |                    |

E. coli (Table 1). Thus, we investigated the functional relationship between carbohydrate metabolism and gonad developmental delay.

Perturbations in Glycolysis Cause the Gonad Developmental Defects in pnc-1 Mutants

Loss of pnc-1 function caused accumulation of glucose as well as glucose 6-phosphate and dihydroxyacetone phosphate (DHAP), which are glycolytic intermediates before the step that requires NAD⁺ (Table 1 and Fig. 8). In contrast, we observed a decrease in 3-phosphoglycerate (3PGA) (0.05 < p < 0.10), subsequent to the NAD⁺-dependent step (Table 1 and Fig. 8). Each of these perturbations is rescued by supplementation with NA (Table 1 and Fig. 8), suggesting that the detected disruption to glycolysis can be rescued by restoring salvage NAD⁺ biosynthesis. Although exacerbation by UV-killed E. coli was statistically detected for only DHAP (Table 1 and Fig. 8), each of these glycolytic intermediates shows trends in the appropriate direction for association with the reproductive delay on UV-killed E. coli (Fig. 8). Pyruvate is an exception to the pattern; its levels are increased in the pnc-1 mutant (Table 1, see “Discussion”). As further evidence for the relevance of the changes in glycolytic metabolite levels, we also compared levels in pnc-1 and NR-supplemented pnc-1. We found that NR supplementation had similar effects to NA supplementation in lowering the levels of a metabolite before the NAD⁺-dependent step and raising the levels of subsequent glycolytic intermediates (Fig. 9).

To investigate the functional phenotypic consequences of the depletion of the late glycolytic metabolites, we added 3PGA and the subsequent metabolite PEP as supplements in the culture media. Both 3PGA and PEP significantly increased the percentage of the pnc-1 population with normal gonad development relative to supplementation of glucose as a control (Fig. 10A). These results suggest that efficient glycolytic output is sufficient to permit normal progression of gonad development in the pnc-1 mutant.

To further investigate the relationship of glycolysis to gonad developmental progression, we also examined the effects of experiments designed to diminish the glycolytic rate. For this experiment we used RNAi of genes encoding the glycolytic enzymes phosphofructokinase (pfk-1.1 and pfk-1.2) and triosephosphate isomerase (tpi-1). Further inhibition of glycolysis would be expected to exacerbate the gonad developmental defects of pnc-1 mutants and cause a gonad developmental delay in wild-type animals. We found that RNAi of each of the three glycolytic enzyme genes causes a gonad developmental delay only in the pnc-1 mutant background (Fig. 10B). These results support a role for compromised glycolysis in contributing to the gonad delay of pnc-1 mutants and suggest that although restoration of glycolysis is sufficient to rescue pnc-1, blocking glycolysis is not sufficient to cause a gonad developmental delay in an otherwise wild-type background.

