

<https://doi.org/10.1038/s42003-024-07089-2>

# miR-124 coordinates metabolic regulators acting at early stages of human neurogenesis



Geurim Son<sup>1</sup>, Yongwoo Na<sup>2,3</sup>, Yongsung Kim<sup>4</sup>, Ji-Hoon Son<sup>1</sup>, Gregory D. Clemenson<sup>4</sup>, Simon T. Schafer<sup>4</sup>, Jong-Yeon Yoo<sup>5</sup>, Sarah L. Parylak<sup>4</sup>, Apua Paquola<sup>4,6,7</sup>, Hyunsu Do<sup>1</sup>, Dayeon Kim<sup>1</sup>, Insook Ahn<sup>1</sup>, Mingyu Ju<sup>1</sup>, Chanhee S. Kang<sup>1</sup>, Younghee Ju<sup>5,8</sup>, Eunji Jung<sup>1</sup>, Aidan H. McDonald<sup>4</sup>, Youngjin Park<sup>9</sup>, Gilhyun Kim<sup>10</sup>, Se-Bum Paik<sup>9,11</sup>, Junho Hur<sup>12</sup>, Joon Kim<sup>1</sup>, Yong-Mahn Han<sup>1</sup>, Seung-Hee Lee<sup>10,11</sup>, Fred H. Gage<sup>4</sup>, Jong-Seo Kim<sup>2,3</sup> & Jinju Han<sup>1,13,14</sup> ✉

Metabolic dysregulation of neurons is associated with diverse human brain disorders. Metabolic reprogramming occurs during neuronal differentiation, but it is not fully understood which molecules regulate metabolic changes at the early stages of neurogenesis. In this study, we report that miR-124 is a driver of metabolic change at the initiating stage of human neurogenesis. Proteome analysis has shown the oxidative phosphorylation pathway to be the most significantly altered among the differentially expressed proteins (DEPs) in the immature neurons after the knockdown of miR-124. In agreement with these proteomics results, miR-124-depleted neurons display mitochondrial dysfunctions, such as decreased mitochondrial membrane potential and cellular respiration. Moreover, morphological analyses of mitochondria in early differentiated neurons after miR-124 knockdown result in smaller and less mature shapes. Lastly, we show the potential of identified DEPs as novel metabolic regulators in early neuronal development by validating the effects of GSTK1 on cellular respiration. GSTK1, which is upregulated most significantly in miR-124 knockdown neurons, reduces the oxygen consumption rate of neural cells. Collectively, our data highlight the roles of miR-124 in coordinating metabolic maturation at the early stages of neurogenesis and provide insights into potential metabolic regulators associated with human brain disorders characterized by metabolic dysfunctions.

Metabolic reprogramming is a common phenomenon in neuronal differentiation. Studies have shown that metabolic reprogramming is essential for efficient energy production in neurons<sup>1–3</sup>. Neural progenitor cells (NPCs) have immature mitochondria with spherical and condensed globular structures and depend mainly on aerobic glycolysis for energy production. As NPCs are differentiated into neurons, mitochondria simultaneously become elongated and result in mature mitochondria with tubular and cristae-rich structures. Neurons utilize oxidative phosphorylation

(OXPHOS) as the major energy-producing machinery in this bioenergetic shift. Neurons require a large amount of energy to function, with one cortical neuron at a resting state using about 4.7 billion ATP molecules per second<sup>4</sup>. Most ATPs are generated by OXPHOS occurring in mitochondria. In contrast to the existing consensus about the mitochondrial dynamics in neuronal differentiation, a recent study has reported that an immediate fragmentation of mitochondria in NPCs after they exit the cell cycle is necessary to differentiate into neurons containing fused mitochondria of

