Mitochondrial function in liver cells is resistant to perturbations in NAD⁺ salvage capacity

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Running title: Role of NAMPT for maintaining liver mitochondrial function

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Abstract
Supplementation with nicotinamide adenine dinucleotide (NAD⁺) precursors such as nicotinamide riboside (NR) has been shown to enhance mitochondrial function in the liver and prevent hepatic lipid accumulation in high-fat diet (HFD)-fed rodents. Hepatocyte-specific knockout of the NAD⁺-synthesizing enzyme nicotinamide phosphoribosyltransferase (NAMPT) reduces liver NAD⁺ levels, but the metabolic phenotype of Nampt-deficient hepatocytes in mice is unknown. Here, we assessed Nampt’s role in maintaining mitochondrial and metabolic functions in the mouse liver. Using the Cre–LoxP system, we generated hepatocyte-specific Nampt knockout (HNKO) mice, having a 50% reduction of liver NAD⁺ levels. We screened the HNKO mice for signs of metabolic dysfunction following 60% HFD feeding for 20 weeks +/− NR supplementation and found that NR increases hepatic NAD⁺ levels without affecting fat mass or glucose tolerance in HNKO or WT animals. High-resolution respirometry revealed that NR supplementation of the HNKO mice did not increase state III respiration, which was observed in WT mice following NR supplementation. Mitochondrial oxygen consumption and fatty-acid oxidation was unaltered in primary HNKO hepatocytes. Mitochondria isolated from whole HNKO livers had only a 20% reduction in NAD⁺, suggesting that the mitochondrial NAD⁺ pool is less affected by
HNKO than the whole-tissue pool. When stimulated with tryptophan in the presence of $^{15}$N-glutamine, HNKO hepatocytes had a higher $^{15}$N-NAD$^+$ enrichment than WT hepatocytes, indicating that HNKO mice compensate through de novo NAD$^+$ synthesis. We conclude that NAMPT-deficient hepatocytes can maintain substantial NAD$^+$ levels and that the Namp knockout has only minor consequences for mitochondrial function in the mouse liver.

Mitochondrial dysfunction is thought to contribute to the development of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) (1, 2). Lipid accumulation increases mitochondrial fatty-acid oxidation capacity, but impaired capacity of the mitochondrial respiratory chain complexes results in increased ROS formation, which may contribute to the liver damage observed in NASH (3, 4). Hence, improving mitochondrial function in patients with NAFLD and NASH represents a potential treatment strategy.

In several animal models of NAFLD, hepatic levels of nicotinamide adenine dinucleotide (NAD$^+$) are reduced (5–10). NAD$^+$ precursor supplementation in a rodent model for NAFLD increases the abundance of enzymes regulating beta-oxidation and mitochondrial respiratory complexes (7). Moreover, this treatment improves mitochondrial function and decreases hepatic lipid accumulation (7). NAD$^+$ precursor supplementation has also been shown to efficiently improve mitochondrial function in multiple organs and cell types (9, 11–15), and has been used to treat rodent models of type 2 diabetes (16, 17). In mitochondria, NAD$^+$ is central for ATP synthesis through oxidative phosphorylation, where NADH serves as an electron donor (18). NAD$^+$ is also used as a precursor for the synthesis of NADP$^+$, which is an important cofactor for biosynthesis and antioxidative defenses (19). However, beneficial effects of NAD$^+$ precursor supplementation may be mediated by increased activity of NAD$^+$-dependent sirtuins (20). Sirtuins (SIRT1-7) comprise a family of deacetylase enzymes of which several regulate mitochondrial biogenesis and function (21).

Sirtuins break down NAD$^+$ to nicotinamide (NAM) and O-acetyl ADP-ribose. As NAM inhibits sirtuin activity, the NAD$^+$ generated from recycled NAM is important for maintaining sirtuin activity (22). NAM and phosphoribosyl pyrophosphate (PRPP) are turned into nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT) (23), and nicotinamide mononucleotide adenylyltransferases (NMNATs) further convert NMN to NAD$^+$ using ATP as a substrate. Supplementation with NMN reduces the adverse effects of obesity, diabetes, and aging in rodents (9, 17, 24). In the liver, NAD$^+$ can also be generated de novo from tryptophan or from nicotinic acid (NA) through the Preiss-Handler pathway (25). Synthesis of NAD$^+$ from tryptophan is a multi-step process requiring 8 separate reactions (26). Synthesis from NA begins with the conversion of NA to nicotinic acid mononucleotide (NAMN) by the action of NA phosphoribosyltransferase (NAPRT) (27). NAMN is then further converted to NA adenine dinucleotide (NAAD) and finally to NAD$^+$ by the action of NMNAT and NAD synthase, respectively (27). These final two steps are also required for NAD$^+$ synthesis from tryptophan (26). As Nadsyn1 expression is restricted to liver, kidney, and small intestine, NAD$^+$ synthesis from NA and tryptophan is limited to these tissues (28). Additionally, NAD$^+$ can be synthesized from nicotinamide riboside (NR), which is converted to NMN by the nicotinamide riboside kinases (NRKs) (29). NR has been widely applied in NAD$^+$ supplementation studies in both rodents (7, 8, 12, 15, 16, 30) and humans (31–35). NMN and NR can both be delivered orally to increase NAD$^+$ levels in peripheral tissues (7, 8, 12, 24). Interestingly, NMN appears to be converted to NR for cellular uptake, indicating that NR is the “transport form” of these NAD$^+$ precursors (29).

It is not known if decreased liver NAD$^+$ content observed in rodent models for NAFLD is due to increased activity of NAD$^+$ consuming enzymes, or
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because of decreased NAD$^+$ biosynthesis. Furthermore, it is not known if low NAD$^+$ levels are a consequence of hepatic lipid accumulation and mitochondrial dysfunction, or if low NAD$^+$ levels precede steatosis development. The NAMPT inhibitor FK866 aggravates the development of steatosis in high-fat diet-fed mice (36), and FK866 increases the susceptibility of human cancer cell lines to oxidative stress (37). Furthermore, hepatocyte-specific knockout of Nampt decreases hepatic NAD$^+$ content, impairs fatty-acid oxidation, and decreases mitochondrial oxygen consumption (38). Enzymatically inactive NAMPT transgenic mice are also more susceptible towards NAFLD development, and inducible hepatocyte-specific knockdown of Nampt impairs liver regeneration (8, 39). Previously, low hepatic NAMPT levels were observed in obese animals (5, 8, 10, 17). This suggests that impaired NAD$^+$ salvage may be an important factor for disease development. However, we and others have observed increased NAD$^+$ and NAMPT levels in livers of high-fat diet (HFD)-fed mice (40), or little or no change in hepatic NAD$^+$ content following long-term HFD feeding (41–43). This suggests that hepatic lipid accumulation per se may not cause a shortage of NAD$^+$ or impair NAD$^+$ salvage capacity. Hence, further investigation into the relationship between mitochondrial function, hepatic lipid accumulation, and NAD$^+$ salvage capacity is warranted.

As the metabolic phenotype of hepatocyte-specific Nampt knockout mice has not been thoroughly investigated (38), we hypothesized that disruption of salvage capacity would impact mitochondrial function, and increase the susceptibility of the liver to lipid accumulation following a HFD. To this end, we generated stable Nampt knockdown Hepa1c1c7 cells and hepatocyte-specific Nampt knockout (HNKO) mice with the aim to determine the role of Nampt for maintaining mitochondrial function in liver cells under normal- and excess energy states. Our data suggest that the liver is capable of maintaining part of the NAD$^+$ pool in the absence of NAMPT, and that deletion of Nampt per se has minor effects on hepatic mitochondrial function.

Results

Nampt knockdown in Hepa1c1c7 cells reduces NAD$^+$ levels without affecting mitochondrial respiratory function

As embryonic deletion of Nampt from hepatocytes reduces hepatic NAD$^+$ content and reduces mitochondrial oxygen consumption rate (38), we investigated whether this could be replicated in a hepatocarcinoma cell line. Stable Nampt knockdown (shNampt) in Hepa1c1c7 cells reduced NAMPT protein abundance (Fig. 1A, p<0.01) and ablated NAMPT activity (Fig. 1B, p<0.05). Knockdown of Nampt reduced NAD$^+$ levels by 60% (Fig. 1C, p<0.01), confirming that salvage of NAM contributes to the maintenance of NAD$^+$ levels in hepatocarcinoma cells. NR treatment increased NAD$^+$ levels in both control and shNampt cells (p<0.01), but NAD$^+$ levels were still lower in shNampt cells after NR treatment. To determine whether reduced cellular NAD$^+$ content affected mitochondrial oxygen consumption, we performed extracellular flux analysis on control and shNampt cells +/- supplementation with NR. Neither knockdown of Nampt nor NR treatment affected basal respiration, oxygen consumption for ATP synthesis, maximal respiratory capacity, or the glycolytic capacity (basal extracellular acidification rate, ECAR) (Fig. 1D-I). Hence, NAMPT contributes to maintaining NAD$^+$ levels in Hepa1c1c7 cells, but appears dispensable for maintaining mitochondrial respiratory function.

Hepatocyte-specific knockout of Nampt does not affect body composition or glucose tolerance

While mitochondrial respiratory capacity was maintained in Hepa1c1c7 cells, previous reports observed impaired mitochondrial function in hepatocytes from hepatocyte-specific Nampt KO mice (38). We next investigated whether this defect in mitochondrial function would make HNKO mice prone to develop obesity and glucose intolerance.
Since male mice are prone to fighting when group-housed, and since prolonged single-housing is also associated with stress and depression (44), the metabolic characterization of HNKO mice was performed in females. HNKO mice did not become obese with age and had a comparable body composition to WT animals at 10-, 55- and 110 weeks of age (Fig. 2A). In young adult female mice (15-27 weeks of age), no significant changes in glucose-, insulin-, or pyruvate tolerance were detected (Fig. 2B-D) indicating that glucose homeostasis was maintained. 55-week-old HNKO mice had a slight shift in glucose tolerance (Fig. 2E, main effect of genotype, p<0.05), but pyruvate tolerance remained unaltered in these mice (Fig. 2F). As the 110-week-old mice were too frail for tolerance tests, we measured circadian oscillations in blood glucose-, lactate- and ketone levels. No differences were observed between WT and HNKO mice (Fig. 2G-I). Liver weight increased independently of genotype with age (Fig. 2J, p<0.05). Protein abundance of NAMPT was equally reduced in HNKO mice of all ages (Fig. 2K), and at most time points, hepatic NAD⁺ levels were approximately 50% lower in HNKO mice (Fig. 2L, p<0.01). Hepatic NAD⁺ levels increased with age regardless of genotype (p<0.05), which could indicate an increased reliance on NAD⁺ synthesis from tryptophan or NA. NADP⁺ levels were not significantly affected at any of the time points (Fig. 2M). Collectively, these data confirm the importance of NAMPT for maintaining hepatic NAD⁺ levels in vivo. However, lack of Nampt does not predispose HNKO mice to the development of obesity, and glucose homeostasis is similarly affected with age between genotypes.