pnc-1 Mutants Are More Sensitive to Disruption of Mitochondrial Function

Our data show that although the pnc-1 mutants have reduced glycolytic output, mitochondrial function is maintained, suggesting that other metabolic changes compensate for reduced glycolytic output to provide fuel for mitochondria. This working model suggests that the pnc-1 mutants would be hypersensitive to disruption in mitochondrial function relative to wild-type animals, which maintain the flexibility of producing energy via glycolysis. We tested this hypothesis using sodium azide to disrupt mitochondrial function in pnc-1 mutants and wild-type animals and via RNAi of genes required for oxidative phosphorylation. We found that pnc-1 mutants paralyze more quickly in response to sodium azide and recover more slowly from sodium azide treatment compared with wild-type animals (Fig. 11, A and B). Muscle function of pnc-1 mutants is also more sensitive than wild type to RNAi of genes encoding electron transport chain and ATP synthase subunits (Fig. 11C).
A Deficit in NAD⁺ Biosynthesis Results in a Reproductive Developmental Delay—NAD⁺ biosynthesis via the salvage pathway is critical for temporal progression of reproductive development in *C. elegans*. Because knock-out of Nampt is embryonic lethal, loss of salvage biosynthesis in mice likely has developmental phenotypes as well (41). We investigated the physiological mechanisms underlying the developmental phenotype of loss of salvage NAD⁺ biosynthesis in *C. elegans*. We were surprised to find that NAD⁺ levels were only mildly reduced in the \textit{pnc-1} mutants. However, our results are consistent with a minor deficit of NAD⁺ bioavailability as a cause of the gonad phenotype based on two lines of investigation. First, every condition predicted to boost NAD⁺ levels ameliorates the gonad delay phenotype, including supplementation with NA, NR, NMN, or NAD⁺ as well as replacing \textit{pnc-1} with NAMPT or reducing PARP function (data presented here and Vrablik \textit{et al.} (12)). We also specifically show that supplementation with NR and loss of \textit{pme-1}/PARP1, which each rescue the gonad delay, simultaneously boost NAD⁺ levels. Second, experiments designed to lower NAD⁺ levels recapitulate the phenotype. Reducing the activity of each of the other enzymes in the salvage/Preiss-Handler pathway mimics the phenotype, suggesting that loss of NAD⁺ itself causes the phenotype as opposed to perturbation of any other specific intermediate metabolite. We also measured NAD⁺ levels in these experiments designed to deplete NAD⁺, and although we did not find a significant difference in average levels, the averages did trend lower, and the minimum and maximum measurements were lower as well. The lack of a significant difference could reflect the experimental protocol involving RNAi that is expected to reduce as opposed to remove gene function, which is achieved with the \textit{pnc-1} null allele (12). Nonetheless, it suggests that the reproductive system is sensitive to even a minor decrease in NAD⁺ availability.

**Role of Salvage Synthesis in Global NAD⁺ Production**—Our data show that loss of \textit{pnc-1} function blocks the recycling of...
NAM produced by NAD⁺ consumers and drastically reduces the production of the precursor NA for NAD⁺ biosynthesis. Nonetheless, the levels of NAD⁺ are only mildly affected. We conclude that salvage biosynthesis makes a significant but minor contribution to the global levels of organismal NAD⁺ in C. elegans. The NAD/NADH ratio is not perturbed by this mild deficit in global levels of NAD⁺. It remains possible that salvage from NAM may make a substantial contribution to NAD⁺ production, but in its absence, compensatory production via other pathways or regulatory feedback on NAD⁺ consumption might maintain NAD⁺ levels. No increase in the mononucleotide forms of NAM or NA were detected in pnc-1 mutants (supplemental Table S1), but these steady-state measurements may not give a full picture of compensatory mechanisms that could be in play. Moreover, other pathways for NAD⁺ biosynthesis in C. elegans have not yet been well defined. For example, whether C. elegans have a functional de novo pathway is not yet clear (12, 15). Thus, these questions remain to be answered, and metabolic flux experiments will be required to investigate hypotheses about compensatory mechanisms. In mammalian systems the need for various NAD⁺ biosynthetic pathways is usually discussed in tissue-specific terms, and the ability of one pathway to compensate for others is also not explored well at the cellular or organism levels. Technical considerations prevent us from measuring NAD⁺ levels in a tissue-specific manner. However, we do not favor a model where the gonad in particular has less NAD⁺ than other tissues. We have previously shown that restoration of NAD⁺ salvage biosynthesis in limited numbers of cells outside the gonad in the pnc-1 mutant can at least partially rescue the gonad delay (14). These results suggest that NAD⁺ biosynthetic intermediates are likely shared liberally between tissues and that the gonad is adept at collecting them if available.

