Rev1 deficiency induces a metabolic shift in MEFs that can be manipulated by the NAD+ precursor nicotinamide riboside

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Published in:
Heliyon

DOI:
10.1016/j.heliyon.2023.e17392

Publication date:
2023

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Rev1 deficiency induces a metabolic shift in MEFs that can be manipulated by the NAD\(^+\) precursor nicotinamide riboside

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**ARTICLE INFO**

Keywords:
- Rev1
- Replication stress
- NAD\(^+\)
- Nicotinamide riboside
- Healthspan
- Autophagy
- Mitochondria

**ABSTRACT**

Replication stress, caused by Rev1 deficiency, is associated with mitochondrial dysfunction, and metabolic stress. However, the overall metabolic alterations and possible interventions to rescue the deficits due to Rev1 loss remain unclear. Here, we report that loss of Rev1 leads to intense changes in metabolites and that this can be manipulated by NAD\(^+\) supplementation. Autophagy decreases in Rev1\(^{-/-}\) mouse embryonic fibroblasts (MEFs) and can be restored by supplementing the NAD\(^+\) precursor nicotinamide riboside (NR). The abnormal mitochondrial morphology in Rev1\(^{-/-}\) MEFs can be partially reversed by NR supplementation, which also protects the mitochondrial cristae from rotenone-induced degeneration. In nematodes rev-1 deficiency causes sensitivity to oxidative stress but this cannot be rescued by NR supplementation. In conclusion, Rev1 deficiency leads to metabolic dysregulation of especially lipid and nucleotide metabolism, impaired autophagy, and mitochondrial anomalies, and all of these phenotypes can be improved by NR replenishment in MEFs.

**Research topic(s)**

CP: Molecular biology.

1. **Introduction**

Maintaining genomic integrity is vital for the survival and health of organisms. The DNA repair machinery is tightly regulated and fixes endogenously and exogenously induced DNA damages. Less efficient or low-fidelity DNA repair is a major driving force for genomic instability, which further leads to aging and aging-related diseases [1]. Insufficient DNA repair such as gene mutations or loss of function also causes replication stress. In the context of UV-induced replication stress, regular replicative polymerases are exchanged

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for specialized translesion DNA synthesis (TLS) polymerases such as REV1, which is recruited to the replication arrest site, and directly interacts with monoubiquitinated PCNA [2]. Replication stress has crucial effects on cell survival, genome stability, mitochondrial maintenance, and human diseases [3,4]. Replication stress via loss of Rev1 is associated with genome instability, mitochondrial alterations (bioenergetics, dynamics, and mitophagy), autophagy, and metabolic stress via alterations in nuclear-mitochondrial communication [5]. Rev1-deficient mice exhibit mild cytopenia and compromised proliferation and viability of hematopoietic stem cells (HSPCs), which is associated with mitochondrial dysfunction [6]. Moreover, DNA replication stress increases the dNTP pool which leads to spontaneous mutations [7,8]. In turn, nucleotide imbalance leads to more replication stress [9], as well as mitochondrial dysfunction [10]. Interestingly, increased dNTP pools or treatment to induce the dNTP pool precursors in cancerous cells is associated with reduced autophagy [11]. Overall, there appear to be strictly regulated mechanisms that ensure communication between DNA metabolism in the nucleus and metabolic processes in the cytoplasm. Interestingly, Rev1 knockout mice have lower lean mass accompanied by glucose intolerance. Furthermore, the mice were characterized by a mitochondrial energy deficit associated with increased anaerobic glycolysis and reduced locomotor activity. NAD+ accompanied by glucose intolerance. Furthermore, the mice were characterized by a mitochondrial energy deficit associated with decreased autophagy [11].

NAD+ plays a critical role in the TCA cycle, oxidative phosphorylation (OXPHOS), and acts as a co-factor for cellular homeostasis [13–15]. The decline in NAD+ leads to altered metabolism in aging and age-related diseases [16–20]. DNA damage repair is an energy-consuming process and the proteins involved in this process are dysregulated in some diseases, such as cancer, neurodegenerative diseases, and diabetes, involving alterations in post-translational modifications including PARylation, acetylation, and deacetylation [21]. One of the NAD+ consumers, Poly[ADP-ribose] polymerase 1 (PARP1), is recruited to DNA damage sites during single and double-strand break repair [22]. Activation of PARP1 confers PARylation of itself and other proteins, affecting the nuclear deacetylase protein, SIRT1, which competes for NAD+ with PARP1 [23]. Hyperactivation of PARP1 increases the catalysis of NAD+ to NAM and ADP ribose. NAM inhibits the expression of the NAD+-dependent deacetylase, SIRT1 [24]. Persistent PARP1 activation along with NAD+ depletion increases PARylation in Xeroderma pigmentosum Group A (XPA) [25], Cockayne syndrome (CS) [26], and Alzheimer’s disease (AD) models [27]; and replenishment of NAD+ by NAD precursors, such as nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN), can reverse these alterations [25,28]. Besides, PARP1 activation also leads to mitochondrial dysfunction [25,29,30].

Our previous study shows that Rev1−/− MEFs display hyperactivation of PARP1, NAD+ depletion, decreased AMPK, SIRT1 expression, and mitochondrial dysfunction [5]. Here, we demonstrate that Rev1 deficiency causes a metabolic shift including decreased NAD+ metabolism as well as increased nucleotide, phospholipids, sphingolipids, and ceramides synthesis. These changes likely influence the dysregulation of mitochondrial homeostasis in Rev1-deficient MEFs since they can be rescued by the replenishment of NR in MEFs. This study provides new insights into the intersection between DNA damage repair, autophagy, and metabolism.

2. Results

2.1. Metabolism is altered in Rev1−/− MEFs

Rev1 deficiency induces replication stress to cause metabolic dysfunction in mice [6,12] suggesting an interaction between genomic instability and cellular metabolism. Therefore, we asked whether Rev1 deficiency would cause dysregulation or changes in specific metabolic pathways. We examined the metabolome of wildtype and Rev1−/− MEFs with and without NR supplementation (Fig. 1).

Multivariate analysis using Partial Least Square Discriminate Analysis (PLS-DA) resulted in the visual separation of the metabolomes in multidimensional space, which clearly showed that the metabolite profiles were different in Rev1−/− compared to wildtype

![Fig. 1. Metabolome analysis of wildtype and Rev1−/− MEFs treated with or without nicotinamide riboside (NR).](image-url)

(A) The four different clusters were plotted. (B) Significantly altered metabolites between wildtype and Rev1 deficient cells were ranked. (C) Ranked metabolites between Rev1−/− MEFs treated with or without NR. Metabolites with importance in the projection (VIP) scores > 1.2 were considered as significant contributors.
Table 1  
Metabolic pathways in mouse embryo fibroblasts changed by Rev1 knockout compared to wild-type controls.