### HFD-induced fat mass accumulation and glucose intolerance develop independently of hepatic Nampt expression

As mice with enzymatically inactive NAMPT in the liver were found to develop hepatic steatosis (8), we challenged HNKO mice with HFD. Furthermore, we included a group of mice receiving NR in the drinking water. To determine if NR was stable in solution, we measured NR stability over one week. Nampt content in the drinking water increased significantly over time (p<0.01, Fig. 3A), but a significant decrease in NR levels was only detected after 6 days. Even then, more than 75% of the NR remained in the solution (p<0.01, Fig. 3A). NR stability was not affected by placing the water bottle in a cage with mice, demonstrating that potential bacterial contamination did not accelerate breakdown. Thus, NR appears stable in drinking water. Female WT and HNKO mice were fed a HFD (60% of total energy content from fat) or a matched low-fat control diet (LFD, 10% of total energy content from fat) for 20 weeks. The presence of NR in the drinking water did not affect mouse water intake (Fig. 3B). Body weight increased rapidly in mice fed a HFD (Fig. 3C, time x diet interaction p<0.01) whereas body weight was unaltered in LFD-fed mice over time (Fig. 3C). Lean mass was not significantly altered between groups throughout the study (Fig. 3D). Fat mass accumulation increased significantly throughout the study in the HFD groups (Fig. 3E, time x diet interaction p<0.01), and HNKO mice accumulated fat mass to the same degree as WT mice. Glucose tolerance was impaired regardless of genotype after both 10 and 19 weeks of HFD feeding (Fig. 3F-G, time x diet interaction p<0.01). No changes in fat mass accumulation or glucose tolerance were detected in NR-supplemented mice regardless of genotype. Hence, neither Nampt knockout nor NR supplementation affected HFD-induced body weight gain or fat mass accumulation.

### Liver mitochondrial function is unaffected following 20 weeks of HFD, but NR supplementation increases State III respiration in a genotype-specific manner

To determine whether mitochondrial respiratory capacity in the liver was affected in HFD/NR-treated HNKO and WT mice, hepatic mitochondrial function was evaluated by high-resolution respirometry. An interaction was observed between genotype and NR for basal respiration (Fig. 4A, interaction p<0.05), and post hoc testing showed decreased oxygen consumption in the NR-treated HNKO mice (p<0.05). When
stimulated with carnitine palmitate, the HFD-fed HNKO groups had lower respiration rates, indicating an impairment of fatty-acid utilization during uncoupled respiration (Fig. 4B, p<0.05). HFD caused an increase in State III respiration compared to LFD groups (respiration rate in response to ADP addition, Fig. 4C, p<0.01). Interestingly, NR treatment increased state III respiration in WT mice, but not in HNKO animals (interaction p<0.05, post hoc test p<0.05). The differences in oxygen consumption persisted following the addition of glutamate and pyruvate (Fig. 4D-E). No change in respiration rate was observed between groups after succinate and FCCP stimulation (Fig. 4F-G), though a borderline effect of genotype was observed between HFD vs. HFD+NR following FCCP stimulation (p=0.06). No change was detected in the CytC/pyruvate ratio between groups, indicating that mitochondria were not damaged (data not shown). Thus, while mitochondrial oxygen consumption was largely maintained in HNKO mice, HNKO mice have minor impairments in fatty-acid handling and a decreased ability to increase respiration in response to NR supplementation.

**HFD-feeding does not affect mitochondrial protein abundance in HNKO mice**

To investigate whether the difference in State III respiration between genotypes following NR stimulation was associated with an altered number of mitochondria, we measured citrate synthase activity in the liver. Citrate synthase activity correlates with mitochondrial content in skeletal muscle (45). We observed decreased citrate synthase activity in HFD-fed mice (Fig. 5A, p<0.01). However, activity was not affected by NR or genotype. NR was shown to increase abundance of the oxidative phosphorylation (OXPHOS) complexes (7), but in our hands levels of OXPHOS complex I, II, IV, and V were not affected by either genotype or NR (Fig. 5B-E). However, complex II abundance was decreased in HFD-fed mice compared to LFD-fed mice (Fig. 5C, p<0.05). Reduced NAD+ content in the cytosol may affect transport into the mitochondria of electrons generated through glycolysis (46). We therefore measured abundance of the malate-aspartate shuttle proteins SLC25A11 and SLC25A13. HFD-diet feeding increased hepatic abundance of SLC25A11, but abundance was not affected by genotype or NR treatment (Fig. 5F, p<0.01). SLC25A13 abundance was not affected by diet, genotype or NR (Fig. 5G). NR has previously been demonstrated to decrease hepatic lipid peroxidation, indicating an attenuation of oxidative stress (7). Abundance of catalase and MnSOD were not affected by diet, genotype or NR (Fig. 5H-I) indicating no changes in oxidative stress from HFD or Nampt knockout. Together, these data indicate that HFD decreases citrate synthase activity and alters the abundance of specific mitochondrial proteins. However, these changes were not affected by NR supplementation, and the genotype differences in state III respiration appear not to be associated with alterations in mitochondrial protein abundances.

**NR decreases LDL+VLDL and HDL content regardless of genotype, but does not affect hepatic triglyceride content**

To investigate whether the impaired oxygen consumption observed in HNKO liver following palmitate stimulation was associated with changes in hepatic lipid metabolism, we quantified hepatic triglyceride levels. A significant increase in triglyceride was observed following HFD feeding independent of genotype (Fig. 6A, p<0.05). Interestingly, NR did not decrease hepatic triglyceride content in this model as previously described (12). HFD feeding significantly increased plasma cholesterol levels regardless of genotype (Fig. 6B, p<0.01). An interaction was observed between NR treatment and genotype (p<0.05), where NR significantly decreased plasma cholesterol levels in HNKO mice (p<0.05) but not in WT mice. NR tended to decrease plasma LDL+VLDL levels (Fig. 6C, p=0.05) and resulted in a small, but significant decrease in plasma HDL levels regardless of genotype (Fig. 6D, p<0.05). We observed a tendency towards decreased hepatic ATGL abundance in HFD-fed mice regardless of
genotype (Fig. 6E, p=0.06), but NR had no significant effects on ATGL abundance. HSL abundance was unaltered between groups (Fig. 6F) and no significant change was observed in pS660, indicating a similar regulation of lipolysis between groups (Fig. 6G). HFD-fed mice had low abundance of hepatic fatty-acid synthase (FASN) compared to LFD-fed mice (Fig. 6H, p<0.01) indicating that control of hepatic lipid synthesis was maintained. No differences in FASN abundance were observed between genotypes or with NR treatment. We found no changes in the abundance of ACACA2, which mediates the final step in beta-oxidation (Fig. 6I), but HFD feeding decreased hepatic abundance of LCAD (Fig. 6J, p<0.01) and borderline increased abundance of MCAD (Fig. 6K, p=0.09). However, no differences were observed for either protein between genotypes or with NR treatment. HFD tended to decrease abundance of the hepatic cholesterol sensor LXRα (Fig. 6L, p=0.06), but no change was observed following NR treatment, even though NR lowered plasma cholesterol levels in HNKO mice. Collectively, our data indicate that the decreased respiration following palmitate stimulation in HNKO mice does not result in severe lipid accumulation, or altered abundance of proteins associated with lipogenesis or lipolysis. Furthermore, we demonstrate that NR reduces plasma HDL content independent of genotype.

**NR supplementation increases hepatic NAD⁺ content and rescues HFD-induced decrease in protein malonylation**

The general lack of effect of NR on fat mass accumulation and hepatic triglyceride content, could be due to lack of effects on hepatic NAD⁺ metabolism. As expected, NAMPT protein abundance was significantly decreased in HNKO mice (Fig. 7A, p<0.01), but was not altered by diet or NR treatment in WT mice. Similarly to chow-fed mice, hepatic NAD⁺ levels were decreased in HNKO mice from both the LFD and HFD groups (Fig. 7B, p<0.01). NR increased hepatic NAD⁺ levels compared to HFD-fed mice (Fig. 7B, p<0.01), but HFD feeding also increased hepatic NAD⁺ levels regardless of genotype compared to LFD-fed mice (Fig. 7B, p<0.01). NADH levels were not affected by NR or genotype (Fig. 7C). NADP⁺ levels decreased in HNKO mice on both LFD and HFD (Fig. 7D, p<0.01), and NR increased NADP⁺ levels in HNKO mice compared to HFD-fed mice (Fig. 7D, interaction p<0.05, KO HFD vs. KO HFD+NR p=0.01). No alterations in NADPH levels could be detected between groups (Fig. 7E).

To identify whether knockout of Nampt or NR supplementation affected activity of NAD⁺-consuming processes, we first attempted to detect formation of poly(ADP)-ribose (PAR) polymers in the liver samples as a measure of PARP activity, but no PAR polymers could be detected in the samples (data not shown). PARP1 abundance was unaltered between groups (Fig. 7F), though a high degree of variance was observed between samples. NR has been demonstrated to increase SIRT1- and SIRT3-mediated deacetylation in skeletal muscle, liver, and brown adipose tissue (12), and HFD causes hyperacetylation of mitochondrial proteins (47). We found increased lysine acetylation following HFD feeding (Fig. 7G, p<0.05), but no effect of genotype or NR supplementation. We also measured the acetylation levels of P65, a subunit of NF-κB, as an indirect measure of SIRT1 activity (48). P65 content was similar between all groups (Fig. 7H), but HFD increased P65 acetylation (Fig. 7I, p<0.01), and acetylation status was not affected by either genotype or by NR. Hence, although NR increased hepatic NAD⁺ content, this did not decrease acetylation of this SIRT1 target. In addition to deacetylation, other sirtuins can remove different acyl-groups such as succinyl- and malonyl from lysine residues (49). In line with previous observations (42), HFD feeding caused a significant decrease in lysine malonylation compared to LFD feeding (Fig. 7I, p<0.01). Interestingly, no differences were observed between malonylation in HNKO and WT mice, but NR supplementation caused a small increase in lysine malonylation (Fig. 7I, p<0.01), indicating either increased malonylation or decreased demalonylation. No change was observed in SIRT5 abundance (Fig. 7K). Collectively, knockout of
\textit{Nampt} has the expected effects on NAD\textsuperscript{+} levels, and NR is able to increase hepatic NAD\textsuperscript{+} concentrations. However, NR affects only lysine malonylation and not lysine acetylation.

The hepatic mitochondrial NAD\textsuperscript{+} pool is less sensitive to \textit{Nampt} ablation than the whole tissue NAD\textsuperscript{+} pool

The respirometry analysis showed only minor alterations in mitochondrial oxygen consumption in HNKO mice, and our data suggest that mitochondrial function is maintained in the absence of \textit{Nampt}. A recent report suggested that mitochondria contain no NAMPT, and instead import NAD\textsuperscript{+} from the cytosol (50). We therefore hypothesized that the mitochondrial NAD\textsuperscript{+} pool could be less sensitive to \textit{Nampt} ablation, which would allow mitochondrial function to be maintained. To this end, we isolated mitochondria from livers of male HNKO mice and WT littersmates. Mitochondrial fractions contained no GAPDH (Fig. 8A, p<0.01) or Lamin A/C (Fig. 8B, p<0.01), indicating that the mitochondrial fraction was free of cytosolic and nuclear proteins. In contrast, the mitochondrial fraction contained an increased abundance of OXPHOS complex I (Fig. 8C, p<0.01). NAMPT was not detected in the livers of HNKO mice (Fig. 8D), and the mitochondrial fraction from WT livers did not contain any NAMPT as expected (50). While total NAD\textsuperscript{+} content was reduced by 50\% in livers from HNKO mice (Fig. 8E, p<0.01), NAD\textsuperscript{+} levels in mitochondria from HNKO mice were only reduced by 20\% (Fig. 8F, p<0.01). The ratio between mitochondrial NAD\textsuperscript{+} and total liver NAD\textsuperscript{+} content was increased in HNKO mice (Fig. 8G, p<0.01), indicating that a larger proportion of the total NAD\textsuperscript{+} pool of HNKO hepatocytes resides in the mitochondria. Tissue NADH levels were reduced by 40\% in HNKO livers (Fig. 8H, p<0.01), whereas levels were unaltered in HNKO mitochondria (Fig. 8I). However, the ratio between mitochondrial and total NADH levels was not significantly increased in HNKO mice (Fig. 8J). NADP\textsuperscript{+} levels were not altered in HNKO mice (Fig. 8K-M). Interestingly, NADPH levels were significantly decreased in HNKO liver tissue (Fig. 8N, p<0.05), but were not significantly altered in the HNKO mitochondria (Fig. 8O). The ratio between mitochondrial NADPH and whole-liver NADPH was not significantly altered (Fig. 8P). Collectively, our data show that the mitochondrial NAD\textsuperscript{+} and NADP pools are less sensitive to \textit{Nampt} ablation compared with whole tissue levels. This provides a possible explanation for how mitochondrial function can be maintained in the absence of \textit{Nampt}.