**pnc-1 Mutants Have a Decrease in NAD⁺ Bioavailability Specifically Outside the Mitochondria**—We suggest that the decrease in NAD⁺ levels in pnc-1 mutants specifically excludes the mitochondria because we detected perturbations in nucleo-cytoplasmic NAD⁺-dependent processes but no perturbation in mitochondrial functions. This conclusion is consistent with previous studies demonstrating that NAD⁺ levels can be regulated independently in specific cellular compartments (22, 42). The cytoplasmic NAD⁺ deficit produced by a lack of salvage
C. elegans NAD⁺ Biosynthesis

How Is Mitochondrial Activity Preserved?—Unlike the other late glycolytic metabolites, pyruvate levels are not reduced in pnc-1 mutants relative to wild-type animals (Table 1). The metabolomics data suggest two explanations for the lack of a decrease in pyruvate even in the presence of reduced glycolytic output. First, lactate levels are reduced (Table 1). Given that cytoplasmic NADH is required to convert pyruvate to lactate, pyruvate levels are likely maintained because of less conversion to lactate, preserving pyruvate to feed mitochondrial metabolism. Second, the metabolomics data are consistent with a potential increase in amino acid catabolism, supplying carbon to the mitochondrial TCA cycle via pyruvate and potentially other metabolites. Both α-ketoglutarate and glutamate, which are used in conversion of glucogenic amino acids to pyruvate, are increased (Table 1). Also consistent with measurements indicating no depletion of pyruvate, the addition of pyruvate as a culture supplement does not rescue the gonad delay (data not shown), suggesting pyruvate is not limiting. Regardless of the mechanism by which mitochondrial function is preserved, the metabolic shift makes the animals hyper-reliant on their mitochondria and thus hypersensitive to disruption in mitochondrial function. Our results suggest that a shift away from glycolysis is not compatible with a normal pace of reproductive development.

Why Is the Reproductive System Sensitive to Reduced Glycolytic Output?—Supplementation with late glycolytic intermediates rescues the pnc-1 mutant gonad delay, suggesting that efficient glycolysis is required for rapid progression of reproductive development. Alternatively or in combination, the secondary effects of inefficient glycolysis must be avoided to support normal reproductive development. For example, inefficient glycolysis combined with an increase in amino acid catabolism to fuel the mitochondria for normal metabolic activity could be incompatible with the need for a high level of biomolecules to maintain the rate of cell division required to form a gonad and populate it with germ cells. Blocking glycolysis is not sufficient to induce a gonad delay phenotype in wild-type animals, suggesting that another effect caused by a lack of NAD⁺ bioavailability contributes to the phenotype as well. For example, inhibition of glycolysis alone may not be sufficient to induce the proposed increase in amino acid metabolism in a normal metabolic background. However, in pnc-1 mutants, which have changes in lipid and nucleic acid metabolism as well and may have perturbations in the pentose phosphate pathway (supplemental Table S1), inhibition of glycolysis is not compatible with a fast pace of reproductive development. We propose that the pnc-1 animals delay the pace of reproductive development in response to the need to compensate for reduced glycolytic output. By shifting resources for cell division to basal metabolic function, the pace of reproductive development cannot be maintained. Also, our data demonstrating that diet can have a large effect on reproductive developmental progression in the

biosynthesis of NAD⁺ affects the efficiency of glycolysis, likely by reducing the activity of the NAD⁺-dependent enzyme glyceraldehyde-3-phosphate dehydrogenase. However, this effect is not carried through to the TCA cycle. Similar to our findings, salvage biosynthesis has cytoplasmic-specific effects in cancer cells. Inhibition of NAMPT activity in human cancer cells inhibits glycolysis at the NAD⁺-dependent step but appears to have little impact on the TCA cycle (7). We conclude that the mitochondria are protected from the effects of loss of salvage NAD⁺ biosynthesis and that NAD⁺ salvage biosynthesis is not critical for maintaining functional levels of NAD⁺ in the mitochondria. The maintenance of mitochondrial activity likely occurs as a result of compensatory metabolic changes that direct carbon for oxidation to the mitochondria through other routes. Consistent with our model of a block in carbohydrate utilization and a potential switch to use of alternative fuels, the major carbohydrate storage molecule, trehalose, is drastically increased in pnc-1 mutants (Table 1).
C. elegans NAD\(^+\) Biosynthesis