<table>
<thead>
<tr>
<th>No.</th>
<th>Pathway Name</th>
<th>Measured Metabolites in the Pathway (N)</th>
<th>Expected Pathway Proportion (P) N/474</th>
<th>Expected Hits in Sample of 80 (P * 80)</th>
<th>Observed Hits in the Top 80 Metabolites</th>
<th>Fold Enrichment (Obs/Exp)</th>
<th>Hypergeometric p Value</th>
<th>Impact (Sum VIP)</th>
<th>Fraction of Impact (VIP) Explained (% of 143.39)</th>
<th>Increased</th>
<th>Decreased</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Purine Metabolism</td>
<td>30</td>
<td>0.06</td>
<td>5.1</td>
<td>10</td>
<td>2.0</td>
<td>0.02</td>
<td>27.3</td>
<td>19%</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Phospholipid Metabolism</td>
<td>75</td>
<td>0.16</td>
<td>12.7</td>
<td>13</td>
<td>1.0</td>
<td>0.51</td>
<td>20.3</td>
<td>14%</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Sphingomyelin Metabolism</td>
<td>38</td>
<td>0.08</td>
<td>6.4</td>
<td>13</td>
<td>2.0</td>
<td>0.01</td>
<td>19.0</td>
<td>13%</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Glycosphingolipid Metabolism</td>
<td>18</td>
<td>0.04</td>
<td>3.0</td>
<td>6</td>
<td>2.0</td>
<td>0.07</td>
<td>15.0</td>
<td>11%</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Glycolysis and Gluconeogenesis</td>
<td>13</td>
<td>0.03</td>
<td>2.2</td>
<td>5</td>
<td>2.3</td>
<td>0.05</td>
<td>9.5</td>
<td>7%</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>Deoxysphingolipids</td>
<td>9</td>
<td>0.02</td>
<td>1.5</td>
<td>6</td>
<td>4.0</td>
<td>0.001</td>
<td>9.4</td>
<td>7%</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Amino-Sugar, Galactose, &amp; Non-Glucose Metabolism</td>
<td>7</td>
<td>0.02</td>
<td>1.2</td>
<td>3</td>
<td>2.5</td>
<td>0.10</td>
<td>5.9</td>
<td>4%</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Fatty Acid Oxidation and Synthesis</td>
<td>32</td>
<td>0.07</td>
<td>5.4</td>
<td>3</td>
<td>0.6</td>
<td>0.93</td>
<td>4.6</td>
<td>3%</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Ceramide Metabolism</td>
<td>28</td>
<td>0.06</td>
<td>4.7</td>
<td>3</td>
<td>0.6</td>
<td>0.88</td>
<td>3.9</td>
<td>3%</td>
<td>3</td>
<td>0</td>
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<tr>
<td>10</td>
<td>Bioamines and Neurotransmitter</td>
<td>9</td>
<td>0.02</td>
<td>1.5</td>
<td>2</td>
<td>1.3</td>
<td>0.47</td>
<td>2.9</td>
<td>2%</td>
<td>2</td>
<td>0</td>
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<tr>
<td>11</td>
<td>Ubiquinone and Dolichol Metabolism</td>
<td>4</td>
<td>0.01</td>
<td>0.7</td>
<td>2</td>
<td>3.0</td>
<td>0.13</td>
<td>2.8</td>
<td>2%</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>Branch Chain Amino Acid Metabolism</td>
<td>11</td>
<td>0.02</td>
<td>1.9</td>
<td>1</td>
<td>0.5</td>
<td>0.87</td>
<td>2.4</td>
<td>2%</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>Eicosanoid and Resolvin Metabolism</td>
<td>14</td>
<td>0.03</td>
<td>2.4</td>
<td>1</td>
<td>0.4</td>
<td>0.93</td>
<td>2.0</td>
<td>1%</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>Pentose Phosphate, Gluconate Metabolism</td>
<td>5</td>
<td>0.01</td>
<td>0.8</td>
<td>1</td>
<td>1.2</td>
<td>0.61</td>
<td>1.8</td>
<td>1%</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>Vitamin D (Calciferol) Metabolism</td>
<td>2</td>
<td>0.004</td>
<td>0.3</td>
<td>1</td>
<td>3.0</td>
<td>0.31</td>
<td>1.7</td>
<td>1%</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>Vitamin B3 (Niacin, NAD+) Metabolism</td>
<td>6</td>
<td>0.01</td>
<td>1.0</td>
<td>1</td>
<td>1.0</td>
<td>0.67</td>
<td>1.6</td>
<td>1%</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>17</td>
<td>Gamma-Glutamyl and other Dipeptides</td>
<td>4</td>
<td>0.01</td>
<td>0.7</td>
<td>1</td>
<td>1.5</td>
<td>0.52</td>
<td>1.3</td>
<td>1%</td>
<td>0</td>
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</tr>
<tr>
<td>18</td>
<td>Cardiolipin Metabolism</td>
<td>3</td>
<td>0.01</td>
<td>0.5</td>
<td>1</td>
<td>2.0</td>
<td>0.43</td>
<td>1.3</td>
<td>1%</td>
<td>1</td>
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</tr>
<tr>
<td>19</td>
<td>Lysine Metabolism</td>
<td>3</td>
<td>0.01</td>
<td>0.5</td>
<td>1</td>
<td>2.0</td>
<td>0.43</td>
<td>1.3</td>
<td>1%</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>Cholesterol, Cortisol, Non-Gonadal Steroid</td>
<td>8</td>
<td>0.02</td>
<td>1.4</td>
<td>1</td>
<td>0.7</td>
<td>0.78</td>
<td>1.2</td>
<td>1%</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* Sphingolipids.
Table 2
Metabolic pathways in Rev1 knockout mouse embryo fibroblasts changed by nicotinamide riboside treatment.