<sup>1</sup>Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Korea. <sup>2</sup>Center for RNA Research, Institute for Basic Science, Seoul, Korea. <sup>3</sup>School of Biological Sciences, Seoul National University, Seoul, Korea. <sup>4</sup>Laboratory of Genetics, Salk Institute for Biological Studies, La Jolla, CA, USA. <sup>5</sup>Department of Biological Sciences, KAIST, Daejeon, Korea. <sup>6</sup>Lieber Institute for Brain Development, Baltimore, MD, USA. <sup>7</sup>Department of Neurology, School of Medicine, Johns Hopkins University, Baltimore, MD, USA. <sup>8</sup>Sovagen.CO., LTD., Daejeon, Korea. <sup>9</sup>Department of Bio and Brain Engineering, KAIST, Daejeon, Korea. <sup>10</sup>Center for Synaptic Brain Dysfunctions, Institute for Basic Science, Daejeon, Korea. <sup>11</sup>Department of Brain and Cognitive Sciences, KAIST, Daejeon, Korea. <sup>12</sup>College of Medicine, Hanyang University, Seoul, Korea. <sup>13</sup>BioMedical Research Center, KAIST, Daejeon, Korea. <sup>14</sup>KAIST Stem Cell Center, KAIST, Daejeon, Korea. ✉e-mail: [jinjuhan@kaist.ac.kr](mailto:jinjuhan@kaist.ac.kr)

complex morphology<sup>5</sup>. In the end, the metabolic shift must be accompanied by neuronal differentiation.

With regards to the gene expression changes in neurogenesis, multi-omics studies show that only several days of neuronal differentiation are required to detect differentially expressed genes (DEGs) of NPCs and neurons<sup>6,7</sup>. These changes also reflect metabolic reprogramming<sup>2,3,7</sup>. The expression level of mitochondrial proteins required for OXPHOS protein complexes is increased with neuronal differentiation. Moreover, the expression levels of glycolytic and TCA cycle-related metabolic genes differ between NPCs and differentiated neurons<sup>3</sup>. The reduction of NPC-enriched metabolic genes such as *LDHA* and *HK2* is essential for the survival of developing neurons<sup>3</sup>. Several protein-coding genes that control metabolic reprogramming during neurogenesis have been identified. For instance, Western blot analyses detecting OXPHOS subunit proteins show an increased level of protein expression in neurogenesis<sup>2,3</sup>. In addition, the expression level of NADH dehydrogenase increased, supporting the expanded surface area of the mitochondrial matrix<sup>7</sup>. The increased level of proteins is required for a higher level of mitochondrial respiration. These previous studies have shown the significant roles of ubiquitously expressed metabolic regulators in neurogenesis. However, further investigation is necessary to identify additional metabolic regulators that may be specifically involved in neurogenesis beyond the well-established roles of ubiquitously expressed metabolic regulators.

The expression level of proteins during neuronal development is largely regulated through the 3' untranslated region (UTR), which is extended with neuronal differentiation<sup>8</sup>. The 3' UTR contains various motifs regulating mRNA stability and translational efficiency<sup>9</sup>. One of the regulatory motifs in the 3' UTR is a microRNA (miRNA) binding site. miRNAs are small, noncoding elements regulating gene expression at the post-transcriptional level by binding to the 3' UTR of target mRNAs. It is known that mRNAs expressed in the brain possess much longer 3' UTR on average than mRNAs expressed in other organs, and the extended 3' UTR of mRNAs in the brain carries conserved miRNA binding sites. It is assumed that miRNAs play essential roles in regulating metabolic gene expression during neuronal development. Although many factors participate in the regulation of intracellular bioenergetics, the regulation of metabolic gene expression incorporating miRNAs in neurogenesis has not been studied extensively.

miR-124, which is the most abundant miRNA in the brain, has been demonstrated to be a key player in brain development and function. In neuronal differentiation, expression of miR-124 is upregulated, as it is known that miR-124 is enriched in neurons<sup>10,11</sup>. A disrupted expression of miR-124 results in the inhibition of neuronal development and survival<sup>12–15</sup>. Alternatively, miR-124 suppression changes the cellular lineage of neural stem cells into non-neuronal cells<sup>12,14,16</sup>. In contrast, overexpression (OE) of miR-124 enhances neuronal differentiation<sup>12,16</sup>. Targets of miR-124 regulating neurogenesis include proteins modulating neuronal gene expression through transcriptional (LHX2, SOX9, and SCP1) and splicing (PTBP1) regulations, post-transcriptional feedback (EFNB1), and chromatin remodeling (BAF53a)<sup>12,13,16–19</sup>. However, the major gene regulatory networks controlled by miR-124 at the early stages of neuronal differentiation of NPCs remain to be elucidated.