Mitochondrial function is maintained in primary hepatocytes with knockout of \textit{Nampt}

We next investigated whether mitochondrial function was preserved in primary mouse hepatocytes from HNKO mice. As several reports have observed enhanced mitochondrial function following NR treatment (12, 13, 15), we also treated cells with NR. No NAMPT protein was detected in primary hepatocytes from HNKO mice (Fig. 9A). Decreased fatty acid oxidation in liver homogenate from hepatocyte-specific \textit{Nampt} knockout mice has been reported previously (38). However, fatty acid oxidation was not different between WT and HNKO primary hepatocytes (Fig. 9B). Primary hepatocytes were incubated with TMRM dye to measure mitochondrial membrane potential, but no difference in fluorescence intensity was detected between genotypes (Fig. 9C). Following 6 hours of NR treatment, NAD\textsuperscript{+} content increased in primary hepatocytes regardless of genotype (Fig. 9D, p<0.01), however, no increase was observed in the mitochondrial NAD\textsuperscript{+} pool (Fig. 9E). Thus, there was a borderline decrease in the mitochondrial/whole cell NAD\textsuperscript{+} ratio after NR treatment compared to untreated cells (Fig. 9F, p=0.08), suggesting that NAD\textsuperscript{+} repletion may primarily increase cytosolic and nuclear NAD\textsuperscript{+} pools. Finally, we found no differences in basal respiration rate, oxygen consumption for ATP synthesis, maximal respiration rate, or basal ECAR with or without 500 \mu M NR (Fig. 9G-L). Thus, in primary hepatocytes, mitochondrial respiratory function, fatty-acid oxidation, and mitochondrial membrane potential.
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are not affected by Nampt knockout or NR stimulation.

**NAMPT is the major contributor for maintaining NAD\(^+\) levels in primary mouse hepatocytes**

Across experiments, HNKO mice maintained 50% of liver NAD\(^+\) levels. To determine whether HNKO mice were more efficient in utilizing other NAD\(^+\) precursors, we measured expression of genes from different NAD\(^+\) synthesizing pathways. Primary hepatocytes from HNKO mice expressed very low levels of Nampt (Fig. 10A). HNKO mice expressed Tdo2, Afmid, Kmo, or Qprt to WT levels (Fig. 10A), suggesting a maintained expression level of de novo synthesis genes. Nrkl expression was also not affected in HNKO hepatocytes (Fig. 10A) and no change was observed in expression of Nadsyn (Fig. 10A). To test the efficiency of NAD\(^+\) synthesis from NAD\(^+\) precursors, we incubated HNKO and WT hepatocytes with different substrates for 6 hours. Across treatments there was an overall reduction in NAD\(^+\) in primary HNKO hepatocytes as assessed by a two-way ANOVA testing genotype vs. treatment (Fig. 10B, main effect p<0.01). NR and NMN significantly increased NAD\(^+\) levels regardless of genotype, though NAD\(^+\) content was still lower in HNKO hepatocytes (Fig. 10B, p<0.05). Treatment with NAM, NA or Trp did not affect NAD\(^+\) levels after 6 hours (Fig. 10B). However, when HNKO hepatocytes were incubated with NA or Trp for 24 and 48 hours, both precursors prevented the decline in NAD\(^+\) observed in the control group (Fig. 10C, p<0.01). These data suggest the different NAD\(^+\) precursors affect the hepatocyte NAD\(^+\) pool in distinct ways. WT and HNKO hepatocytes were subsequently incubated for 48 hours with the NAMPT inhibitor, FK866, or the NADSYN inhibitor, azaserine, which blocks synthesis from Trp and NA (51). Regardless of genotype, NAD\(^+\) content declined over time (Fig. 10D, p<0.01). NAD\(^+\) levels were reduced in HNKO hepatocytes at all investigated time points (Fig. 10D, p<0.01). FK866 treatment significantly reduced NAD\(^+\) levels in WT hepatocytes over time (Fig. 10D, p<0.01), but not in HNKO hepatocytes. After 48 hours NAD\(^+\) levels in FK866-treated WT hepatocytes were similar to NAD\(^+\) levels in HNKO hepatocytes, suggesting that NAMPT is the primary contributor to the hepatic NAD\(^+\) pool in vitro. Azaserine did not significantly affect NAD\(^+\) levels regardless of genotype, indicating that the contribution of the de novo pathway was reduced in culture. When WT and HNKO hepatocytes were stimulated with NAD\(^+\) precursors following 48h of FK866 treatment, NR and NA caused an almost 3-fold increase in NAD\(^+\) content independent of genotype (Fig. 10E, p<0.01). Trp caused a minor increase in NAD\(^+\) content when tested alone against FK866 with a two-way ANOVA (p<0.01), but the increase was much smaller than for both NA and NR. No genotype-specific differences were observed in response to stimulation with the various precursors, indicating that HNKO hepatocytes do not utilize NA or Trp for NAD\(^+\) synthesis to a greater extend compared with WT cells. To directly demonstrate whether HNKO hepatocytes rely on de novo synthesis for maintaining NAD\(^+\) levels, we incubated HNKO and WT hepatocytes with 500 μM tryptophan and \(^{15}\)N-glutamine for up to 24 hours. This results in \(^{15}\)N-labelling of NAD\(^+\) produced through NADSYN1 (27). To prevent the confounding factor of NAD\(^+\) salvage in WT hepatocytes, the experiment was also carried out in the presence of FK866. A main effect of genotype was observed for endogenous \(^{14}\)N-NAD\(^+\) levels (Fig. 10F, p<0.05), though \(^{14}\)N-NAD\(^+\) levels declined throughout the experiment (P<0.05). \(^{15}\)N-NAD\(^+\) could be detected at all time points, but levels peaked after 6 hours (Fig. 10G, p<0.05). Enrichment of \(^{15}\)N-NAD\(^+\) increased over time (Fig. 10H, p<0.01), and was significantly higher in HNKO mice (p<0.05). No effect of FK866 was observed, indicating that salvage of \(^{15}\)N NAD\(^+\) was not a major factor in WT mice. Through MS/MS analysis of NAD\(^+\), enrichment occurred at the carboxamide nitrogen of the NAM ring of NAD\(^+\) (data not shown) as expected for de novo synthesis of NAD\(^+\). Collectively, these data indicate that hepatocytes from HNKO mice can compensate for
the lack of NAMPT by increasing the amount of NAD\(^+\) produced from Trp.

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**Discussion**

As dietary NAD\(^+\) precursor supplementation can alleviate symptoms of a wide range of diseases and conditions, it is essential to investigate the underlying mechanisms associated with NAD\(^+\) deficiency. We demonstrate that while NAMPT contributes significantly to the hepatic NAD\(^+\) pool, hepatocytes are capable of maintaining a substantial fraction of NAD\(^+\) in the absence of *Nampt* through the *de novo* pathway. We also show that deletion of *Nampt* in the liver causes no obvious phenotype in the adult animal, and that HNKO mice are not susceptible to diet-induced fat mass accumulation. Hence, under normal culturing/feeding conditions, ablation of NAMPT-mediated NAD\(^+\) salvage appears to have only minor metabolic consequences.

NAD\(^+\) levels were significantly decreased in young and adult HNKO mice. These results are in line with previously published results in hepatocyte-specific *Nampt* knockout mice and mice expressing enzymatically inactive NAMPT in the liver (8, 38). In contrast, inducible knockout of *Nampt* in liver does not affect NAD\(^+\) levels (39). HNKO mice resembled WT littermates in both body composition and in the various tolerance tests. At 110 weeks of age, mice maintained normoglycemia, indicating that HNKO mice are not prone to age-induced loss of glucose control. A surprising finding was that hepatic NAD\(^+\) content increased with age for both HNKO and WT mice, which is in contrast to several reports (8, 52, 53). This may be a consequence of the continuous backcrossing of our model on to the C57BL/6JBomTac background, done to avoid the *Nnt* mutation present in other C57BL6/J strains (54). We have previously reported that the hepatic NAMPT/NAD\(^+\) levels in C57BL/6JBomTac mice are resistant to prolonged high-fat diet feeding (42), and this strain may therefore also be resistant to aging-induced decreases in hepatic NAD\(^+\) levels. In HNKO mice, the increased NAD\(^+\) levels may reflect an increased efficiency of the *de novo* synthesis pathway with time compared to young mice, adapting to a life-long shortage of NAD\(^+\) in the liver.

The mitochondrial NAD\(^+\) pool of HNKO mice was less affected than the total NAD\(^+\) pool, suggesting that cytosolic- and nuclear NAD\(^+\) pools are primary sites of NAD\(^+\) depletion, but also that the mitochondrial NAD\(^+\) pool is prioritized over the cytosolic/nuclear NAD\(^+\) pool. A recent study show that mitochondria are unable to utilize NAM for NAD\(^+\) synthesis (50). Instead, mitochondria were found to import NAD\(^+\) from the cytosol. It would be of interest to determine whether the mitochondrial NAD\(^+\) pool can be maintained if NAD\(^+\) levels in the cytosol/nucleus are further depleted. Interestingly, decreased oxygen consumption was observed in isolated mitochondria from liver-specific *Nampt* knockout mice following stimulation with palmitoylcarnitine (38). We also observed decreased oxygen consumption following palmitate-carnitine stimulation of whole liver from HNKO mice. Our data therefore suggest that a decrease in hepatocyte NAD\(^+\) content may be associated with a minor impairment of fatty acid oxidation. However, no decrease in fatty acid oxidation was observed in HNKO primary hepatocytes. Moreover, no HNKO-specific reductions in abundance of proteins associated with fatty-acid oxidation were observed, indicating that decreased oxidation might be a result of decreased pathway activity rather than decreased protein abundance.