<table>
<thead>
<tr>
<th>No.</th>
<th>Pathway Name</th>
<th>Measured Metabolites in the Pathway (N)</th>
<th>Expected Pathway Proportion (P) N/474</th>
<th>Expected Hits in Sample of 70 (P * 70)</th>
<th>Observed Hits in the Top 70 Metabolites</th>
<th>Fold Enrichment (Obs/Exp)</th>
<th>Hypergeometric p Value</th>
<th>Impact (Sum VIP)</th>
<th>Fraction of Impact (VIP) Explained (% of 134.86)</th>
<th>Increased</th>
<th>Decreased</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phospholipid Metabolism</td>
<td>75</td>
<td>0.16</td>
<td>11.1</td>
<td>17</td>
<td>1.5</td>
<td>0.0</td>
<td>30.3</td>
<td>23%</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>Vitamin B3 (Niacin, NAD+) Metabolism</td>
<td>6</td>
<td>0.01</td>
<td>0.9</td>
<td>4</td>
<td>4.5</td>
<td>0.0</td>
<td>16.1</td>
<td>12%</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Purine Metabolism</td>
<td>30</td>
<td>0.06</td>
<td>4.4</td>
<td>7</td>
<td>1.6</td>
<td>0.1</td>
<td>14.7</td>
<td>11%</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Eicosanoid and Resolvins Metabolism</td>
<td>14</td>
<td>0.03</td>
<td>2.1</td>
<td>6</td>
<td>2.9</td>
<td>0.0</td>
<td>12.1</td>
<td>9%</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Pyrimidine Metabolism</td>
<td>25</td>
<td>0.05</td>
<td>3.7</td>
<td>5</td>
<td>1.4</td>
<td>0.3</td>
<td>7.9</td>
<td>6%</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>Benzoate and Indole Metabolism</td>
<td>22</td>
<td>0.05</td>
<td>3.2</td>
<td>4</td>
<td>1.2</td>
<td>0.4</td>
<td>7.6</td>
<td>6%</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Glycosphingolipid Metabolism</td>
<td>18</td>
<td>0.04</td>
<td>2.7</td>
<td>4</td>
<td>1.5</td>
<td>0.3</td>
<td>6.2</td>
<td>5%</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>Fatty Acid Oxidation and Synthesis</td>
<td>32</td>
<td>0.07</td>
<td>4.7</td>
<td>3</td>
<td>0.6</td>
<td>0.9</td>
<td>5.6</td>
<td>4%</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Pentose Phosphate, Gluconate Metabolism</td>
<td>5</td>
<td>0.01</td>
<td>0.7</td>
<td>2</td>
<td>2.7</td>
<td>0.2</td>
<td>4.9</td>
<td>4%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>Glycolysis and Gluconeogenesis Metabolism</td>
<td>13</td>
<td>0.03</td>
<td>1.9</td>
<td>3</td>
<td>1.6</td>
<td>0.3</td>
<td>4.6</td>
<td>3%</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>Sphingomyelin Metabolism</td>
<td>38</td>
<td>0.08</td>
<td>5.6</td>
<td>3</td>
<td>0.5</td>
<td>0.9</td>
<td>4.4</td>
<td>3%</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>Ceramide Metabolism</td>
<td>28</td>
<td>0.06</td>
<td>4.1</td>
<td>2</td>
<td>0.5</td>
<td>0.9</td>
<td>3.5</td>
<td>3%</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>Cholesterol, Cortisol, Non-Gonadal Steroid Metabolism</td>
<td>8</td>
<td>0.02</td>
<td>1.2</td>
<td>2</td>
<td>1.7</td>
<td>0.3</td>
<td>3.5</td>
<td>3%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>Deoxyxphospholipids</td>
<td>9</td>
<td>0.02</td>
<td>1.3</td>
<td>2</td>
<td>1.5</td>
<td>0.4</td>
<td>3.3</td>
<td>2%</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>Bioamines and Neurotransmitter Metabolism</td>
<td>9</td>
<td>0.02</td>
<td>1.3</td>
<td>2</td>
<td>1.5</td>
<td>0.4</td>
<td>2.8</td>
<td>2%</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>1-Carbon, Folate Metabolism</td>
<td>9</td>
<td>0.02</td>
<td>1.3</td>
<td>1</td>
<td>0.8</td>
<td>0.8</td>
<td>2.5</td>
<td>2%</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>GABA, Glutamate, Arginine, Ornithine, Proline Metabolism</td>
<td>7</td>
<td>0.02</td>
<td>1.0</td>
<td>1</td>
<td>1.0</td>
<td>0.7</td>
<td>2.4</td>
<td>2%</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>18</td>
<td>Nitric Oxide, Superoxide, Peroxide Metabolism</td>
<td>4</td>
<td>0.01</td>
<td>0.6</td>
<td>1</td>
<td>1.7</td>
<td>0.5</td>
<td>1.3</td>
<td>1%</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>Endocannabinoid Metabolism</td>
<td>3</td>
<td>0.01</td>
<td>0.4</td>
<td>1</td>
<td>2.3</td>
<td>0.4</td>
<td>1.2</td>
<td>1%</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
cells (Fig. 1A). NR treatment had a significant effect on the metabolite profiles in both the Rev1\(^{-/-}\) and wildtype cells (Fig. 1A).

We found that Rev1\(^{-/-}\) cells show significantly altered metabolites mainly in purine, pyrimidine, phospholipid, sphingolipid, glucose, and vitamin metabolic pathways, as compared to wildtype cells (Table 1). Twenty-one metabolic pathways were significantly altered in Rev1\(^{-/-}\) cells. Purine metabolism was the most changed. About 19% of the total metabolic impact was contributed by changes in purines. All 10 of 10 purines that were changed were increased. These included increased guanosine monophosphate, adenosine monophosphate, inosine monophosphate, adenosine, guanosine, adenosine diphosphate, and guanosine diphosphate (Fig. 1B, Table 1). Several of these metabolites have been linked to protection of glial cells during glucose deprivation and mitochondrial respiratory chain inhibition [31]. Rev1 KO MEF cells showed increased deoxynucleotide metabolites such as dAMP and dGMP, which may be cytotoxic [32]. The pyrimidine metabolites, uridine monophosphate, uridine diphosphate, uridine, and thiamine were all increased, while deoxyuridine was decreased (Fig. 1B, Supplementary Tables S1 and S2).