pnc-1 mutant is consistent with this model. For example, growth on the normal live HT115 E. coli strain results in a poorly penetrant reproductive delay, whereas growth on conditions with UV-killed food where some nutrients, including NA produced by the E. coli, are likely destroyed or eliminated results in a penetrant phenotype. It is worth noting that embryogenesis, another highly active period in terms of cell division, is not affected by loss of salvage NAD\(^+\) biosynthesis, suggesting that either other pathways for biosynthesis of NAD\(^+\) are sufficient to maintain NAD\(^+\) levels during embryonic development, which we have not yet tested, or the metabolic requirements in terms of the need for efficient glycolysis differs embryonically and post-embryonically. Experiments to examine metabolic profiles at developmental stages will be required to answer these questions.

In summary, we found that the effects on basal metabolic pathways mediate the developmental phenotype in the reproductive system upon the inhibition of salvage NAD\(^+\) biosynthesis. This result was surprising because the effects on NAD\(^+\) consumer activity have been the primary focus for probing the functional consequences of manipulation of NAD\(^+\) bioavailability (43–46). Such studies have demonstrated the importance of regulation of NAD\(^+\) consumers, and our experiments also suggest that manipulation of salvage synthesis functionally impacts sirtuin activity. However, the phenotypic effects on reproductive development were not mediated by regulation of NAD\(^+\) consumers. Instead, upon loss of salvage NAD\(^+\) biosynthesis in C. elegans perturbation of the elegant interplay of basic metabolic pathways is the underlying cause of the reproductive development phenotype. From a therapeutic standpoint, our results are consistent with efforts aimed at using salvage NAD\(^+\) biosynthesis as a target to compromise metabolism in cancer cells, where cytoplasmic metabolic pathways are often relatively more important than mitochondrial metabolism.

Author Contributions—W. H.-R. and W. W. conceived and coordinated the study. W. W. prepared the samples, coordinated the metabolomics analysis with Metabolon, and analyzed the results for Figs. 1, B, C, and D, 5C, 7, and 8. W. W. also performed the experiments in Figs. 2, A and C, 3A and C, 4, and 11C and analyzed the results. M. R. M. performed the experiments in Figs. 1E, 2, B and D, 3B, and 9 and analyzed the results. I. D. and B. P. B. performed and analyzed the experiments in Fig. 6. S. E. L. performed and analyzed the experiments in Fig. 5, A and B. J. F. G., M. S., K. K., and A. C. D. performed the experiments in Fig. 10, A and B, with supervision and technical assistance from W. W. M. J. P. performed the experiments in Fig. 11, A and B. W. H.-R. wrote the paper. All authors reviewed the results and approved the manuscript.

Acknowledgments—We thank the Pennsylvania State Metabolomics Core Facility of Huck Institutes of the Life Sciences for technical assistance and advice. We thank Anne Hart for the sirtuin triple mutant strains. tm alleles were provided by the Mitani laboratory through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology of Japan, Japan. Other strains were provided by the Caenorhabditis Genetics Center, which is funded by National Institutes of Health Office of Research Infrastructure Programs (Grant P40 OD010440).