Here, we report a novel role for miR-124 driving metabolic reprogramming in human neurogenesis. Increasing its expression along with neuronal differentiation<sup>11</sup>, miR-124 is essential for the morphological and electrical maturation of human neurons. For a systemic approach to analyzing the changes in neuronal differentiation by miR-124, we analyzed the neuronal proteome after depleting miR-124 and obtained a list of differentially expressed proteins (DEPs). DEPs indicated an association between defective metabolism in developing neurons and miR-124 depletion. Decreased mitochondrial membrane potential (MMP), mitochondrial size, and cellular respiration validate the metabolic dysfunctions of neurons resulting from the miR-124 knockdown. Furthermore, we show the potential of DEPs as novel metabolic regulators by showing that GSTK1 (also known as DsbA-L) – one of the upregulated proteins in

miR-124-depleted neurons – reduces cellular respiration, corresponding to the results of miR-124 depletion. Our findings demonstrate an essential role of miR-124 in coordinating metabolic reprogramming at the initiating stages of neurogenesis.

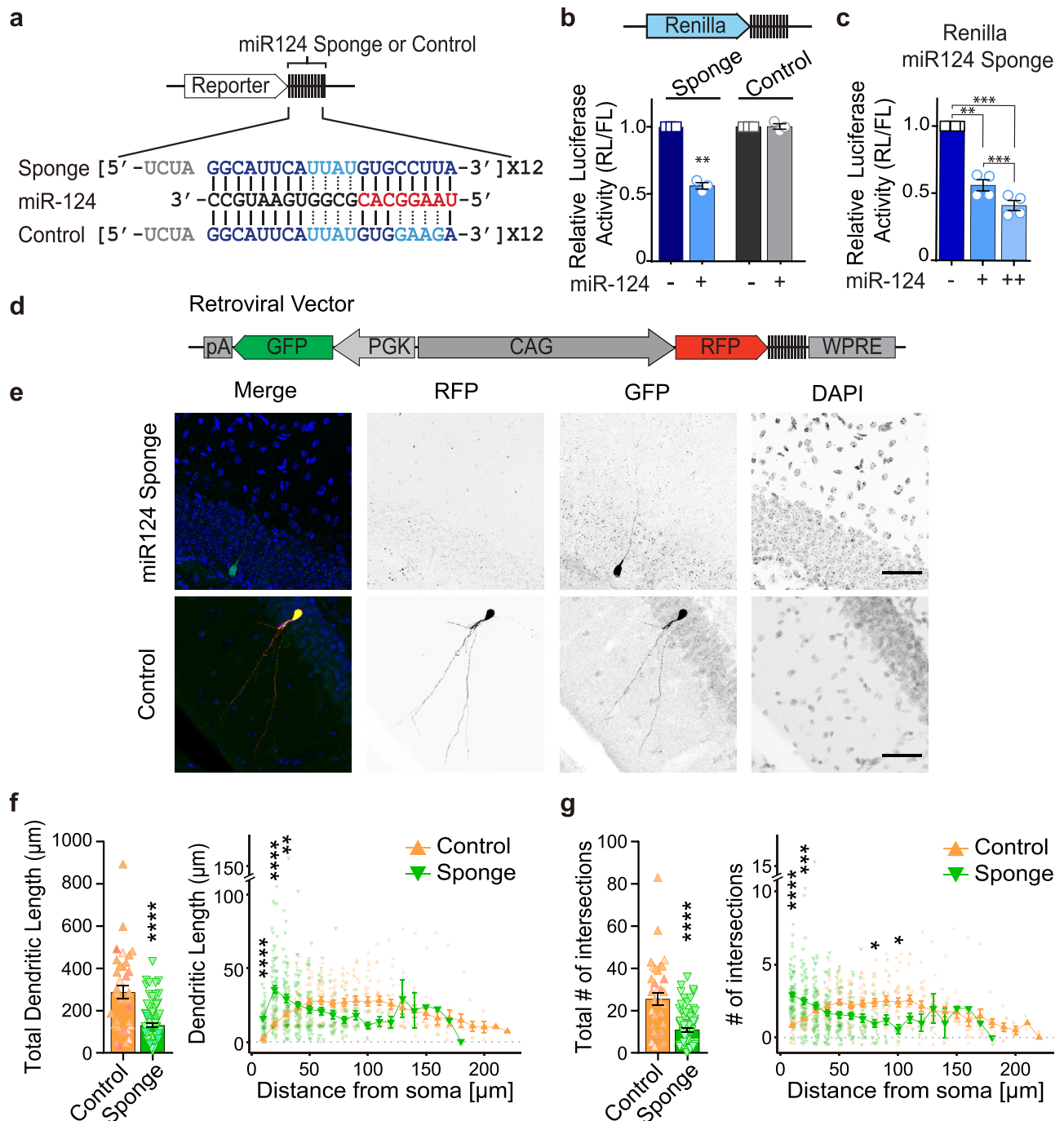
## Results

### Impairment of human neurogenesis by miR-124 suppression

To investigate major signaling pathways affected by miR-124 in early neuronal differentiation, we sought to suppress miR-124 during neurogenesis. To do so, we employed the miRNA sponge system to inhibit miR-124 function<sup>20,21</sup>. A miR-124 sponge construct was designed by inserting 12 tandem miR-124 binding motifs with mismatches in the middle (Fig. 1a). The sponges were then inserted downstream of a reporter gene. The control construct for the sponge, possesses four