Four classes of sphingolipids contributed over 34% of the metabolic impact in Rev1\(^{-/-}\) cells. These included changes in sphingomyelins (13%), glycosphingolipids (11%), deoxysphingolipids (7%), and ceramides (3%) (Table 1). All 13 of 13 sphingomyelins that were changed were increased. These included Smd18:1/20:0 OH, Smd18:1/16:1 OH, Smd18:1/22:0, and Smd18:1/16:0 OH. We also found increases in deoxyxosphingolipids like deoxyceramide 18:1/24:1, and ceramides like ceramide d18:1/24:1. Increased deoxydihydroceramide is associated with diabetic neuropathy in type 2 diabetes and obesity [33]. Furthermore, increased plasma levels of select deoxy-ceramide and ceramide species are associated with diabetic neuropathy in type 1 diabetes [34]. Interestingly, Rev1-deficient mice revealed increased body weight, reduction of lean mass, and increased glucose intolerance [12]. Of the 4 classes of sphingolipids changed by Rev1 knockout, only the glycosphingolipids were decreased, including the dihexosylceramides DHC18:1/20:0, and DHC18:1/24:0 (Fig. 1B, Supplementary Table S2). Dihexosylceramides, including lactosylceramide are enriched in diabetic neuropathy [33].

![Figure 2](image-url)

Fig. 2. Autophagy and mitophagy defects in Rev1\(^{-/-}\) MEFs.
(A) Wildtype and Rev1\(^{-/-}\) MEFs were treated with or without NR. LC3 was used as a marker for autophagy. N = 3. The full, non-adjusted image is shown in Supplementary Fig. 2A. (B) To measure mitophagy we used a pH-dependent fluorescence assay based on the coral-derived protein Keima. Wildtype and Rev1\(^{-/-}\) MEFs integrated with mt-mKeima were treated with 20 μM FCCP to induce mitophagy. Cells were analyzed by flow cytometry. (C) Quantification of LC3-II/ACTIN in panel A. (D,E) Quantification of LC3-II/Actin ratios in panel A. (F-H) Quantification of LC3-II/I ratios in panel A. (I) Quantification of mitophagic cells in panel B. N = 3. Data is presented as mean ± SEM. *p < 0.05; **p < 0.01.
Fig. 3. Electron microscope analysis of MEFs treated with rotenone and/or NR.
Wildtype and Rev1<sup>−/−</sup> MEFs were treated with NR or rotenone or the combination of NR or rotenone. NR (3 mM, 18 h), rotenone (Rot, 4 μM, 12 h), and quantification of mitochondrial area, length and cristae length was done using Image J. The average mitochondria area, the average mitochondria length and the average of mitochondria cristae length per cell was obtained by counting three cells under different conditions individually. Randomly ten images were used for mitochondrial area and length quantification, and twenty images were used for the quantification of cristae length. (A), (B), (C), (D), (E): The average mitochondria area was quantified under different conditions. (F), (G), (H), (I), (J): The average mitochondria length was quantified under different conditions. (K), (L), (M), (N), (O): The average mitochondria cristae length was quantified under different conditions. (P) The representative electron microscopy images were shown under different conditions. Scale bar, 2 μm. Data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ****p < 0.0001.
in membrane lipid rafts and play essential roles in innate immunity through neutrophil function, and adaptive immunity through T cell selection [35].

Changes in phospholipid metabolism contributed 14% of the metabolic impact (Table 1, Supplementary Table S2). Twelve of the 13 (92%) phospholipids that were changed in Rev1+/− cells were increased. These included several phosphoinositol lipids involved in calcium signaling like PI36:2, PI36:4, PI38:4, and PI36:1, and bis(monoacylglycerol) phosphates like BMP18:1/22:6, and BMP18:1/22:5. BMP lipids are involved in lysosomal trafficking and are upregulated in secreted exosomes in response to defects in autophagy [36].

Our results show that specific glucose, vitamins, and amino acid metabolites increase in Rev1+/− compared to wildtype MEFs. These include hexose monophosphates and disaccharides, prostaglandin, octanoylcarnitine, vitamin D3, N-acetyl-glucosamine-1-phosphate, and 1-aspartic acid. However, other metabolites associated with glucose, NAD+, and vitamin metabolism were decreased in Rev1+/− MEFs, including phosphoenolpyruvate, isovalerylcarnitine, hexose diphosphate, erythrose 4-phosphate, dihydroxyacetone, and 1-methyl nicotinamide (Fig. 1B, Supplementary Table S2).

Phospholipid metabolism was the top pathway that was most changed by NR treatment, contributing over 22% of the metabolic impact (Table 2, Supplementary Table S3). Treating Rev1+/− cells with NR decreased several phosphoinositol (PI) phospholipids that were previously elevated such as PI34:1, PI36:1, and PI36:2. NR treatment also increased CDP-choline levels that were previously decreased in Rev1−/− MEFs, 1-methylnicotinamide as a marker of vitamin B3 (Niacin, NAD+) metabolism, was significantly decreased (Table 1, Fig. 1B, Supplementary Table S2). Interestingly, the premature aging syndrome Werner knockout cells also show decreased 1-methylnicotinamide metabolite compared to wildtype [19]. Furthermore, Rev1 KO mice show signs of premature aging and shorter lifespan than their wildtype littermates [6]. Supplementation with NR increased three of six measured metabolites in vitamin B3 metabolism. Increased NR metabolites were nicotinic acid, niacinamide, and NAD+ (Table 1). NADH in comparison, was decreased after NR supplementation. Pentose phosphate and gluconate metabolism were decreased and fatty acid oxidation and synthesis were increased (Table 1). The pentose phosphate pathway is a pathway used for NADPH production and biosynthesis of fatty acids and the nucleotide precursor ribose [37]. The five most significantly altered metabolites between Rev1−/− cells treated with and without NR supplementation were nicotinic acid, niacinamide, xanthosine, CDP-choline, and sedoheptulose (Fig. 1C). Pyridine metabolites were decreased and eicosanoid and resolvin metabolites were increased (Table 2) in Rev−/− MEF cells treated with NR compared to the untreated controls.

In conclusion, we found that Rev1 deficiency causes comprehensive alterations in cellular metabolites, particularly in nucleotide (purines and pyrimidines), lipid metabolites, and NAD+ metabolism. The Rev1−/− cells compared to wildtype have several features of metabolic syndrome. This includes elevations in purines, phospholipids, sphingolipids, and cholesterol. Interestingly, NR supplementation could restore many of the metabolite changes observed in Rev1−/− cells but not all.