References

1. Houtkooper, R. H., Pirinen, E., and Auwerx, J. (2012) Sirtuins as regulators of metabolism and healthspan. Nat. Rev. Mol. Cell Biol. 13, 225–238
2. Sauve, A. A., and Youn, D. Y. (2012) Sirtuins: NAD\(^+\)-dependent deacetylase mechanism and regulation. Curr. Opin. Chem. Biol. 16, 535–543
3. Cantó, C., and Auwerx, J. (2011) NAD\(^+\) as a signaling molecule modulating metabolism. Cold Spring Harb. Symp. Quant. Biol. 76, 291–298
4. Chiarugi, A., Dölle, C., Felici, R., and Ziegler, M. (2012) The NAD metabolome: a key determinant of cancer cell biology. Nat. Rev. Cancer 12, 741–752
5. Dölle, C., Skoge, R. H., Vanlinden, M. R., and Ziegler, M. (2013) NAD biosynthesis in humans: enzymes, metabolites, and therapeutic aspects. Curr. Top. Med. Chem. 13, 2907–2917
6. Mouchiroud, L., Houtkooper, R. H., and Auwerx, J. (2013) NAD\(^+\) metabolism: a therapeutic target for age-related metabolic disease. Crit. Rev. Biochem. Mol. Biol. 48, 397–408
7. Tan, B., Young, D. A., Lu, Z. H., Wang, T., Meier, T. I., Shepard, R. L., Roth, K., Zhai, Y., Huss, K., Kuo, M. S., Gillig, J., Parthasarathy, S., Burkholler, T. P., Smith, M. C., Geeganege, S., and Zhao, G. (2013) Pharmacological inhibition of nicotinamide phosphoribosyltransferase (NAMPT), an enzyme essential for NAD\(^+\) biosynthesis, in human cancer cells: metabolic basis and potential clinical implications. J. Biol. Chem. 288, 3500–3511
8. Cea, M., Cagnetta, A., Fulciniti, M., Tai, Y.-T., Hideshima, T., Chauhan, D., Roccaro, A., Sacco, A., Calimeri, T., Cottini, F., Jakubikova, J., Kong, S.-Y., Patrone, F., Nencioni, A., Golbi, M., Richardson, P., Munshi, N., and Anderson, K. C. (2012) Targeting NAD\(^+\) salvage pathway induces autophagy in multiple myeloma cells via miTORC1 and extracellular signal-regulated kinase (ERK1/2) inhibition. Blood 120, 3519–3529
9. Sampath, D., Zabka, T. S., Misner, D. L., O’Brien, T., and Dragovich, P. S. (2015) Inhibition of nicotinamide phosphoribosyltransferase (NAMPT) as a therapeutic strategy in cancer. Pharmacol. Ther. 151, 16–31
10. Fu, L., Doreswamy, V., and Prakash, R. (2014) The biochemical pathways of central nervous system neural degeneration in niacin deficiency. Neural Regen. Res. 9, 1509–1513
11. Porter, R. M., and Anstey, A. (2014) Evidence and conjecture about mechanisms of cutaneous disease in photodermatology. Exp. Dermatol. 23, 543–546
12. Vrablik, T. L., Huang, L., Lange, S. E., and Hanna-Rose, W. (2009) Nicotinamide modulation of NAD\(^+\) biosynthesis and nicotinamide levels separately affect reproductive development and cell survival in C. elegans. Development 136, 3637–3646
13. Vrablik, T. L., Wang, W., Upadhyay, A., and Hanna-Rose, W. (2011) Muscle type-specific responses to NAD\(^+\) salvage biosynthesis promote muscle function in Caenorhabditis elegans. Dev. Biol. 349, 387–394
14. Crook, M., Mcreynolds, M. R., Wang, W., and Hanna-Rose, W. (2014) An NAD\(^+\) biosynthetic pathway enzyme functions cell non-autonomously in C. elegans development. Dev. Dyn. 243, 965–976
15. Gossmann, T. I., Ziegler, M., Puntervoll, P., de Figueiredo, L. F., Schuster, S., and Heiland, I. (2012) NAD\(^+\) biosynthesis and salvage: a phylogenetic perspective. FEBS J. 279, 3355–3363
16. Rongvai, A., András, F., Van Gool, F., and Leo, O. (2003) Reconstructing eukaryotic NAD metabolism. Bioessays 25, 683–690
17. Preiss, J., and Handler, P. (1958) Biosynthesis of diphosphoryridine nucleotide. I. Identification of intermediates. J. Biol. Chem. 233, 488–492
18. Preiss, J., and Handler, P. (1958) Biosynthesis of diphosphoryridine nucleotide. II. Enzymic aspects. J. Biol. Chem. 233, 493–500
19. Bieganowski, P., and Brenner, C. (2004) Discoveries of nicotinamide riboside as a nutrient and conserved NRK genes establish a Preiss-Handler pathway. Curr. Biol. 14, 233, 709–712
20. Tempel, W., Rabeh, W. M., Bogan, K. L., Bellen, P., Wojcik, M., Seidle, H. F., Nedylkova, L., Yang, T., Sauve, A. A., Park, H.-W., and Brenner, C. (2007) Nicotinamide riboside kinase structure reveals new pathways to NAD\(^+\). PloS Biol. 5, e263
21. Magni, G., Amici, A., Emanuelli, M., Raffaelli, N., and Ruggieri, S. (1999) Enzymology of NAD\(^+\) synthesis. Adv. Enzymol. Relat. Areas Mol. Biol. 73, 135–182
22. Nikiforov, A., Dölle, C., Niere, M., and Ziegler, M. (2011) Pathways and
subcellular compartmentalization of NAD biosynthesis in human cells: from entry of extracellular precursors to mitochondrial NAD generation. *J. Biol. Chem.* **286**, 21767–21778.