Stimulation with different NAD\(^+\) precursors showed differential effects on hepatocyte NAD\(^+\) levels. NR and NMN induced a rapid increase in cellular NAD\(^+\) levels. The ability of NR to increase cellular NAD\(^+\) levels has been demonstrated across multiple cell lines (12) and in primary mouse hepatocytes (29). NA and Trp were not efficient precursors for NAD\(^+\) after 6 hours, but prevented a decline in NAD\(^+\) content in HNKO mice after 24 and 48 hours of incubation. NA and Trp may not be capable of increasing NAD\(^+\) levels after 6 hours due to the culturing media used, as this media contains...
50 µM Trp and 200 nM of NAM and NA, respectively. The presence of these metabolites may mask true precursor utilization capacities in the stimulated wells. When cells were stimulated with NA and tryptophan for 6 hours following 48 hours of NAD\(^+\) depletion and without replacing the media, NA was as potent as NR, and caused a much higher increase in NAD\(^+\) content than Trp. Moreover, we found that tryptophan contributes to the maintenance of the NAD\(^+\) pool, but that it is not efficient at increasing total NAD\(^+\) content. This is in line with a recent report, demonstrating that primary hepatocytes can utilize Trp to generate NAD\(^+\), but to a much lower degree than full liver (55). However, the labeling experiment also demonstrates a higher enrichment of NADSYN-produced NAD\(^+\) in HNKO hepatocytes, indicating that this pathway may be the primary way of compensation for the lack of NAM salvage in HNKO mice.

The NADSYN inhibitor azaserine did not affect NAD\(^+\) content (51), suggesting that an acute inhibition of the de novo pathway causes no immediate decline in NAD\(^+\) content. We demonstrate the importance of the NAD\(^+\) salvage pathway in primary hepatocytes by showing that FK866 treatment depleted NAD\(^+\) levels in WT hepatocytes after 48 hours, whereas no effect was observed in HNKO hepatocytes. This is in contrast to human primary hepatocytes that demonstrated resistance to FK866 depletion (56). In a previous study, mouse hepatocytes maintained 50% of NAD\(^+\) following 30 hours of FK866 stimulation (29). This resistance to full depletion could arise from efficient ‘detoxification’ of FK866 in primary hepatocytes, or from a lower turnover of NAD\(^+\). Primary mouse hepatocytes may require little new NAD\(^+\) synthesis in the absence of stress. However, we found that FK866 treatment accelerated the loss of NAD\(^+\), demonstrating the importance of NAMPT for maintaining NAD\(^+\) levels over time.

NAD\(^+\) levels increased independent of genotype in response to HFD feeding, and this was associated with a significant increase in triglyceride levels in both genotypes. Thus, in contrast to several published studies (5–10, 17), fat accumulation was positively associated with NAD\(^+\) levels. This was especially true for the HNKO mice which increased hepatic triglyceride content by 3 fold in response to HFD feeding. Female mice on a C57BL/6J BomTac background have been shown to only develop hepatic steatosis when housed under thermoneutral conditions (57). The choice of mouse strain for hepatic steatosis models should therefore be carefully considered. HFD feeding did, however, induce severe fat mass accumulation in our experiment indicating that this model can be used to mimic obesity development.

The HFD induced changes in liver protein abundance and modified the level of specific posttranslational modifications. HFD feeding decreased CS activity and OXPHOS complex II abundance, and increased lysine acetylation and decreased lysine malonylation. Both of the latter effects have been reported before (42, 47). NR supplementation did not decrease hepatic lysine acetylation, suggesting that sirtuin activity may not have been increased despite increased NAD\(^+\) levels. Furthermore, NR supplementation did not correct the HFD-induced increase in P65 acetylation, suggesting SIRT1 activity was not increased. However, NR supplementation partially corrected the HFD-induced decrease in lysine malonylation. Future investigations should determine how HFD affects the protein-specific malonylation patterns in the liver, and define the mechanism by which NR causes increased malonylation. As SIRT5 is an NAD\(^+\)-dependent demalonylase (49) it seems counterintuitive that increased NAD\(^+\) levels would cause increased malonylation. Currently it is not known whether increased malonylation may be related to putative beneficial effects of NR.

NR was stable in the drinking water, and oral NR administration significantly increased hepatic NAD\(^+\) and NADP\(^+\) levels. However, NR did not prevent fat mass accumulation or improve glucose tolerance as previously reported (7, 12), but decreased plasma HDL and LDL/VLDL levels as
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reported earlier (12). However, dietary NR supplementation for 12 weeks in obese, older, and dyslipidemic men was recently shown to not affect plasma levels of LDL, HDL, or total cholesterol (32). Intriguingly, our data revealed that while NR increased oxygen consumption following induction of State III respiration in WT mice, this was not observed in HNKO mice. No genotype effects were observed following FCCP stimulation, demonstrating that maximal uncoupled respiratory capacity was not compromised in the HNKO liver. At present, we have no explanation for this observation, but since NR increased NAD$^+$ levels in both genotypes, the underlying cause may be unrelated to reduced NAD$^+$ content induced by knockout of Nampt.

In conclusion, NAMPT plays an important role to maintain NAD$^+$ levels in hepatocytes. However, lack of NAMPT has only minor effects on mitochondrial function measured either in vitro or ex vivo. While NR was able to increase hepatic NAD$^+$ levels in both cell cultures and whole liver, and showed positive effects on the blood lipid profile in response to HFD feeding, it did not significantly affect hepatic triglyceride content, glucose tolerance, or whole-body fat mass accumulation in HFD-fed mice. Future studies will be directed towards clarifying the contribution of individual hepatic NAD$^+$ salvage pathways as well as the de novo synthesis pathway in response to dietary interventions that exert a more profound metabolic stress on the liver.

Experimental Procedures

Ethical Approval
Animal experiments were performed in accordance with the European directive 2010/63/EU of the European Parliament and of the Council for the protection of animals used for scientific purposes. Ethical approval was given by the Danish Animal Experiments Inspectorate (#2012-15-2934-307 and 2015-15-0201-00796).

Chemicals and reagents
Unless otherwise noted, all chemicals and reagents were purchased from Merck (Kenilworth, NJ, USA).

Cell Culture Experiments

Generation and culturing of Nampt knockdown cell line
Hepa1c1c7 cells were transfected with lentivirus carrying a shRNA construct to generate a stable Nampt KD cell line (shNampt, RMM3981-201824136, Open Biosystems Dharmacon, Lafayette, CO, USA) or a non-sense shRNA control cell line (sh-Nonsense, SHC202, Sigma) as previously described (58). Cells were cultured in Minimum Essential Medium Eagle Alpha (MEMα, 12561, ThermoFisher, Waltham, MA, USA) with 1% penicillin streptomycin (P/S) (15070063, ThermoFisher), 10% fetal bovine serum (FBS, F7524) and 2.5 µg/mL puromycin for selection, at 37°C with 5% CO$_2$. Cells were transfected in passage 4. Experiments were performed from passage 7 to 20.

Nicotinamide riboside supplementation
For NAD$^+$ measurement experiments, 500,000 cells/well of each cell line were seeded in 6-well plates and allowed to attach overnight. The medium was changed to serum-free MEMα (12561, ThermoFisher) with 1% P/S +/- 500 µM nicotinamide riboside (NR) (Chromadex, Irvine, CA, USA) dissolved in PBS. After ~24h incubation, media was removed and cells were rinsed once in PBS. Subsequently, cells were added 200 µL of trypsin (Trypsin-EDTA, Gibco) and incubated at 37°C for 10 min. Trypsin was neutralized with MEMα with 10% serum and cells were centrifuged for 5 min at 200 g. The media was removed and the pellets were rinsed once in PBS. Subsequently, cells were re-suspended in 200 µL of trypsin (Trypsin-EDTA, Gibco) and incubated at 37°C for 10 min. Trypsin was neutralized with MEMα with 10% serum and cells were centrifuged for 5 min at 200 g. The media was removed and the pellets were rinsed once in PBS and centrifuged for 5 min at 200 g. NAD$^+$ levels were measured using resazurin conversion (described below). NAD$^+$ concentration was normalized to protein concentration, determined from the cell pellets following NAD$^+$ extraction. Proteins were extracted by adding 100 µL 2% Sodium dodecyl sulphate (SDS) and heating them.
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to 95°C for 5 min, after which protein concentration was determined using the Bicinchoninic Acid Assay (BCA) (23227, ThermoFisher Scientific).

**Extracellular flux analysis**
Mitochondrial function was assessed using the XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent, Santa Clara, CA, USA). SeaHorse plates were coated with Corning Matrigel Basement Membrane Matrix (#356234, BD Biosciences, Franklin Lakes, NJ, USA), diluted 1:10 in MEMα with 10% FBS. 10 µL were added to each well for 5 min at room temperature, before plates were dried for 20 min at 37°C. 30,000 cells/well of shNampt and shNonsense cells (22-24 wells pr. Group) were seeded in MEMα with 10% FBS, 1% P/S, and puromycin (2.5 µg/mL), +/- 500 µM NR dissolved in PBS, for 20h at 37°C with 5% CO₂. 4 wells containing only media were included for background measurement. The SeaHorse XF96 Sensor Cartridge was hydrated with Seahorse XF Calibrant Solution (100840-000, Agilent) and was incubated overnight at 37°C without CO₂ in the XF Prep Station. On the day of measurement, 5.6 mM glucose and 1 mM pyruvate was added to the Seahorse XF Assay Medium (102365-100, Agilent), heated to 37°C, and pH-adjusted to 7.4 using 1 M HCl. Following incubation, each well was washed in 180 µL Seahorse XF Assay Medium and incubated 1h in 180 µL assay medium +/- 500 µM NR in the XF Prep Station at 37°C without CO₂. Cells were stimulated sequentially with 1 µM oligomycin, 0.2 µM FCCP (optimized for maximum oxygen consumption rate) and finally 1 µM Antimycin A + 1 µM rotenone, delivered through the sensor cartridge injection ports in XF Assay Medium. All oxygen consumption rates (OCR) were subtracted non-mitochondrial respiration, measured as average OCR following rotenone and antimycin A addition. Basal respiration was calculated as an average of OCR prior to oligomycin addition. Oxygen consumption for ATP synthesis was calculated by subtracting OCR following oligomycin addition from basal respiration rate. Maximal respiration was calculated as an average of OCR following FCCP addition. Basal extracellular acidification rate (ECAR) was calculated as an average of ECAR prior to oligomycin addition.