different nucleotides within the seed sequences of miR-124; the remaining sequences were identical to the miR-124 sponge (Fig. 1a). The luciferase activity from the miR-124 sponge construct was significantly decreased compared to the control construct when miR-124 was overexpressed, indicating the responsiveness of the miR-124 sponge to miR-124 (Fig. 1b). Additionally, the miR-124 sponge was responsive to a varying level of miR-124 expression (Fig. 1c).

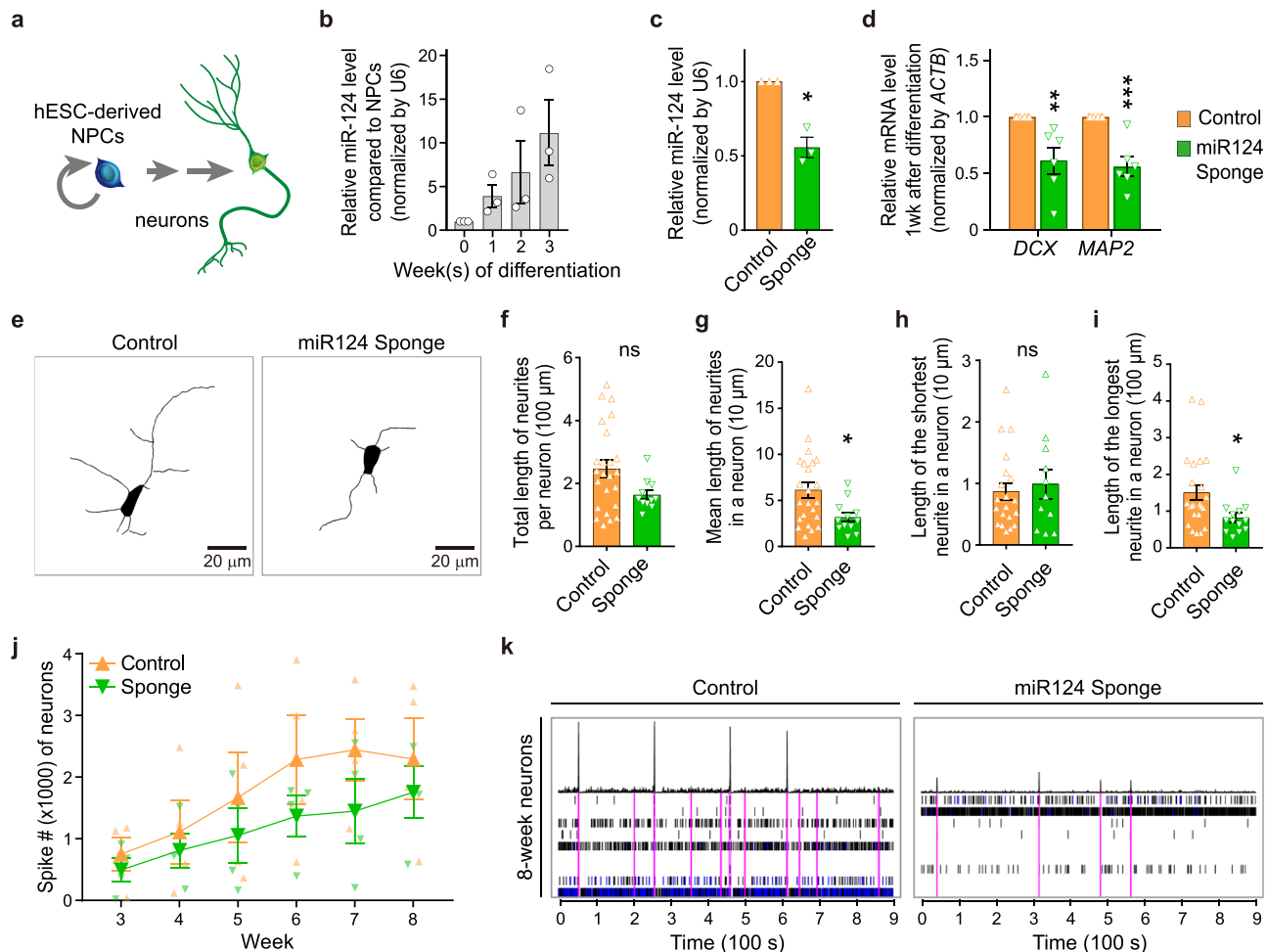
Next, we produced a retrovirus expressing miR-124 sponge to determine whether the miR-124 sponge physiologically affected neuronal development in vivo. In this case, the expression of RFP by retrovirus is decreased along with miR-124 expression, although GFP is expressed constitutively. On the other hand, both GFP and RFP are expressed together with the retrovirus carrying the control sponge sequences. The retrovirus was injected into the region where NPCs reside in the adult hippocampus of the mouse brain. In the hippocampus where miR-124 sponge was introduced 3 weeks post-injection, only GFP-positive cells were observed, in contrast to control neurons that expressed both GFP and RFP. The GFP-positive cells expressing miR-124 sponge were validated as neurons, as they expressed neuronal markers (PROX1 and TUJ1) (Fig. S1a). Then, the morphological changes of newborn neurons were analyzed. We measured the dendritic length and complexity of newborn neurons using GFP signal and found that the knockdown of miR-124 in vivo resulted in a significant reduction in dendritic length and arborization (Fig. 1d–g, S1b). The results showed that the miR-124 sponge adequately suppressed miR-124 in developing neurons and impaired neuronal development.