23. Houkooper, R. H., Cantó, C., Wanders, R. J., and Auwerx, J. (2010) The secret life of NAD⁺: an old metabolite controlling new metabolic signaling pathways. *Endocr. Rev.* **31**, 194–223.

24. Di Lisa, F., and Ziegler, M. (2001) Pathophysiological relevance of mitochondria in NAD⁺ metabolism. *FEBS Lett.* **492**, 4–8.

25. Di Lisa, F., Menabò, R., Canto, M., Barile, M., and Bernardi, P. (2001) Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD⁺ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. *J. Biol. Chem.* **276**, 2571–2575.

26. Alano, C. C., Tran, A., Tao, R., Ying, W., Karliner, J. S., and Swanson, R. A. (2007) Differences among cell types in NAD⁺ compartmentalization: a comparison of neurons, astrocytes, and cardiac myocytes. *J. Neurosci. Res.* **85**, 3378–3385.

27. Zhang, T., Berrocal, J. G., Frizzell, K. M., Gamble, M. J., DuMond, M. E., Krishnakumar, R., Yang, T., Sauve, A. A., and Kraus, W. L. (2009) Enzymes in the NAD⁺ salvage pathway regulate SIRT1 activity at target gene promoters. *J. Biol. Chem.* **284**, 20408–20417.

28. Huang, L., and Hanna-Rose, W. (2006) EGF signaling overcomes a uterine cell death associated with temporal mis-coordination of organogenesis within the *C. elegans* egg-laying apparatus. *Dev. Biol.* **300**, 599–611.

29. Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71–94.

30. Berdichevsky, A., Viswanathan, M., Horvitz, H. R., and Guarante, L. (2006) *C. elegans* Sir-2.1 interacts with 14–3–3 proteins to activate DAF-16 and extend life span. *Cell* **125**, 1165–1177.

31. Lu, W., Clasquin, M. F., Melamud, E., Amador-Noguez, D., Caudy, A. A., and Rabinowitz, J. D. (2010) Metabolomic analysis via reversed-phase ion-pairing liquid chromatography coupled to a stand alone orbitrap mass spectrometer. *Anal. Chem.* **82**, 3212–3221.

32. Timmons, L., and Fire, A. (1998) Specific interference by ingested dsRNA. *Nature* **395**, 854.

33. Ahringer, J., ed (April 6, 2006) *Reverse genetics*. *WormBook*, The *C. elegans* Research Community Edition, WormBook, 10.1895/wormbook.1.47.1.