**Animal experiments**

**Mouse generation and maintenance**
Hepatocyte-specific Nampt knockout (HNKO) mice were generated by breeding homozygous floxed animals (15) with mice heterozygous for the cre recombinase gene which was driven by the serum albumin promoter (Alb-cre). Both lines were continuously backcrossed to the C57BL/6JBonTac background, and breeding was maintained by pairing Namptfl/fl, cre-/- (HNKO) males with Namptfl/fl (wild type (WT)) littermate females. For the aging cohort, female HNKO mice or WT littermates between 10 weeks of age and 110 weeks of age were used. Mice were group housed and had access to ad libitum chow (1310, Altromin, Lage, Germany) and water. Mice were housed in a temperature-controlled environment (22 ±1°C) with 12-hour light/dark cycles. In the “55-week-old” group, mice were between 53 and 58 weeks of age, and in the “110-week-old” group, mice were between 99 and 113 weeks of age. Body compositions were obtained by nuclear magnetic resonance (NMR) scanning (EchoMRI 4-1, EchoMRI, Houston, TX USA). Before oral glucose tolerance tests (OGTT), mice were fasted for 5.5 hours, from Zeitgeber time (ZT) 2.5 to 8. Basal blood glucose levels were measured using Contour Blood Glucose Meter (Bayer, Leverkusen, Germany). Mice were given an oral gavage of 2 g glucose pr. kg body weight. Blood glucose levels were measured after 20, 40, 60, 90, and 120 min following the gavage. For the insulin tolerance test (ITT), mice were fasted similarly as for the OGTT, and were given an intraperitoneal dose of 0.75 units of insulin pr. kg body weight, diluted in gelofusine (B. Braun, Melsungen, Germany). Mice were given an oral gavage of 2 g glucose pr. kg body weight. Blood glucose levels were measured after 20, 40, 60, 90, and 120 min following the gavage. For the insulin tolerance test (ITT), mice were fasted similarly as for the OGTT, and were given an intraperitoneal dose of 0.75 units of insulin pr. kg body weight, diluted in gelofusine (B. Braun, Melsungen, Germany). Blood glucose levels were measured immediately before injection, and 20, 40, 60, 90, and 120 min after. For the pyruvate tolerance test, mice were fasted for 18 hours, beginning at ZT 10. Mice were given an intraperitoneal injection dosed as 20 µL/g body...
weight of a pyruvate-lactate solution in saline (0.1 g lactate/mL and 10 mg pyruvate/mL) (59). Blood glucose levels were measured before and 20, 40, 60, and 90 min after the injection. For the 55-week-old group, a 120 min time point was included. The 110-week-old group was deemed too frail for tolerance tests, so blood glucose-, lactate- and ketone bodies were measured at ZT 3, 9, 15, and 21 during the light/dark cycle over 2 weeks. Lactate levels were measured using a Lactate Pro 2 (HaB Direct, Southam, United Kingdom) and ketone bodies were measured using Freestyle Optium Neo (Abbot, Chicago, IL, USA). All blood measurements were performed on blood from the tail vein, obtained by making a small incision with a 25G needle. Mice were sedated with pentobarbital (0.1 mg/g body weight, diluted in saline) and killed by cardiac puncture, livers were snap-frozen in liquid nitrogen and stored at -80°C until use for NAD⁺ measurements and Western blot analysis.

Mitochondria Isolation
Mitochondria were isolated from the liver of female WT and HNKO mice (11-15 weeks of age), sacrificed by cervical dislocation. The right liver lobe was transferred to a buffer containing 100 mM sucrose, 100 mM KCl, 50 mM Tris-HCl, 1 mM KH₂PO₄, 0.1 mM EGTA and 0.2% BSA. Samples were placed on wet ice throughout the isolation protocol, and mitochondria were isolated following a procedure adapted from muscle (60). Following isolation, the mitochondrial fraction was distributed into 4 tubes, and centrifuged at 7,000 g for 4 min. The supernatant was discarded and the pellets were stored at -80 °C.

High-fat diet experiments
Female HNKO and WT mice, (14-18 weeks of age), were randomized into three diet groups. Mice were fed a 60% HFD (D12492, Research Diets, New Brunswick, NJ, USA) or a sucrose-matched low-fat diet (LFD, D12450J, Research Diets). Half of the HFD-fed mice received NR (Chromadex, Irvine, CA, USA) in the drinking water, which was changed every 2-3 days. NR was supplied to deliver 400 mg/kg body weight/day, dosed as 3.33 g/L drinking water. This was based on previous experiments measuring water intake in HFD-fed mice (data not shown). Mice were fed this diet for 20 weeks, and body composition was assessed every fourth week by NMR (EchoMRITM). The experiment was repeated twice to obtain a sufficient number of animals in each group. After 9 weeks, water intake was measured for a subset of mice. The water bottle was weighed 5 times over the course of 1 week, and the average water intake pr. mouse pr. day was calculated from these measurements. After 10 and 19 weeks of HFD/LFD feeding, mice were subjected to an OGTT as described above. On the day of sacrifice, mice were sedated with pentobarbital and killed by cardiac puncture. Part of the liver was transferred to BIOPS buffer (as described below) for respirometry measurement, whereas the rest was snap-frozen in liquid nitrogen. Before further processing, frozen livers were crushed in liquid nitrogen using mortar and pestle.

Respirometry measurement
High-resolution respirometry was performed as previously described (45), with minor modifications. Following dissection, one piece of liver was transferred to ice-cold BIOPS buffer (containing 2.77 mM CaK₂EGTA, 7.23 mM K₂EGTA, 5.77 mM Na₂ATP, 6.56 mM MgCl₂·6H₂O, 20 mM Taurine, 15 mM Na₂Phosphocreatine, 20 mM Imidazole, 0.5 mM Dithiothreitol, 50 mM MES, pH 7.1). The liver piece was cut into smaller pieces, app. 0.5x0.5 mm, in ice-cold MiR05 buffer (containing 0.5 mM EGTA, 3 mM MgCl₂·6H₂O, 60 mM K-lactonionate, 20 mM Taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM Sucrose, 1 g/L BSA, pH=7.1). Tissues were not treated with permeabilisation reagents as this was previously demonstrated to be unnecessary for liver tissue (61). The liver pieces were transferred to fresh respiration buffer on ice for 10 min before being weighed and transferred to the Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). Respirometry measures were performed in MiR05 buffer at 37°C in hyper oxygenated conditions in
triplicates. Oxygen flux was processed by DatLab software (Oroboros Instruments). Mitochondrial respiratory function was assessed by the sequential addition of mitochondrial substrates. State II respiration was assessed by the addition of 2 mM malate. Then 25 µM palmitate-carnitine was added to determine fatty-acid oxidation. State III respiration was achieved by the addition of ADP-Mg (5 mM) followed by the addition of 10 mM glutamate and 5 mM pyruvate (Complex I substrates). 10 µM Cytochrome c was added to assess the integrity of the outer mitochondrial membrane. 10 mM succinate was added to assess maximal complex I+II linked respiration, and finally 0.25 µM cyanide p-trifluorome thoxyphenylhydrazon (FCCP) was added to achieve maximal uncoupled respiration.

**Primary hepatocyte experiments**

**Isolation and culturing of primary mouse hepatocytes**

Isolation of primary mouse hepatocytes was based on two protocols (62, 63). Male HNKO or WT mice (3-7 months of age) were sedated using avertin, prepared from a stock of 1 g/mL tribromoethanol in T-amyl alcohol, diluted 1:20 in saline, and dosed as 10 µL/g body weight. An incision was made into the abdomen and the cava vein was localized. A 24G catheter (1962025, Mediq Danmark, Denmark) was inserted into the inferior vena cava and fixed with a suture and a vein clamp. The catheter was connected to a peristaltic pump (520s, Watson Marlow, MA, USA). The portal vein was cut and the liver was perfused with 50 mL Hank's buffered saline solution without calcium and magnesium (HBSS, 14170112, Thermofisher), supplemented with 76 mg/L EGTA and 10 mM HEPES, heated to 42°C at 3.5 mL/min. After this, the liver was perfused with 50 mL Williams E medium (32551020, Thermofisher) supplemented with 400 mg/L collagenase (C5138, Sigma). Hepatocytes were dispensed in a petri dish containing plating medium (Minimum Essential Medium Eagle-199 (MEM-199) (41150, Thermofisher), 10% FBS, 1% P/S), and the cell suspension was passed through a 70 µm strainer (352350, Corning, Corning, NY, USA). Cells were centrifuged at 3x50 g for 2 min and between each step, the pellet was washed in plating medium.

Following the washes, the cell pellet was dissolved in plating medium and viability was assessed (NucleoCounter NC-200, ChemoMetec). 500,000 living cells/well were seeded in 12-well plates, 1 mio. living cells/well were seeded in 6-well plates, and 50,000 living cells/well were seeded in 96-well plates. All plates were collagen coated. Cells were allowed minimum 2 hours to attach. Medium was changed to culture medium (MEM-199 w. 0.5% FBS, 1% P/S, 1 µM dexamethasone and 1 nM insulin) and the cells were incubated overnight at 37°C with 5% CO₂.

**NAD⁺ precursor stimulation experiments**

On the day following seeding in a 6-well plate, the medium was changed to fresh culture medium, added PBS, or 500 µM of either NR, nicotinamide mononucleotide (NMN), NAM, tryptophan, NA, 10 µM FK866 or 10 µM azaserine. Cells were stimulated for 6, 24, or 48 hours, after which cells were harvested as described for Hepa1c1c7 cells. NAD⁺ levels were quantified using resazurin conversion, as described below. For the FK866 NAD⁺ depletion experiment, media was removed on the day following isolation, and replaced with culture medium supplemented with 10 µM FK866. Cells were incubated for 48 hours, after which each well was added NR, NA or Trp to a final concentration of 500 µM (or an equal volume of DMSO for the control wells), still in the presence of FK866. Cells were stimulated for an additional 6 hours, after which cells were harvested as described for Hepa1c1c7 cells.

**Fatty-acid oxidation detection**

Primary mouse hepatocytes from HNKO and WT mice were plated at a density of 50,000 cells/well in a collagen-coated 96-well plate. ¹⁴C-labeled palmitate:BSA complexes were prepared by evaporating the ethanol from a 0.1 µCi/µL palmitic acid solution (55 µCi/mmol, MC 121, Moravek, Brea, CA, USA) under a nitrogen stream, using 1.4 µL/well. The dried palmitate stock was subsequently added 5 µL/well of 1 mM palmitate:BSA prepared as previously described (64), and the solution was incubated for 30 min at 37 °C. 100 µL/well of glucose-free DMEM was
added to the vial, and part of the media was supplemented with 500 µM of NR. The media was subsequently added to the hepatocytes, and a glass-fiber filter 96-well plate (6005177, Perkin Elmer, Waltham, MA, USA) added 30 µL 1 M NaOH/well was clamped on top of the culture plate, separated by a custom-made perforated rubber insert. The back of the filter plate was sealed with a back cover seal (6005199, Perkin Elmer) and the plates were wrapped in parafilm. The cells were incubated at 37 °C for 4 hours, and the filter plate was recovered to detect 14CO2 release. The filter plate was dried at 50°C for one hour. Each well of the filter plate was added 50 µL Microscint-20 (6013621, Perkin Elmer). The plate was sealed with a Topseal (6050185, Perkin Elmer) and the plate was counted on a TopCount NXT scintillation counter (Perkin Elmer). Counts from wells with cells stimulated with media without 14C palmitate were subtracted as background. Wells with 14C-media but without cells were included as an internal control, and showed background level CPM on the filter (data not shown).

**Tetramethylrhodamine (TMRM) staining of primary hepatocytes**

Two days following seeding in a 12-well plate, medium was changed to culture medium with 500 µM NR. Cells were stimulated for 1 hour. TMRM (T668, Thermofisher) was added from a 1 µM stock in PBS to a final concentration of 50 nM, and cells were incubated for 20 min at 37°C. Cells were washed in PBS, and fluorescence was detected using a Hidex Sense plate reader (Excitation 544 nm, Emission 575 nm; Hidex, Finland). Values were corrected by subtracting fluorescence from hepatocytes without TMRM addition.

**Oxygen consumption measurements in primary hepatocytes**

Oxygen consumption was measured using the XF96 Extracellular Flux Analyzer as described for Hepa1c1c7 cells, with minor modifications. Plates were collagen-coated by adding 50 µL collagen solution (0.1 mg/mL in PBS) pr. well, and incubating the plate for 5 min at room temperature (RT). Liquid was discarded and the plate was left to dry overnight at RT. 40,000 cells pr. well were seeded in plating medium. After 2 hours, medium was changed to culture medium, +/- 500 µM NR. Cells were incubated overnight and on the day of the experiment, incubated for 1 hour in SeaHorse medium, prepared as described for Hepa1c1c7 cells, +/- 500 µM NR. Measurement and data analysis was performed as described in for Hepa1c1c7 cells.