2.2. Compromised autophagy is rescued by NR supplementation in MEFs

Our metabolomics analysis indicates that Rev1−/− MEFs show metabolic alterations in nucleotide and lipid biosynthesis, and these alterations might affect mitochondrial functions. The depletion of NAD+ metabolites decreases autophagy and mitophagy, which has been implicated in aging and aging-related diseases [38] and is also observed in Rev1−/− MEFs [5]; (Fig. 1). Given that the NAD+ metabolism is augmented in Rev1−/− cells after NR supplementation, we asked whether the increase of NAD+ metabolites would rescue the decreased autophagy/mitophagy (Fig. 2). We examined autophagy by detecting the protein expression of the autophagy marker LC3, and quantifying the ratio of LC3-II to LC3-I in wildtype and Rev1−/− MEF cells. Rev1−/− cells showed a decrease in LC3-II expression and LC3-II/LC3-I ratio compared to wildtype cells, indicating compromised autophagy (Fig. 2A,C,F). The autophagy defects were reversed by supplementing with 3 mM of NR for 30 h (Fig. 2D,E,G,H). There is no significant difference of NR supplementation on autophagy in wildtype MEFs under the experimental conditions used in this study (Fig. 2D,G). Next, we evaluated mitophagy using the mt-mKeima [39]. Rev1−/− cells show a decreased proportion of mitophagic cells compared to wild-type cells (Fig. 2B,I).

Taken together, we show that replication stress-mediated dysregulation of NAD+ metabolism impairs autophagy and mitophagy in Rev1−/− MEFs. This can be reversed by supplementation of NR via remodeling NAD+ metabolism.

2.3. Impaired mitochondrial dynamics is reversed by NR supplementation in MEFs

Defective autophagy and mitophagy can cause the accumulation of dysfunctional mitochondria, which can influence the pathogenesis of many age-related diseases. This will worsen if there is an increase in dysfunctional mitochondria. Mitochondrial dynamics comprises mitochondrial morphology such as number, size, and cristae [40]. Our previous work showed that mitochondrial function was impaired and mitochondria were more fragmented or elongated in Rev1−/− MEFs and murine hepatocytes [5]. To further investigate the mechanisms underlying dysfunctional mitochondria, we examined mitochondrial morphological parameters changes using transmission electron microscopy (TEM). Mitochondrial parameters in Rev1−/− cells compared to wildtype cells show that mitochondrial area, length, and length of cristae decreased significantly (Fig. 3A,F,K). These results suggest that Rev1-deficient cells have smaller mitochondria with fewer cristae compared to wildtype MEFs. The smaller and fragmented mitochondria are in concert with the increased DRP1 phosphorylation and activation in Rev1−/− MEFs [5], which contributes to the enhancement of fission in these cells. In wildtype MEFs supplementation with 3 mM NR for 30 h showed a significant decrease in cristae length (Fig. 3B,G,L). In Rev1−/− MEFs, there was a decrease in mitochondrial number (data not shown), length and area of mitochondria as well as the length of cristae (Fig. 3C,H,M). In Rev1−/− MEFs, mitochondrial length decreased after NR treatment (Fig. 3H) whereas as mitochondrial area significantly increased after NR treatment (Fig. 3C). To investigate the benefits of NR supplementation for mitochondrial protection,
the Complex I inhibitor rotenone was used to stress the cells. After treatment with 4 μM rotenone for 12 h, we did not detect any significant effects on wildtype cells except for the decreased cristae length compared to the untreated control (Fig. 3D,I,N). On the other hand, the Rev1−/− cells show increased mitochondrial area (Fig. 3E) and a significant decrease in mitochondrial length and cristae length after rotenone treatment (Fig. 3J,O). Rev1−/− cells pretreated with 3 mM NR for 18 h and then treated with 4 μM rotenone show a significant recovery of mitochondrial area and cristae length compared to rotenone treatment in Rev1−/− cells (Fig. 3E,O). Our results show that mitochondrial morphology is altered in Rev1-deficient MEFs and mitochondrial morphology changes in Rev1-deficient cells is more sensitive to rotenone treatment. Interestingly, rotenone-induced mitochondria morphology changes such as mitochondria area and length of cristae can be reversed by NR supplementation in Rev1−/− MEFs.

2.4. Rev1 deficiency does not affect lifespan and healthspan in C. elegans

C. elegans has been used as a model system for studies on aging, age-related diseases, and mechanisms of longevity in recent decades. This is based on its physiological aging characteristics. Therefore, we used this model to investigate role of Rev1 deficiency in vivo. In C. elegans models, NAD⁺ depletion, mitochondrial dysfunctions, and alteration of mitochondrial dynamics affect lifespan and healthspan [29]. Our previous work also shows a shortened lifespan in Rev1−/− mice compared to their wildtype siblings [6]. Our metabolomics results show decreased NAD⁺ metabolites in Rev1−/− MEFs and that supplementation of NAD⁺ precursor restores NAD⁺ metabolites (Fig. 1A, C). Based on these results and the observation that NR supplementation partly suppresses changes in mitochondrial morphology in Rev1−/− MEF cells (Fig. 3E,O), we further asked whether Rev1 deficiency would affect life- and healthspan in a nematode model. The rev-1 gene in C. elegans is a homolog of mouse Rev1, which is involved in TLS upon UV damage [41, 42]. We used a rev-1 C. elegans mutant carrying a deletion of rev-1 (exon 2). This strain is sensitive to UV irradiation [42] (Fig. 4 A and B) and undergoes replication stress upon UV damage. Since unrepaired DNA damage induces PARP1 and depletes NAD⁺ pools, we treated

![Fig. 4. Lifespan and healthspan of wildtype N2 and rev-1-deficient C. elegans.](image-url)
nematodes with NR to investigate if the UV sensitivity of rev-1 nematodes could be suppressed by increasing NAD$^+$ levels. We used two different protocols, without NR pretreatment (Fig. 4A) and with NR pretreatment (Fig. 4B). Our results show that rev-1 deficiency sensitizes nematodes to UV damage and that NR supplementation does not suppress this phenotype (Fig. 4A and B). This result suggests that unrepaired UV damage does not deplete NAD$^+$ levels, at least not enough, to cause a shorter lifespan induced by NAD$^+$ supplementation in the rev-1 nematode model.

To investigate if Rev1 and NAD$^+$ levels regulate lifespan we followed the lifespan of N2 and rev-1 nematodes from the L4 stage untreated or treated with 5 mM of NR (Fig. 4C and D). There was no significant difference in the lifespan of N2 nematodes treated with NR, compared to untreated controls (Fig. 4C and D). Paraquat is known to induce oxidative stress and induce replication stress. Accordingly, treatment with paraquat shortened the lifespan of both N2 and rev-1-deficient nematodes and this could not be suppressed by NR supplementation (Fig. 4E and F). Along these lines, we did not detect any differences in healthspan of N2 and rev-1-deficient nematodes treated with paraquat and NR (Fig. 4G–J). Overall, our results show that Rev1 deficiency does not shorten the lifespan in nematodes as it does in mice.