34. French, J. B., Cen, Y., Vrablik, T. L., Xu, P., Allen, E., Hanna-Rose, W., and Sauve, A. A. (2010) Characterization of nicotinamidases: steady state kinetic parameters, classwide inhibition by nicotinaldehydes, and catalytic mechanism. *Biochemistry* **49**, 10421–10439.

35. Cantó, C., Houkooper, R. H., Pirinen, E., Youn, D. Y., Ooesterveer, M. H., Cen, Y., Fernandez-Marcos, P. J., Yamamoto, H., Andreux, P. A., Cettour-Rose, P., Gademann, K., Rinsch, C., Schoonjans, K., Sauve, A. A., and Auwerx, J. (2012) The NAD⁺ precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet-induced obesity. *Cell Metab.* **15**, 838–847.

36. Mouchiroud, L., Houkooper, R. H., Moullan, N., Katsyuba, E., Ryu, D., Cantó, C., Mottis, A., Jo, Y. S., Viswanathan, M., Schoonjans, K., Guarante, L., and Auwerx, J. (2013) The NAD⁺/sirtuin pathway modulates longevity through activation of mitochondrial UPR and FOXO signaling. *Cell* **154**, 430–441.

37. Sauve, A. A., Cellic, L. Avalos, J., Deng, H., Boeke, J. D., and Schramm, V. L. (2001) Chemistry of gene silencing: the mechanism of NAD⁺-dependent deacetylation reactions. *Biochemistry* **40**, 15456–15463.

38. Sauve, A. A., and Schramm, V. L. (2003) Sir2 regulation by nicotinamide results from switching between base exchange and deacetylation chemistry. *Biochemistry* **42**, 9249–9256.

39. Jackson, M. D., Schmidt, M. T., Oppenheimer, N. J., and Denu, J. M. (2003) Mechanism of nicotinamide inhibition and transglycosidation by Sir2 histone/protein deacetylases. *J. Biol. Chem.* **278**, 50985–50998.

40. Denu, J. M. (2005) Vitamin B3 and sirtuin function. *Trends Biochem. Sci.* **30**, 479–483.

41. Revollo, J. R., Körner, A., Mills, K. F., Satoh, A., Wang, T., Garten, A., Dasgupta, B., Sasaki, Y., Wolberger, C., Townsend, R. R., Milbrandt, J., Kiess, W., and Imai, S. (2007) Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab.* **6**, 363–375.

42. Pittelli, M., Formentini, L., Faraco, G., Lapucci, A., Rapizzi, E., Cialdai, F., Romano, G., Monetti, G., Moroni, F., and Chiarugi, A. (2010) Inhibition of nicotinamide phosphoribosyltransferase: cellular bioenergetics reveals a mitochondrial insensitive NAD pool. *J. Biol. Chem.* **285**, 34106–34114.

43. Imai, S., and Guarante, L. (2014) NAD⁺ and sirtuins in aging and disease. *Trends Cell Biol.* **24**, 464–471.

44. Chi, Y., and Sauve, A. A. (2013) Nicotinamide riboside, a trace nutrient in foods, is a vitamin B3 with effects on energy metabolism and neuroprotection. *Curr. Opin. Clin. Nutr. Metab. Care.* **16**, 657–661.

45. Ruggieri, S., Orsomando, G., Sorci, L., and Raffaelli, N. (2015) Regulation of NAD biosynthetic enzymes modulates NAD-sensing processes to shape mammalian cell physiology under varying biological cues. *Biochim. Biophys. Acta* **1854**, 1138–1149.

46. Kim, H.-J., Oh, G.-S., Choe, S.-K., Kwak, T. H., Park, R., and So, H.-S. (2014) NAD⁺ metabolism in age-related hearing loss. *Aging Dis.* **5**, 150–159.