**Mitochondria Isolation**

Mitochondria were isolated from primary mouse hepatocytes seeded in 6-well plates, following 6 hours of stimulation in culture medium with 500 µM NR or PBS. 500,000 cells pr. condition were recovered (for total NAD+ measurement) and mitochondria were isolated from the remaining 500,000 cells, following the protocol described above for full liver. The final mitochondrial fraction was split into two vials for further analysis.

**Measurement of NR stability in solution**

**Sample Collection**

1.7 g of NR was dissolved in water bottles used for drinking water in mouse experiments. Two bottles were placed in empty cages and 4 were placed in separate cages each containing 3 female C57BL/6J BomTac mice to account for possible increased NR breakdown due to bacterial contamination. NR water was sampled from bottles after 0h, 4h, 8h, and once every following day for a total of 6 days. Immediately after collection, samples were transferred to -80°C where they were stored prior to analysis.

**Materials for LC-MS analysis**

Unless otherwise stated, all reagents were HPLC grade or higher. Nicotinamide-d4 (NAM-d4) (catalog#: D-3457) and nicotinic acid-d4 (NA-d4) (catalog#: D-4368) were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). NR-13Cd was synthesized as described (33) and kindly provided by Prof. Marie Migaud (University of South Alabama, USA). N1-methylnicotinamide-d3 (MeNAM-d3) was prepared as described using non-labeled NAM (33). Non-labeled NAM (catalog#:
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72340), NA (catalog#: 72309), LCMS grade water (catalog#: 1.15333), 2-propanol (catalog#: 1.02781), and acetonitrile (catalog#: 1.00029), ammonium acetate (catalog#: 5.33004), and formic acid (catalog#: 5.33002) were purchased from Merck (Darmstadt, Germany). Hexakis (1H, 1H, 2H-perfluorothoxy) phosphazene (catalog#: PC1165) was purchased from Apollo Scientific Ltd (Stockport, UK).

Sample and Standard Preparation

Samples were thawed on wet ice after storage at -80°C and diluted 1/10,000 in LCMS grade water. 12 μL of diluted sample was combined with internal standard solution (290 pmol of NR-13C,d, NAM-d4, NA-d4, and 15 pmol of MeNAM-d3) in 48 μL of LCMS grade water in a Verex autosampler vial (Catalog#: AR0-3920-12, Phenomenex ApS, Denmark). Vials were capped with Verex Cert+MSQ PTFE caps (catalog#: AR0-8952-12, Phenomenex ApS, Denmark). Standards were composed such that the final volume and internal standard concentrations were the same as for samples. Final standard concentrations were 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μM. Quality controls (QCs) were composed in triplicate at final concentrations of 0.063, 0.63, and 6.25 μM in the same manner as standards. Samples were randomized prior to analysis. All samples, standards, and QCs were placed in a Dionex Ultimate 3000 autosampler pre-chilled to 6°C and injected (10 μL) for analysis.

LC-MS

Analytes were separated in a similar fashion as described (65) with slight modification. These modifications include a faster flow rate (0.55 mL/min) and a modified gradient between mobile phase A (10 mM ammonium acetate + 0.1% (v/v) formic acid) and B (0.1% formic acid in acetonitrile). In the modified gradient, the mobile phase was held at 5% B for 1.8 min followed by a linear increase to 64.1% B over 1.2 min. The column was washed at 90% B for 2 min and then re-equilibrated to starting conditions (5% B) for 1.3 min. Analytes eluted in the following order at the designated retention times: MeNAM (0.87 min), NA (3.22 min), NR (3.31 min), and NAM (3.53 min). Before beginning analysis, the instrument was calibrated by directly infusion of sodium formate. Calibration was modeled with HPC mode. Calibrant was also infused at the beginning of each analysis. A lock mass (hexakis (1H, 1H, 2H-perfluorothoxy) phosphazene in 2-propanol (0.1 mg/mL)) was employed to improve mass accuracy over the duration of analysis. Analytes were ionized via electrospray and detected in positive, full scan (m/z: 50 - 1000) ion mode with a Bruker Impact II Q-TOF. The instrument was operated using the following conditions: cone voltage = 45 kV, collision energy = 7 eV, nebulizer gas flow = 10 L/h, nebulizer gas temperature = 220°C, nebulizer pressure = 2 bar, and scan rate = 3 Hz.

Quantitation and Accuracy

Analytes were identified based upon exact m/z ratio (+/- 0.01 Da) and retention times (+/- 0.2 min). Analytes m/z ratios were 137.0709, 124.0393, 255.0975, and 123.0553 for MeNAM, NA, NR, and NAM, respectively. QCs were injected at equal intervals during the analysis to test precision and accuracy with deviations from theoretical concentrations of ≤20% deemed acceptable. QCs at all concentration levels in all analytes except NA passed. The lowest QC was undetectable for NA; all other concentration levels passed tolerance for NA. Samples were quantified using an internally controlled standard curve (1/x weighted). Micromolar amounts were corrected for dilution.

15N-enrichment of NAD+

On the day following primary hepatocyte isolation, medium was removed and replaced with glutamine-free MEM-199 (M2154, Sigma) supplemented with 0.1 g/L 15N-glutamine (490024, Sigma) and 500 μM tryptophan. Cells were incubated and harvested as described for Hepa1c1c7 cells after 2, 6, and 24h. Cell pellets were stored at -80 °C prior to analysis. Prior to extraction, internal standard (heavy labeled yeast) was composed by incubating yeast in the presence of [U-13C]-D-glucose and 0.3 mM adenine and absence of tryptophan as previously described (65). 0.125% of the total preparation was dosed into each

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sample, standard, and quality control sample in a 10 µL volume while samples rested on dry ice. Cells were extracted following (55) with slight modification. Cells were suspended in 1 mL of dry ice chilled methanol/water (80/20% v/v) (both LCMS grade) with constant shaking (highest energy setting) at 4 °C using a Heidolph Multi Reax system (Heidolph Instruments GmbH & CO. KG, Schwabach, Germany) for 15 minutes. Any non-suspended samples were suspended with vigorous, repeated pipetting. All samples were allowed to rest on dry ice for 20 minutes. The supernatants of centrifuged samples (10,000 x g, 4 C, 5 minutes) were transferred to fresh tubes and dried via speed vacuum with a Gene Vac EZ 2 system for no more than 4 hours. Samples were re-suspended in 50 µL of LCMS grade water and centrifuged ((20,238 x g, 22 °C, 10 min). Clarified supernatants were transferred to autosampler vials and placed in a pre-chilled (6 °C) autosampler in a random order. A standard curve with nine calibration points (see (65) for concentrations) and nine quality control samples (final concentrations of 0.225, 7.5, and 75 µM, prepared in triplicate at each concentration) were composed by extracting 10 µL after freezing in tubes on dry ice in the same manner as samples. An internal standard blank containing heavy labeled yeast and a blank containing only extraction solvent were also prepared.

Analytes were separated using liquid chromatography conditions as described (33) for the alkaline separation. MS conditions were exactly as described above except that the nebulizer gas temperature was 350°C rather than 220°C. 14N labeled NAD+ was detected using its theoretical m/z ratio of 664.1164 ± 0.005 Da and its retention time (5.9 ± 0.2 min). 15N labeled NAD+ was quantified as described (65). Accuracy and precision were determined as described above. 15N labeled NAD+ was detected using its theoretical m/z ratio of 665.11405 ± 0.005 Da and co-elution with 14N labeled NAD+. 15N labeled NAD+ was corrected for natural isotopic distribution, isotope skewing (15N abundance of 0.004), and the purity of 15N-glutamine (2/98% [14N1]-glutamine/[15N1]-glutamine) (66). The concentration of labeled NAD was then determined by multiplying the ratio of 15N-NAD+/14N-NAD+ (enrichment) by the concentration of non-labeled NAD+. The location of the labeled 15N on the molecule was determined by fragmenting doubly labeled NAD+ (m/z: 332.5618) using MS/MS (quadrupole resolution = 3 Da, collision energy = 20 eV). The enrichment of 15N NAM produced by fragmenting NAD+ was identical to intact NAD+ (data not shown).

Molecular and biochemical analyses

Western blot analysis
To determine protein abundance in cell cultures, to cells were harvested and centrifuged as for the NAD+ measurement experiments. Pellets were lysed in 200 µL lysis buffer (42). For tissue samples, approximately 20 mg pulverized tissue was homogenized in 500 µL lysis buffer using a Tissuelyser II (Qiagen, Germany), 2x30 sec of 30 Hz. Lysates were incubated end-over-end for 45 min at 4°C, and were centrifuged for 10 min at 16,000 g at 4°C. Following freezing at -80°C, protein concentration was determined using BCA. 20 µg of protein lysate was loaded on acrylamide SDS-page gels and subjected to SDS-PAGE and transfer as previously described (42). On each gel, an internal control of mixed samples was loaded on each side for normalization, and for evaluation of transfer quality. Proteins were subjected to immunoblotting with antibodies against NAMPT (372A, Bethyl lab, Montgomery, TX, USA), Mn-SOD (06-984, Millipore, Burlington, MA, USA), Acetyl Lysine (9441s, Cell Signaling), Malonyl Lysine (14942, Cell Signaling), Fatty acid synthase (3189, Cell Signaling), ATGL (3189, Cell Signaling), HSL (4107, Cell Signaling), pHSL s660 (372A, Bethyl lab, Montgomery, TX, USA), HSL (4107, Cell Signaling), pHSL s660 (4126, Cell signaling), Catalase (AF3398, R&D Systems, Minneapolis, MN, USA), P65 Lys310 (3045, Cell Signaling), PARP1 (2118, Cell Signaling), Lamin A/C (2032s, Cell Signaling), GAPDH (2118, Cell Signaling), SIRT5 (878, Cell Signaling), GAPDH (2118, Cell Signaling), PARP1...
(9542, Cell Signaling), Poly(ADP-ribose) (ALX-210-890A-0100, Enzo Life Sciences, Farmingdale, NY, USA), SLC25A11 (Ab80464, Abcam), SLC25A13 (Ab156010, Abcam). Bands were developed and quantified as previously described (42). For each gel, band volumes were normalized to the average band volume of the internal controls to account for differences in transfer efficiency.

**NAMPT activity assay**

The conversion of $^{14}$C-labeled nicotinamide to $^{14}$C-NMN, can be measured by precipitation of $^{14}$C-NMN in acetone on filter paper, through scintillation counting. Cell pellets were harvested with trypsin as described above, and 100 µL phosphate buffer (0.01 M Na$_2$HPO$_4$/NaH$_2$PO$_4$ buffer (pH=7.4)) containing protease inhibitor (Sigma) was added to each tube. Samples were further processed and NAMPT activity was determined on 30 µg protein as previously described (15, 67, 68). Background DPM from scintillation fluid was subtracted from each measurement.

**Citrate synthase activity**

Liver lysates, prepared in lysis buffer, were diluted 1:10 in 0.1 M Tris-HCl (pH=8.1). Diluted lysate (10 µL) was added in duplicates in a 96-well plate, along with 150 µL reaction mix containing 44 µg/ml 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 333.3 µg/ml acetyl-CoA in 0.1 M TRIS-HCl. Absorbance at 412 nm was measured for 5 min in a Hidex Sense plate reader (Hidex, Finland) to assess background activity (1 measurement pr. 30 seconds), and for additional 5 min after the addition of 10 µL, 10 mM oxaloacetate. Citrate synthase activity was calculated in the linear range of absorbance increase and was normalized to protein content of the lysate.