To validate the expression pattern of miR-124 during human neurogenesis, we generated NPCs from human embryonic stem cells (hESCs) and subsequently differentiated these NPCs into neurons (Fig. S2). Real-time PCR results indicated that the expression level of miR-124 increased upon neuronal differentiation (Fig. 2a, b). To confirm that the downregulation of miR-124 hinders neuronal development in human, miR-124 was knocked down in human NPCs and then the cells were induced to neuronal differentiation. For a long-lasting inhibition of miR-124 expression during human neurogenesis, the miR-124 sponge and control sequences were inserted into lentiviruses. The miR-124 sponge successfully suppressed the expression of miR-124 in developing human neurons (Fig. 2c) and significantly reduced the expression levels of two neuronal genes, *DCX* and *MAP2*, indicating that downregulation of miR-124 impairs neuronal differentiation (Fig. 2d). Additionally, knockdown (KD) of miR-124 altered the morphological and electrophysiological features of developing neurons (Fig. 2e–k). In miR-124 KD neurons, total neurite length was slightly shorter and mean neurite length was significantly reduced compared to control neurons within one week of differentiation (Fig. 2f, g). Additionally, although the shortest neurites were similar in length, the longest neurites of miR-124 KD neurons were dramatically shorter than those of control neurons (Fig. 2h, i). The number of spikes measured by a microelectrode array (MEA) was significantly less in miR-124 KD neurons throughout the period of 8-week neuronal differentiation (Fig. 2j). The number of network bursts was also significantly reduced in the miR-124 KD group, shown by a comparison of the raster plots at week-8 (Fig. 2k). Overall, results indicate that miR-124 KD at the initial stage of neurogenesis leads to a delayed or permanently dysregulated neuronal differentiation.