3. Discussion

Here we report that loss of Rev1 causes significant changes in the metabolome. These alterations include changes in nucleotide/lipid metabolism, and decreased NAD$^+$ metabolism. The increased nucleotide biosynthesis is in line with the increased replication stress in Rev1$^{-/-}$ cells. Besides, this is also an explanation of why autophagy is compromised in Rev1 knockout cells as dNTP supplementation decreases autophagy induction in cancer cells [11]. Decreased NAD$^+$ metabolites are involved in aging and aging-related diseases, such as Alzheimer’s disease, cancer, diabetes, ataxia-telangiectasia, and WRN syndrome [17,43]. Here, we reveal that NAD$^+$ augmentation by supplementation of NR results in enhanced NAD$^+$ metabolism including a decrease in pyrimidine, phospholipids, sphingolipids, and ceramides metabolism. Sphingolipids are not only involved in cell membrane structure formation, but also play crucial roles in cell signaling, mitochondrial dynamics, and mitophagy process [44]. Besides, many sphingolipid metabolic pathway enzymes are translocated to mitochondria [44]. The increased phospholipids, sphingomyelin, and ceramides in Rev1$^{-/-}$ cells were widely observed in numerous metabolic and inflammatory disorders [45,46]. SM(d18:1/16:0) (a specific sphingomyelin species) is accumulated in diabetic kidney accompanied by decreased AMPK activity and PGC-1α protein expression [47]. While suppression of sphingomyelin synthases relieves the inhibition of AMPK and PGC-1α and the exogenous addition of SM(d18:1/16:0) metabolite in vivo increases glucose consumption and lactate production [47]. This links increased sphingomyelin to decreased AMPK phosphorylation [5].

Increased genomic stress due to loss of Rev1, exacerbates the utilization of energy metabolites, such as NAD$^+$ and ATP, which is likely the reason why Rev1$^{-/-}$ MEFs show a decrease in NAD$^+$ metabolites compared to wildtype cells (Fig. 1, [5]). Depletion of Rev1 has been shown to alter NAD$^+$ levels, which can affect mitochondrial function, and autophagy/mitophagy; while NAD$^+$ supplementation can reverse the mitochondrial respiratory and autophagy defects (Fig. 2, [5]). Increasing NAD$^+$ levels in WRN also show improved mitophagy [38]. NAD$^+$ is an essential cofactor in many processes, and imbalances in the NAD$^+$ level decrease SIRT1 level and AMPK activity through hyperactivation of PARP1 and impairment of mitochondrial dynamics [5]. AMPK controls energy expenditure by increasing cellular NAD$^+$ levels to augment SIRT1 activity [48]. Rev1$^{-/-}$ MEFs show several changes in mitochondrial morphology and NR supplementation suppresses some of these phenotypes (Fig. 3P). Unlike mice, in C. elegans life and healthspan were not affected by Rev1 deficiency. DNA damage decreased life- and healthspan of Rev1-deficient nematodes but NR supplementation did not suppress these phenotypes (Fig. 4). Also, NR supplementation does not rescue the metabolic phenotype of female Rev1 KO mice measured as improvement of adiposity or glucose tolerance. These results suggest that Rev1-mediated NAD$^+$ depletion is tissue and/or cell type specific [12]. These results reveal an interesting and complex interaction between nuclear and mitochondrial phenomena that involves the controlled action of cellular metabolites.

In conclusion, we show that in MEFs, Rev1 deficiency causes metabolic changes involving mitochondrial dysfunction, which can be manipulated by increasing NAD$^+$ levels. In nematodes, rev-1 deficiency and NR supplementation do not affect life- and healthspan as shown in mice [6]. This suggests significantly different mechanisms underlying the role of Rev1 in longevity in mice and nematodes. This study provides new insights into the intersection between DNA damage repair, autophagy, and metabolism. However, the underlying mechanisms remain unclear. In MEFs we have shown that replication stress via Rev1-deficiency contributes to metabolic stress caused by compromised mitochondrial function via this PARP-NAD$^+$-SIRT1-PGC1α axis [5].

4. STAR★Methods

4.1. Experimental model and subject details

4.1.1. Cell culture

Wildtype and Rev1$^{-/-}$ MEFs have been described previously [6,49]. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) plus 10% (v/v) fetal bovine serum (Invitrogen) and 100 U/mL penicillin/streptomycin (Thermo Fisher Scientific, #15140122), in an incubator with 5% CO$_2$ at 37 °C.

4.1.2. Antibodies

LC3B/MAP1LC3B (Novus Biologicals, #NB100-2220) (1:1000); pan-Actin (Thermo Fisher Scientific, #MA5-11869) (1:10,000); AMPKa (Cell signaling, #2532) (1:1000); Phospho-AMPKa (Thr172) (Cell signaling, #2531) (1:1000); DRP1 (Cell signaling, #8570)
1.1000); Phospho-DRP1 (Ser616) (Cell signaling, #3455) (1:1000).

4.1.3. Western blot

MEFs were lysed and resuspended in RIPA buffer (Sigma) supplemented with cComplete™, EDTA-free Protease Inhibitor Cocktail (Sigma), and PhosSTOP phosphatase inhibitor Cocktail (Sigma). The cell suspension was incubated on ice by shaking on a shaker for 30 min. Subsequently, the cell suspension was centrifuged at 15,000 G-force for 15 min, and the supernatant was mixed with NuPAGE® LDS Sample Buffer (4 ×) (Thermo Fisher Scientific). Samples for gel electrophoresis were denatured at 95°C for 5 min and 20–30 µg protein was resolved by NuPAGE™ Novex™ 4–12% Bis-Tris Protein Gels, 1.0 mm (Thermo Fisher Scientific) according to the manufacturer’s instructions.