**NAD(H)/NADP(H) measurement**

NAD$^+$, NADH, NADP$^+$, and NADPH levels were determined using an enzymatic cycling assay, as previously described (42). 10-20 mg of frozen liver was homogenized in 400 µL of either 0.6 M perchloric acid (for NAD$^+$) or 0.1 M NaOH (for NADH) with a TissueLyser II (Qiagen, Germany). The NADH extracts were incubated at 70°C for 10 min, and both fractions were centrifuged for 3 min at 13,000 g. Supernatants were recovered. The NAD$^+$ extracts were diluted 1:1500 in 100 mM Na$_2$HPO$_4$ buffer (pH=8) and the NADH extracts were diluted in 1:500 in 10 mM Tris (pH=6). For NADP$^+$ measurements extracts were diluted 1:100 and for NADPH 1:1200 in the same buffers as above. 100 µL of the diluted extracts were pipetted into a white 96-well plate, and NAD$^+$/NADH levels were determined as previously described (42) and normalized to tissue weight. For NAD$^+$/NADH measurements on cells and mitochondria, one pellet was homogenized in 100 µL of 0.6 M perchloric acid or 0.1 M NaOH, and NAD$^+$/NADH was extracted and measured as for liver tissue. NAD$^+$/NADH levels were normalized to protein concentration extracted from the cell pellet. Total protein was extracted by heating the pellet to 95°C in 100 µL 0.2 M NaOH for 5 min. The solution was then centrifuged at 16,000 g for 5 min, and protein concentration of the supernatant was determined by the BCA assay. For mitochondrial pellets isolated from whole liver, 400 µL of 0.6 M perchloric acid/0.1 M NaOH was added to each pellet and homogenized using using a TissueLyser II (Qiagen, Germany). NAD$^+$/NADH was extracted and measured as for liver tissue. Total protein was extracted from the mitochondrial pellet by adding 100 µL 2% SDS and 200 µL 0.2 M NaOH to each pellet for 5 min at 95 °C followed by 5 min of centrifugation at 16,000 g. For comparison, NAD$^+$ and NADH content from whole tissue from the mitochondria isolation experiment were also normalized to protein content, extracted by the same protocol.

**Quantitative real-time PCR**

RNA from 15 mg liver was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). Samples were homogenized using a TissueLyser II (Qiagen). RNA concentration was determined on a Nanodrop 8000 (Thermo Scientific, MA, USA). RNA quality was assessed by 260 nm/280 nm ratio, and for all samples the ratio was above 1.8. RNA was reverse transcribed to cDNA using iScript cDNA synthesis...
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kit (170-8891, Biorad, Hercules, CA, USA). Reverse transcription was performed on C1000 Thermal Cycler (Invitrogen), using the following protocol: 5 min at 25°C, 40 min at 42°C and 5 min at 85°C. Real-time PCR was performed on a CFX96 real time system (Invitrogen) using SYBR green PCR master mix (600882, AH diagnostics, Aarhus, Denmark). The qPCR was run according to the following protocol: 3 min at 95°C for denaturation, 40 cycles of 95°C for 5 sec and 60°C for 10 sec for amplification. A melting curve was generated by gradual heating from 65°C to 95°C, and was used to verify specificity of the reaction. The reaction was run on 2 µL cDNA template and 300 nM sense and antisense oligonucleotides in a 10 µL reaction. Relative expression was determined from the Cq value and from a standard curve made by serial dilution of a mix of samples. Expression was normalized to ssDNA concentration determined using Qubit ssDNA assay kit (qQ10212, Invitrogen). The Nampt primer sequences were used as previously described (69). The remaining primer sequences were obtained from PrimerBank (70). The following primer sequences were used: Nampt: Forward: 5'-TGCCGTGAAAAGAAGACAGA-3', Reverse: 5'-ACTTCTTTGGCCTCCTGGGAT-3'. Nrkl (PrimerBank ID: 21703978a1): Forward: 5'-CTATTGGAATTGGTGGTGTGAC-3', Reverse: 5'-CAACAGGAAACTGCTGACATCAT-3'. Tdo2 (Primerbank ID: 31982697a1): Forward: 5'- ATGAGTGGGTGCCCGTTTG-3', Reverse: 5'-GGCTCTGTTTACACCAGTGGAG-3'. Afmid (PrimerBank ID: 21746157a1): Forward: 5'-TTGGAACCTCCTGACAGATAGG-3', Reverse: 5'-CAGTTTTCCCTCCCTGACATC-3'. Kmo (PrimerBank ID: 19520730a1): Forward: 5'-ATGGCATCGTCTGATACTCAGG-3', Reverse: 5'-CCCTAGCTTCGTACACATCAACT-3'. Qprt (PrimerBank ID: 19526852a1): Forward: 5'-CCGGGCCCTCATTTGTCATC-3', Reverse: 5'-GGTGTTAAGAGCCACCCCGT-3'. Nadsyn (PrimerBank ID: 26338135a1): Forward: 5'-AATATGCGGCTATGGATGTTGG-3', Reverse: 5'-GGAGCGAATTCAGAGACT-3'.

Biochemical measurements

Hepatic triglyceride content was quantified using a commercial kit (KA0847, Abnova, Taipei, Taiwan). Triglycerides were extracted from 40-50 mg of pulverized tissue, added 350 µL ethanol KOH (2 parts ethanol, 1 part 30% KOH). Tissues were incubated overnight at 55°C. Each sample was added 50% ethanol up to a final volume of 1 mL. Samples were centrifuged for 5 min at 13,000 g. 200 µL was recovered and diluted 1:50 in water prior to measurement as per the manufacturer’s instructions. Absorbance at 570 nm was measured using a Hidex Sense (Hidex, Finland). HDL/LDL plasma levels were determined by a kit (ab65390, Abcam, Cambridge, UK) according to the manufacturer’s instructions. Total plasma cholesterol levels were determined by a kit (CH200, Randox, Crumlin, United Kingdom).

Statistics

Data are presented as means ± SD. Data sets were tested for equal variance using either F-test (for comparing 2 groups) or Brown-Forsythe test (for more than 2 groups). In cases of unequal variance, data were log-transformed prior to further analysis. Data were analyzed using unpaired t-test (when comparing two groups), 1-way analysis of variance (ANOVA, when comparing more than two groups) or, 2-, or 3-way ANOVAs (when comparing 2 or more groups subjected to 2 or more treatments) with Sidak’s multiple comparison test post hoc. Data from the study with NR supplementation was analyzed by two, two-way ANOVAs; one analysis to determine genotype differences in response to the HFD, and one analysis to determine genotype differences in response to NR treatment. All statistical analysis was performed using Sigmaplot 13.0 (Systat Software Inc, San Jose, CA, USA), Graphpad Prism 8 (Graphpad Software, San Diego, CA, USA), and IBM SPSS Statistics 25 (SPSS, Chicago, IL, USA). Statistical significance was set at p<0.05. All figures were produced using Graphpad Prism 8.

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Conflicts of interest
The authors declare that they have no conflicts of interest with the contents of this article

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**Legends**

**Figure 1**
NAMPT is important for maintaining NAD⁺ levels in Hepa1c1c7 cells, but is not essential for mitochondrial function. (A) NAMPT abundance (n=6), (B) NAMPT activity (n=3) and (C) NAD⁺ levels (n=4) in Hepa1c1c7 cells, expressing either a shNonsense or shNampt construct. (D-I) Mitochondrial oxygen consumption and extracellular acidification rate as measured by extracellular flux analysis (n=6). n indicates number of experiments. */** indicate effects of genotype, p<0.05/0.01, respectively. */** indicate effects of NR treatment, p<0.05/0.01, respectively.

**Figure 2**
Role of NAMPT for maintaining liver mitochondrial function

Hepatocyte-specific knockout of Nampt (HNKO) causes no whole-body phenotype. (A) Body composition of female mice at 10-, 55-, and 110 weeks of age, n=9. (B) Oral glucose tolerance, (C) insulin tolerance and (D) pyruvate tolerance tests performed on HNKO and WT mice of 15-, 17- and 27 weeks of age. (E) Oral glucose tolerance and (F) Pyruvate tolerance tests performed on 55-week-old HNKO mice and WT littermates, n=8-9. Circadian oscillations of (G) blood glucose, (H) lactate and (I) ketone bodies in 110-week-old HNKO and WT mice (n=6-9). (J) Liver weight from HNKO and WT mice at 55- and 110 weeks of age, n=3-9. n indicates number of mice pr. genotype. */** indicate effects of genotype, p<0.05/0.01, respectively.

Figure 3
HFD-induced obesity development in HNKO mice is indistinguishable from WT littermates. (A) NR stability and NAM formation in drinking water from cages with or without mice (n=2-4). Female HNKO mice at ~15W of age were fed a low-fat diet (LFD) or a 60% high-fat diet (HFD) +/- 400 µg nicotinamide riboside (NR) pr. g body weight pr. day for 20 weeks. (B) Average drinking intake pr. mouse pr. day after 9 weeks of HFD-feeding and NR supplementation, measured over 7 days. (C-E) Throughout the experiment, mice were weighed and NMR scanned to assess body composition. (F-G) Glucose tolerance was evaluated after 10 and 19 weeks of HFD-feeding. n=6-8 pr. group. */** indicate effects of time, p<0.05/0.01, respectively. *## indicate effects of diet, p<0.05/0.01, respectively.

Figure 4
Hepatocyte-specific knockout of Nampt has no effect on mitochondrial respiratory function, but reduces the ability of NR to increase State III respiration. Mitochondrial function was evaluated by high-resolution respirometry in liver tissue, where mitochondrial substrates were added in succession. Following (A) Basal stimulation, liver tissues were stimulated with (B) Carnitine-Palmitate, (C) ADP+Mg, (D) Glutamate, (E) Pyruvate, (F) Succinate, and (G) FCCP. n=6-8 pr. group. */*** indicate effects of genotype, p<0.05/0.01, respectively. *## indicate effects of diet, p<0.05/0.01, respectively. $/$$ indicate effects of NR within the HFD groups, p<0.05/p<0.01, respectively.

Figure 5
HNKO mice have no alterations in abundance of the mitochondrial OXPHOS complexes. WT and HNKO mice were fed a low-fat diet (LFD) or high-fat diet (HFD) with or without NR supplementation for 20 weeks. To determine if NR affected mitochondrial function, (A) citrate synthase activity, and abundance of (B) complex I, (C) complex II, (D) complex IV, (E) complex V, (F) SLC25A11, and (G) SLC25A13, were determined. To determine whether redox defenses were affected by NR treatment, abundance of (H) catalase and (I) MnSOD were determined. n=6-8 pr. group. */*** indicate effects of genotype, p<0.05/0.01, respectively. *## indicates effects of diet, p<0.05/0.01, respectively. $/$$ indicates effects of NR within the HFD groups, p<0.05/p<0.01, respectively.