**Fig. 1 | The miRNA-124 sponge system successfully suppresses adult hippocampal neurogenesis.** **a** The miRNA-124 sponge contains 12 repeats of the sequences complementary to the miR-124 seed region (indicated in red) downstream of a reporter gene (*Renilla* luciferase or RFP). For the control, four nucleotides of the miR-124 sponge construct have been mutated (indicated in orange) to mismatch the miR-124 seed-binding region. **b** *Renilla* luciferase activity of the miR-124 sponge construct suppressed with miR-124 overexpression (OE) in HEK293T cells. For both miR-124 sponge and control, the relative level of *Renilla* luciferase activity to firefly luciferase activity was compared with or without miR-124 OE ( $n = 3$ , two-tailed paired  $t$ -test,  $**p$ -value = 0.0029, error bar = s.e.m.). **c** Experiment conducted in panel 1b repeated with a higher level of miR-124 OE. Substantial amounts of miR-124 OE led to further suppression of *Renilla* luciferase activity, indicating a dose-dependent reaction ( $n = 4$ , two-tailed paired  $t$ -test,  $**p = 0.0018$  (– vs. +),  $***p = 0.0006$  (– vs. ++),  $***p = 0.0002$  (+ vs. ++), error

bar = s.e.m.). **d** Retrovirus was infected to mouse hippocampus for Sholl analysis. GFP is constantly expressed, but translation of RFP is suppressed when miR-124 binds to miR-124 sponge sequences. **e** Representative images of panels **f** and **g**. RFP translation was not suppressed in the control, so co-expressed with GFP when infected. Mouse hippocampus was imaged three weeks post-infection. Scale bar = 50 μm. **f, g** Sholl analysis results show significantly shorter dendritic length of miR-124-depleted neurons and significantly less number of intersections in miR-124-depleted neurons (Control  $n = 34$  from 5 mice, Sponge  $n = 83$  from 4 mice; error bar = s.e.m.; two-tailed unpaired  $t$ -test (bar graphs),  $****p$ -value < 0.0001; two-way ANOVA Bonferroni's multiple comparisons test (line graphs), dendritic length:  $****$ adj  $p$ -value < 0.0001 and  $**$ adj  $p$ -value = 0.0065, intersections:  $****$ adj  $p$ -value < 0.0001,  $***$ adj  $p$ -value = 0.0005,  $*$ adj  $p$ -value (80 μm) = 0.0188, and  $*$ adj  $p$ -value (100 μm) = 0.0494).



**Fig. 2 | miR-124 knockdown causes defects in neuronal differentiation of human NPCs.** **a** Scheme of neuronal differentiation using human embryonic stem cell (hESC)-derived neural progenitor cells (NPCs) to study neurogenesis. **b** The expression level of mature miR-124 relative to U6 increases during human neurogenesis ( $n = 3$ , Kruskal-Wallis one-way ANOVA,  $p$ -value = 0.0198, error bar = s.e.m.). **c** The miR-124 sponge decreases the expression level of mature miR-124. Relative miR-124 level was measured by real time PCR in neurons differentiated for 6 days after miR-124 sponge infection ( $n = 3$ , two-tailed paired  $t$ -test,  $*p$ -value = 0.0233, error bar = s.e.m.). **d** The expression of neuronal genes, *DCX* and *MAP2*, is decreased in miR-124-depleted day-6 neurons ( $n = 6$ , two-tailed unpaired  $t$ -test,  $**p$ -value (*DCX*) = 0.0083,  $***p$ -value (*MAP2*) = 0.0005, error bar = s.e.m.). **e** Representative images of day-6 neurons with or without miR-124 function. miR-124 KD reduces the neurite complexities of early differentiating neurons. **f–i** Neurite