4.1.4. Metabolome sample preparation and analysis

For the metabolomics study, 300,000 cells/15 cm dishes were plated on Day 0 (Non-critical cells) labeled as “Wildtype C57BL/6 (‘B6’) MEFs without NR”, “Wildtype B6 with NR”, “Rev1−/− MEFs without NR” and “Rev1−/− MEFs with NR” 7 dishes for each experimental group were used as biological replicates. Cells were treated with 3 mM NR on day 1 and collected on day 4, from 6 biological replicates; a 7th replicate plate from each experimental group was processed for cell count using trypan blue viability after trypsination. The rest of the 6 groups of cells were washed with 1 × PBS and the 1 × PBS was aspirated completely. Subsequently, dishes with cells were frozen and stored at −80°C, which were kept on ice. Next, cells were harvested by scraping and transferred into pre-labeled 2 mL Eppendorf tubes, vortexed and then snap-frozen in liquid nitrogen and vortexed again. This freeze-thaw cycle was repeated three times. 50 µL from each sample was collected and stored at −80°C for Pico green assay. Remaining samples are centrifuged at 15,000 G for 10 min at 4°C to precipitate proteins, DNA, RNA, and glycans. The supernatant extract from each plate was split into two (0.9 mL each) tubes and stored in labeled screw-cap cryotubes at −80°C until they were processed at Naviaux Lab. Metabolomics was performed by high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described [50]. Cell biomass was normalized by DNA content quantified by Pico green assay. Metabolite peak areas under the curve (AUCs) were log2 transformed for statistical analysis. Metabolites were scored according to their variable importance in the projection (VIP) scores using the PLS-DA model. Metabolites with VIP scores >1.2 were considered significant. Significant metabolites were aggregated into biochemical pathways and ranked by the sum of the VIP scores. Metabolomics data normalized for cell DNA content are reported in Supplementary Table S1.

4.1.5. Lentivirus packaging and infection

pHAGE-mt-mKeima was a gift from Richard Youle (Addgene plasmid, #131626). 293T cells are seeded in a 10 cm² dish. The next day, 293T cells reach 80% confluency which is suitable for transfection. For one 10 cm dish, mix 500 µL Opti-MEM™ Reduced Serum Medium (Thermo Fisher Scientific, #31985062) with plasmids (9 µg pHAC-mt-mKeima, 4.5 µg pMDLg/pRRE, 4.5 µg pRSV-Rev, and 4.5 µg pMD2.G); mix 500 µL Opti-MEM with 67.5 µg PEI (polyethylenimine, transfection reagent, 1 µg/µL). Gently add the diluted PEI dropwise to the diluted DNA while gently swirling the tube with diluted DNA. Incubate the mixture for 15–20 min at room temperature. Then, the even transfection mixture is added dropwise to the 293T cell culture in a 10 cm² dish. The following morning, carefully replace the media with 10 mL of DMEM complete medium. Viral particles can be harvested at 48 h post-transfection. Collect and filter medium through a 0.45 µm sterile PES syringe filter. The viral supernatant can be stored at 4°C for a short time, but it should be aliquoted and snap-frozen in liquid nitrogen and stored at −80°C as soon as possible to avoid loss of titer for long-term storage. For lentivirus infection, wildtype and Rev1−/− cells are seeded one day before infection (Day −1). The next day (Day 0), mt-mKeima lentivirus is diluted with DMEM complete medium and added to cells. After 8 h, change medium with DMEM complete medium. Day 1, add 1.5 µg/mL puromycin to select the positive cells for 48 h. Finally, the stable mt-mKeima-integrated wildtype or Rev1−/− MEFs are generated.

4.1.6. Detection of mitophagy with FACS

To measure mitophagy we used a pH-dependent fluorescence assay based on the coral-derived protein Keima, which can be targeted to the mitochondrial matrix (mt-mKeima). The mt-mKeima probe enables differential imaging of mitochondria in the cytosol and mitochondria in autophagosomes/lysosomes, thus reflecting the underlying level of mitophagy. Mitophagy in Rev1−/− and wildtype MEF cells expressing mt-mKeima were evaluated by a FACS-based method. mt-mKeima-integrated wildtype or Rev1−/− MEFs were seeded in 12-well plates at a density of 5 × 10⁴ cells/well and cultured for 24 h. Then, cells were treated with either 20 µM FCCP (Sigma-Aldrich, #C2920) or the same volume of DMSO for 3 h. Cells were trypsinized and mixed in 1 mL 1 × cold PBS to ensure singlets and put on ice. Wildtype or Rev1−/− MEFs without mt-mKeima integration were used as negative cells to set the gate for mt-mKeima positive cell detection by using the CytoFLEX S Flow Cytometer. The Violet610-A fluorescent channel is used to detect the green signals irradiated by 405 nm laser, while the ECD-A channel is used to detect the red signals irradiated by 561 nm laser. The mitophagy level is indicated by the ratio of ECD-A (561 nm)/Violet610-A (405 nm). The population with a higher ratio is designated as “high gate”, while the population with a lower ratio as “low gate”. A detailed protocol is available [51]. The ratio of Red to Green fluorescence intensity was used to denote mitophagy positive cells.

4.1.7. TEM samples preparation and analysis

Wildtype and Rev1−/− MEF cells were seeded on plastic coverslips in 24-well plate at a concentration of 2 × 10³ cells per well and
cultured for 24 h at 37 °C in 5% CO2 incubator. Next day, wells were either supplemented with (“NR”) or without (“Ctrl”) 3 mM NR for 30 h; wells were treated with rotenone (either 2 μM or 4 μM) for 12 h (“Rot 2 μM” or “Rot 4 μM”); wells were supplemented with 3 mM NR for 18 h followed by rotenone treatment (either 2 μM or 4 μM) for the last 12 h of the total 30 h corresponding to approximate two cell divisions (“NR + Rot 2 μM” or “NR + Rot 4 μM”); wells were supplemented with 3 mM NR for 18 h followed by rotenone treatment (either 2 μM or 4 μM) combined with 3 mM NR for the last 12 h of 30 h (“NR + (NR + Rot 2 μM)” or “NR + (NR + Rot 4 μM)”). After 30 h, cells were washed twice with 1 × DPBS and fixed with 2% glutaraldehyde/0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 1 h. Thin sections were prepared by the Core Facility for Integrated Microscopy at the University of Copenhagen. All of the specimens are examined using a Transmission Electron Microscope Philips CM100, FEI. The effect of 3 mM and 5 mM NR treatment on mitochondrial function of MEFs has been described in Fakouri et al., [4]; data not shown.

4.1.8. Preparation of E. coli strain and plating on NGM plates for feeding C. elegans

The N2 Bristol strain was used as wildtype control. N2 (wildtype strain) and rev-1(if207)-deficient nematodes were obtained from Marcel Tijsterman (Leiden University, NL) [42].