Figure 6
HNKO mice have no alterations in abundance of proteins associated with oxidative defenses or lipid metabolism. To determine if NR supplementation affected hepatic lipid metabolism, (A) hepatic triglyceride content was determined. To determine the effects of NR supplementation on plasma cholesterol composition, (B) total plasma cholesterol levels, (C) plasma LDL+VLDL levels and (D) plasma HDL levels were determined. To investigate if NR treatment increased abundance of lipases, hepatic (E) ATGL abundance, (F) HSL abundance and (G) phosphorylation of S660 on HSL were determined. Hepatic content of (H) Fatty acid synthase, (I) ACA2, (J) LCAD, (K) MCAD, and (L) LXRα were also determined. n=6-8 pr. group. */*** indicate effects of genotype, p<0.05/0.01, respectively. *## indicate effects of diet, p<0.05/0.01, respectively. $/$$ indicate effects of NR within the HFD groups, p<0.05/p<0.01, respectively.
effects of NR within the HFD groups, p<0.05/p<0.01, respectively.

**Figure 7**
NR treatment increases hepatic NAD+ levels and increases lysine malonylation. WT and HNKO mice were fed a low-fat diet (LFD) or high-fat diet (HFD) with or without NR supplementation for 20 weeks. To determine the effects of HFD-feeding and NR supplementation in HNKO mice, hepatic (A), NAMPT abundance, (B) NAD+ content, (C) NADH content, (D) NADP+, and (E) NADPH content were determined. (F) PARP1 abundance was quantified to investigate whether HFD-feeding or NAMPT knockout affected poly(ADP-ribose) polymer formation. To investigate indications of altered sirtuin activity, (G) total lysine acetylation was determined. (H) Total hepatic P65 content and (I) acetylation of Lysine310 on P65 were determined as an indication of SIRT1 activity. (J) Total lysine malonylation and (K) SIRT5 abundance were also determined to investigate whether SIRT5 activity was affected. n=6-8 pr. group. */** indicate effects of genotype, p<0.05/0.01, respectively. #/## indicate effects of diet, p<0.05/0.01, respectively. $/$$ indicate effects of NR within the HFD groups, p<0.05/p<0.01, respectively.

**Figure 8**
The mitochondrial NAD pool is prioritized in response to hepatocyte-specific knockout of Nampt. To verify the purity of the mitochondrial fraction, the mitochondrial fraction was investigated for presence of (A) GAPDH, (B) LAMIN A/C, and (C) Complex I by immunoblotting (n=6-7). Furthermore, the mitochondrial fraction was investigated for the presence of (D) NAMPT (n=6-7). NAD+ content was determined for (E) whole liver tissue and (F) the mitochondrial fraction, and was used to calculate (G) the ratio between the mitochondrial NAD+ pool and the total NAD+ pool (n=5-6). This was also done for (K-M) NADPH and (N-P) NADP+. n indicates number of animals. */*** indicate effects of genotype, p<0.05/0.01, respectively. */## indicate effects of compartment, p<0.05/0.01, respectively.

**Figure 9**
Mitochondrial respiratory capacity is maintained in HNKO hepatocytes. (A) NAMPT abundance in primary hepatocytes from WT and HNKO mice n=4. (B) CO2-release from primary mouse hepatocytes from WT and HNKO mice following 4 hours of stimulation with 14C-palmitate (n=4). (C) Tetramethylrhodamine (TMRM) fluorescence was used as a measure of mitochondrial membrane potential, n=6. NAD+ content was quantified in (D) primary mouse hepatocytes and in (E) hepatocyte mitochondria following 6 hours of NR stimulation, n=5. (F) Ratio of mitochondrial to whole-cell NAD+ content following 6 hours of NR stimulation. (G-L) Oxygen consumption and respiratory capacity of primary mouse hepatocytes, measured by the Seahorse XF system (n=6). n indicates number of experiments. */** indicate effects of genotype, p<0.05/0.01, respectively. */## indicate effects of NR treatment, p<0.05/0.01, respectively.

**Figure 10**
NAMPT is an important contributor to the maintenance of NAD+ levels in primary mouse hepatocytes (A) mRNA level of enzymes associated with NAD+ synthesis were determined in primary mouse hepatocytes, n=4. (B) NAD+ content in primary mouse hepatocytes from HNKO mice and WT littermates stimulated for 6 hours with various NAD+ precursors (NR: nicotinamide riboside, NMN: nicotinamide mononucleotide, NAM: nicotinamide, NA: nicotinic Acid, Trp: tryptophan, n=4) (C) NAD+ content in primary mouse hepatocytes from WT and HNKO mice stimulated for 24 or 48 hours with DMSO, 500 µM Trp or 500 µM NA, n=4-5. (D) NAD+ content in primary mouse hepatocytes from WT and HNKO mice following 6, 24, and 48 hours of incubation with the NAMPT inhibitor FK866 (10 µM), the NAD synthase inhibitor azaserine (10 µM) or
Role of NAMPT for maintaining liver mitochondrial function

DMSO, n=4. (E) NAD⁺ content in primary hepatocytes from WT and HNKO mice, following 48 hours of 10 µM FK866 treatment and 6 hours of stimulation with 500 µM of NR, NA or Trp, n=3. To determine the contribution of de novo synthesis, primary hepatocytes from WT and HNKO mice were stimulated with 500 µM Trp in the presence of 15N-glutamine to label NAD⁺ produced by NAD⁺ synthase. Cells were incubated with DMSO or 10 µM FK866 to prevent recycling of 15N-NAD⁺. (F) 14N-NAD⁺ is endogenous NAD⁺ or NAD⁺ produced independently of NADSYN1, whereas (G) 15N-NAD⁺ is NAD⁺ produced by amidation through NADSYN1. (H) Enrichment indicates the ratio of of 15N-NAD⁺ to 14N-NAD⁺, n=3. * indicates number of experiments. */** indicate effects of genotype, p<0.05/0.01, respectively. */** indicate effects of treatment, p<0.05/0.01, respectively. $/$$ indicates effects of time, p<0.05/0.01 respectively.
Figure 1

A) NAMPT

B) NAMPT activity

C) NAD⁺ content

D) Basal respiration

E) ATP synthesis

F) Maximal respiration

G) Basal ECAR

H) Oxygen consumption

I) ECAR

1: Oligomycin
2: FCCP
3: Antimycin A + Rotenon
**Figure 2**

**A** Body composition and weight

- **Female mice**
- **Weight (g)**
- **Body Weight (g)**
- **Lean Mass (g)**
- **Fat Mass (g)**

10 weeks of age | WT | HNKO | WT | HNKO | WT | HNKO | WT | HNKO
---|---|---|---|---|---|---|---|---
55 weeks of age
110 weeks of age

**B** Oral glucose tolerance test

- **15 weeks of age**
- **Blood Glucose (mM)**

**C** Insulin tolerance test

- **17 weeks of age**
- **Blood Glucose (mM)**

**D** Pyruvate tolerance test

- **27 weeks of age**
- **Blood Glucose (mM)**

**E** Oral glucose tolerance test

- **55 weeks of age**
- **Genotype p<0.05**
- **Blood Glucose (mM)**

**F** Pyruvate tolerance test

- **55 weeks of age**
- **Blood Glucose (mM)**

**G** Blood glucose

- **110 weeks of age**
- **ZT**
- **Blood Glucose (mM)**

**H** Blood lactate

- **110 weeks of age**
- **ZT**
- **Blood Lactate (mM)**

**I** Blood ketone

- **110 weeks of age**
- **ZT**
- **Blood Ketone (mM)**

**J** Liver weight

- **55 weeks**
- **110 weeks**
- **Weight (g)**

**K** Liver NAMPT abundance

- **Abundance norm. to IC**
- **Age (weeks)**

**L** Liver NAD\(^+\) content

- **pmol/mg tissue**
- **Age (weeks)**
Figure 4

A  Basal
B  Carnitine Palmitate
C  ADP+Mg
D  Glutamate
E  Pyruvate
F  Succinate
G  FCCP

Respiration Rate (pmol O2/mg tissue/s)

LFD  HFD  HFD + NR

WT  HNKO

*  **  #  ##
**Figure 5**

A. Citrate synthase activity

B. Complex I protein abundance

C. Complex II protein abundance

D. Complex IV protein abundance

E. Complex V protein abundance

F. SLC25A11 protein abundance

G. SLC25A13 protein abundance

H. Catalase protein abundance

I. MnSOD protein abundance

- **Citrate synthase activity**: Measured in μmol/min/g protein.
- **Complex I protein abundance**: Relative abundance to IC [AU].
- **Complex II protein abundance**: Relative abundance to IC [AU].
- **Complex IV protein abundance**: Relative abundance to IC [AU].
- **Complex V protein abundance**: Relative abundance to IC [AU].
- **SLC25A11 protein abundance**: Relative abundance to IC [AU].
- **Catalase protein abundance**: Relative abundance to IC [AU].
- **MnSOD protein abundance**: Relative abundance to IC [AU].

**Proteins and Conditions**

- **LFD (Low Fat Diet)**
- **HFD (High Fat Diet)**
- **HFD + NR (High Fat Diet + Nutrient Restriction)**

**Band Locations**

- **20 kDa**
- **70 kDa**
- **37 kDa**
- **75 kDa**
- **25 kDa**
- **50 kDa**

**Significance**

- **##**: p < 0.01
- **#**: p < 0.05
Figure 6

A. Hepatic triglyceride content

B. Plasma cholesterol

C. Plasma HDL

D. Plasma LDL+VLDL

E. ATGL protein abundance

F. HSL protein abundance

G. pHSL S660/HSL Ratio

H. Fatty Acid Synthase

I. ACAA2 protein abundance

J. LCAD protein abundance

K. MCAD protein abundance

L. LXRα protein abundance

Legend:
- LFD
- HFD
- HFD + NR

Notes:
- **p=0.05**
- **##**
- **##**

Data points for each condition are shown with error bars indicating variability.
Figure 7

A. Hepatic NAMPT

B. Hepatic NAD\(^{+}\) levels

C. Hepatic NADH levels

D. Hepatic NADP levels

E. Hepatic NADPH levels

F. PARP1 protein abundance

G. Acetyl lysine

H. P65 protein abundance

I. ac-K310 P65/P65 ratio

J. Malonyl lysine

K. SIRT5 protein abundance
Figure 8

A. GAPDH

B. Lamin A/C

C. Complex I

D. NAMPT

E. Tissue NAD^+ (pmol/mg protein)

F. Mitochondrial NAD^+ (pmol/mg protein)

G. Mitochondria/Liver NAD^+ ratio

H. Tissue NADH (pmol/mg protein)

I. Mitochondrial NADH (pmol/mg protein)

J. Mitochondria/Liver NADH ratio

K. Tissue NADP^+ (pmol/mg protein)

L. Mitochondrial NADP^+ (pmol/mg protein)

M. Mitochondria/Liver NADP^+ ratio

N. Tissue NADPH (pmol/mg protein)

O. Mitochondrial NADPH (pmol/mg protein)

P. Mitochondria/Liver NADPH ratio
Figure 9

A. NAMPT

B. Primary hepatocytes FAO CO₂ Capture
4h stim with 5 nmol palmitate

C. Mitochondrial membrane potential

D. Whole cell NAD⁺
Primary hepatocytes 6h stim

E. Mitochondrial NAD⁺
Primary hepatocytes 6h stim

F. Mitochondria/whole cell NAD⁺ ratio

G. Basal respiration

H. ATP synthesis

I. Maximal respiration

J. Basal ECAR

K. Oxygen consumption
Primary hepatocytes

L. ECAR
Primary hepatocytes
Mitochondrial function in liver cells is resistant to perturbations in NAD⁺ salvage capacity
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