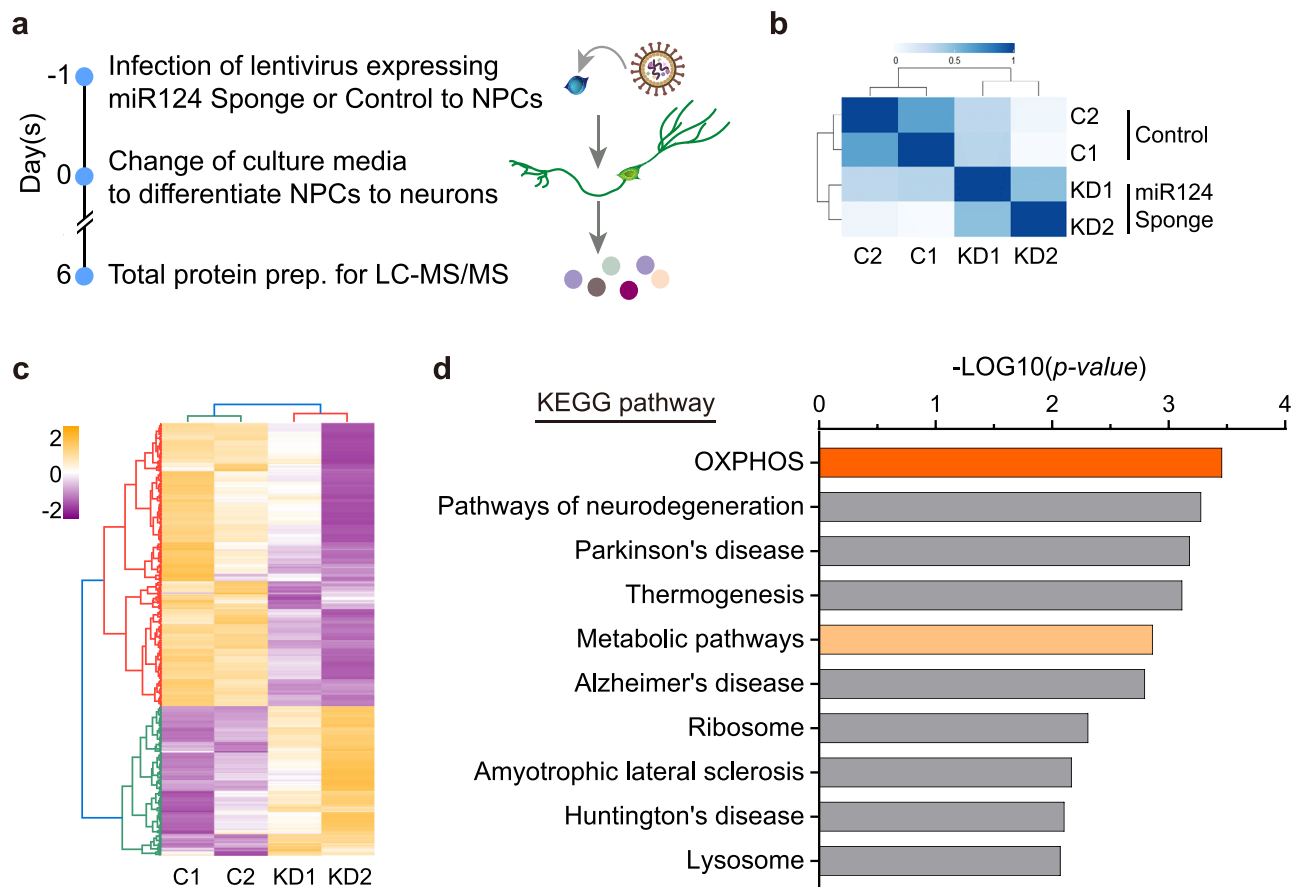
length analyzed by using a FIJI plugin NeuronJ ( $n = 3$ , two-tailed unpaired  $t$ -test, error bar = s.e.m.). **f** Total length of neurites per neuron (24 Control neurons, 11 Sponge neurons, ns = non-significant,  $p$ -value = 0.0563). **g** Mean length of neurite in a neuron (23 Control neurons, 12 Sponge neurons,  $*p$ -value = 0.0184). **h** Length of the shortest neurite in a neuron (20 Control neurons, 11 Sponge neurons, ns = non-significant,  $p$ -value = 0.6466). **i** Length of the longest neurite in a neuron (23 Control neurons, 13 Sponge neurons,  $*p$ -value = 0.0283). **j, k** miR-124 KD suppresses electrophysiological maturation. Electrophysiological properties of neurons with or without miR-124 were measured via microelectrode array for 8 weeks. **j** Number of spikes from week-3 to -8 neurons ( $n = 4$ , two-way ANOVA,  $*p$ -value = 0.0423, error bar = s.e.m.). **k** Representative raster plots presenting network bursts in neurons differentiated for 8 weeks.