Glycerol stock of OP50-1 E. coli strain (from CGC C. elegans library) resistant to streptomycin was streaked on an LB plate prepared with 50 μg/mL streptomycin (Sigma) and incubated overnight at 37 °C for single colony formation. The next day a single colony was picked and stirred in 100 mL of LB medium with 50 μg/mL of streptomycin and left at 37 °C for 12–14 h. 100 μL of the culture was added in the center of NGM 35 × 15 mm plates, or 200 μL in 60 mm NGM plates and plates were incubated for 2 days at room temperature to allow the bacterial lawn to grow.

4.1.9. Progeny survival in response to UV-C

A synchronous population of nematodes was made and about 30 L4 stage nematodes transferred to separate plates for each condition, which were treated with or without NR for a concentration of 5 mM NR. The following day in the young adult stage the plates were exposed to different doses of UV-C and 5 nematodes were picked randomly for each condition and put on separate plates which had been prepared either with or without NR according to the condition. After 24 h of laying eggs on the plates the adult nematodes were removed. After a further 24 h the amount of unhatched eggs and progeny was counted for each plate and the survival rate was calculated as

\[
\frac{\text{progeny}}{\text{progeny-eggs}}
\]

4.1.10. Synchronization of C. elegans

Wildtype and mutant nematodes were picked and transferred to five freshly seeded NGM plates (10 nematodes per plate) and incubated at 20 °C for 4–5 h to allow them to lay eggs. Next, adult nematodes were removed, and eggs were left on the continuation of the life cycle. In the next two days, they were incubated at 20 °C. From the synchronized nematodes, 12–15 L4 larval stage nematodes were picked per plate, and for each experimental group, three replicates were conducted.

4.1.11. Lifespan and healthspan assay

L4 larvae of N2 and rev-1(if207) mutant strains were transferred on to NGM plates with the OP50-1 bacterial strain at 20 °C. The plates were prepared either with FUDR (0.1 mg/mL) only or with FUDR (0.1 mg/mL) and 1 mM, 5 mM or 10 mM concentration of NR. Triplicates consisting of 12–15 L4 stage nematodes each were transferred to each experimental group, for both N2 and rev-1-deficient nematodes. The next day, nematodes were gently touched with a pick (made by using platinum wire), and the observation of whether they can move or not was used to score them as alive or dead. The scoring was done every alternative day until all experimental groups had no nematodes alive. Healthspan examination of C. elegans was performed described in Ref. [29] with nematodes on NGM with OP50-1 bacteria strain at 20 °C. Three NGM plates with 25 nematodes each were synchronized and left for three days to reach day 1 of adulthood. Examination of C. elegans healthspan was measured on synchronized nematodes by counting pharyngeal pumping for 20 s each time, five times per nematode, and five different nematodes every group. This was repeated until less than five nematodes were able to pump. We measured healthspan by counting pharyngeal pumping for 20 s, five times per nematode, in five different nematodes. NR supplementation was given on day 1, just before the measurement of pumping rates. We followed the healthspan of N2 and rev-1-deficient nematodes from day 1 of the adult stage in C. elegans, in the presence or absence of a 5 mM NR for 7 days (day 2-day 8) during which pumping can be easily recorded. The NAD + dependency and experimental conditions for NR treatment that improves the average lifespan and healthspan were determined on nematodes carrying mutated dhs-3 gene.

4.2. Lifespan

A synchronous population of N2 and rev-1(if207) L4 nematodes was prepared. Plates were prepared with water, NR, paraquat and NR + paraquat at concentrations of 0.35 nM paraquat and 5 mM NR in the total volume of the growth medium in the plates. Around 30 L4 nematodes were placed on each plate, 3 plates per condition per genotype. Every second day the nematodes were moved to new plates which had been freshly prepared to the corresponding condition and simultaneously scored for whether they were alive.

4.3. Healthspan

4.3.1. 4 Days

NGM plates were treated with water, NR, paraquat or NR + paraquat respectively. Concentrations of NR and paraquat were 5 mM
and 2 mM in the total volume of the growth medium in the plate. Around 25–30 nematodes from a synchronous population of L4 nematodes were put on each plate, 3 plates per condition per genotype. The following day (day 1) adult 5 random nematodes on each plate were selected and the pumping of the pharynx was counted for 30 s and noted down. At day 2 adult the nematodes were transferred to freshly prepared plates with their corresponding conditions. Counting of the pumping was done every day from day 1 to day 4.

4.3.2. 10 Days
The 10 day healthspan was done simultaneously with the lifespan and was done the same way as for the 4 day healthspan besides the counting of additional days.

4.4. Statistical analysis
The difference between two groups was analyzed with unpaired Student’s t-test. For the TEM images, data are shown as mean ± SEM. Group differences were analyzed with one-way or two-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test for multiple groups. All of the bar charts are prepared by using GraphPad Prism 8. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. A multiple regression line analysis was performed for the healthspan of C. elegans.

Author contribution statement
Sharath Anugula, Zhiquan Li, Yuan Li, Alexander Hendriksen, Peter Bjarn Christensen, Lin Wang, Jonathan M. Monk, Robert K. Naviaux: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Niels de Wind, Vilhelm A. Bohr, Claus Desler, Lene Juel Rasmussen: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement
Dr Lene Juel Rasmussen was supported by Nordea-fonden, Olav Thon Stiftelsen, Novo Nordisk Fonden (NNF17OC0027812).

Data availability statement
Data included in article/supp. material/referenced in article.

Declaration of competing interest
The authors have no Declaration of Interest.

Acknowledgment
We thank the technical support for confocal microscopy and transmission electron microscope (TEM) at the Core Facility for Integrated Microscopy (CFIM) and that for FACS at the Flow Cytometry & Single Cell Core Facility at the Health and Medical Faculty at the University of Copenhagen. This work was supported by research grants from Nordea-fonden (L.J.R.), Olav Thon Foundation (L.J. R.), Novo Nordisk Foundation NNF17OC0027812 (L.J.R.). L.J.R. is a member of the Clinical Academic Group: Recovery Capacity After Acute Illness in An Aging Population (RECAP). We acknowledge support from the Intramural Program of the National Institute on Aging, National Institutes of Health, USA. Nicotinamide (NR) was obtained from Chromadex Corp., which has a CRADA with Vilhelm Bohr. Support for the metabolomics studies was provided in part by the UCSD Christini Fund and the UCSD Mitochondrial Disease Research Fund.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17392.

References