### Metabolic dysfunctions of developing neurons by miR-124 suppression

To assess the intracellular changes resulting from miR-124 KD during human neurogenesis, the proteome of miR-124 depleted neurons and the control neurons were analyzed by performing a tandem mass tag (TMT) liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Fig. 3a, Supplementary Table S1). Proteins detected from the proteomic analysis of the biological duplicate were clustered by their experimental conditions (Fig. 3b), indicating that the experiments were well controlled. We identified 593 DEPs that are common between both biological replicates of miR-124 KD and control neurons (Fig. 3c, Supplementary Table S2). Then, with the list of DEPs, a gene ontology (GO) analysis was carried out using the database for annotation, visualization and integrated discovery (DAVID) bioinformatics resource<sup>22,23</sup> (Supplementary Table S3). Through the DAVID GO analysis, it was discovered that the top annotated term of the KEGG pathway analysis

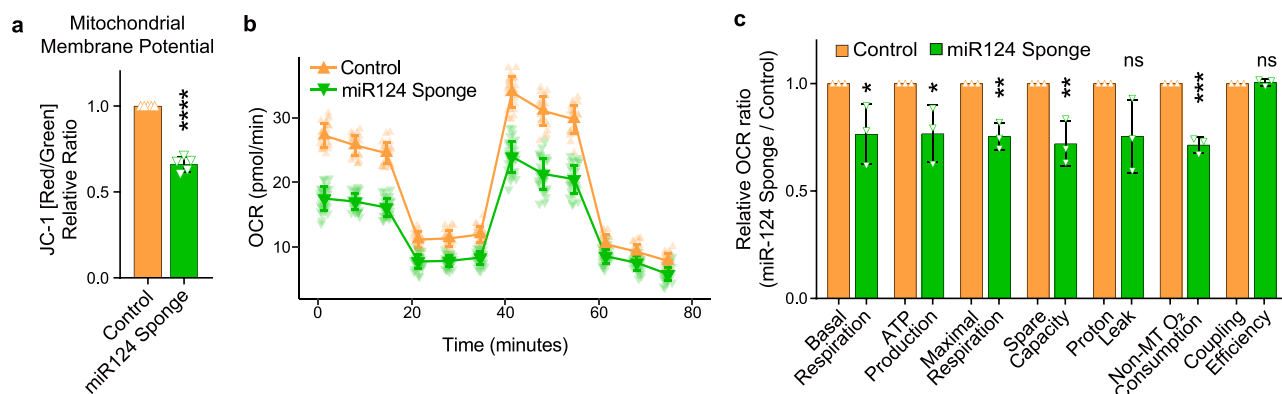
was OXPHOS, a major metabolic pathway that neurons utilize to produce energy (Fig. 3d). Interestingly, the annotated term relating to metabolism such as 'metabolic pathways' was also identified significantly (Fig. 3d).

To validate the results from the proteome analyses, in view of metabolic changes by miR-124 KD, the MMPs of miR-124-depleted neurons were measured. Neurons were incubated with JC-1 dyes, which turn red in hyperpolarized mitochondria with high MMP and green in depolarized mitochondria with low MMP. The red-to-green ratio of the JC-1 dyes in neurons, which is measured by flow cytometry, showed a significant decrease in MMP in miR-124 KD neurons compared to the control neurons (Fig. 4a). This result indicates that mitochondria in miR-124 KD neurons are relatively depolarized compared to the control and implies that the mitochondrial function, more specifically the OXPHOS (as indicated by GO analysis) may be defective due to an improper formation of the proton gradient across the inner mitochondrial membrane.



**Fig. 3 | Proteomics analysis reveals an association of miR-124 with cellular metabolism.** **a** Scheme of a TMT LC-MS/MS proteomic analysis. **b** Hierarchical clustering of proteomic analysis results with Pearson correlation distance.

**c** Heatmap of 593 DEPs ( $-1.3 < FC < +1.3$ ) clustered with Pearson correlation distance. **d** Gene ontology analysis with KEGG pathway annotations using 593 DEPs used in panel 3c.



**Fig. 4 | Effects of miR-124 on the functional features of mitochondria on day-6 neurons.** **a** miR-124 sponge neurons show depolarized mitochondria, indicated by lower ratio of red-to-green JC-1 dyes representing lower MMP ( $n = 5$ , two-tailed unpaired t-test, \*\*\*\* $p$ -value  $< 0.0001$ , error bar = stdev). Gating strategy is provided in Supplemental Information. **b**, **c** Mitochondrial respiration of early neurons. Seahorse Cell Mito Stress test was performed using neurons differentiated for six days after miR-124 knockdown. **b** One representative result from three biological experiments is depicted. Cellular respiration is decreased in miR-124 KD neurons

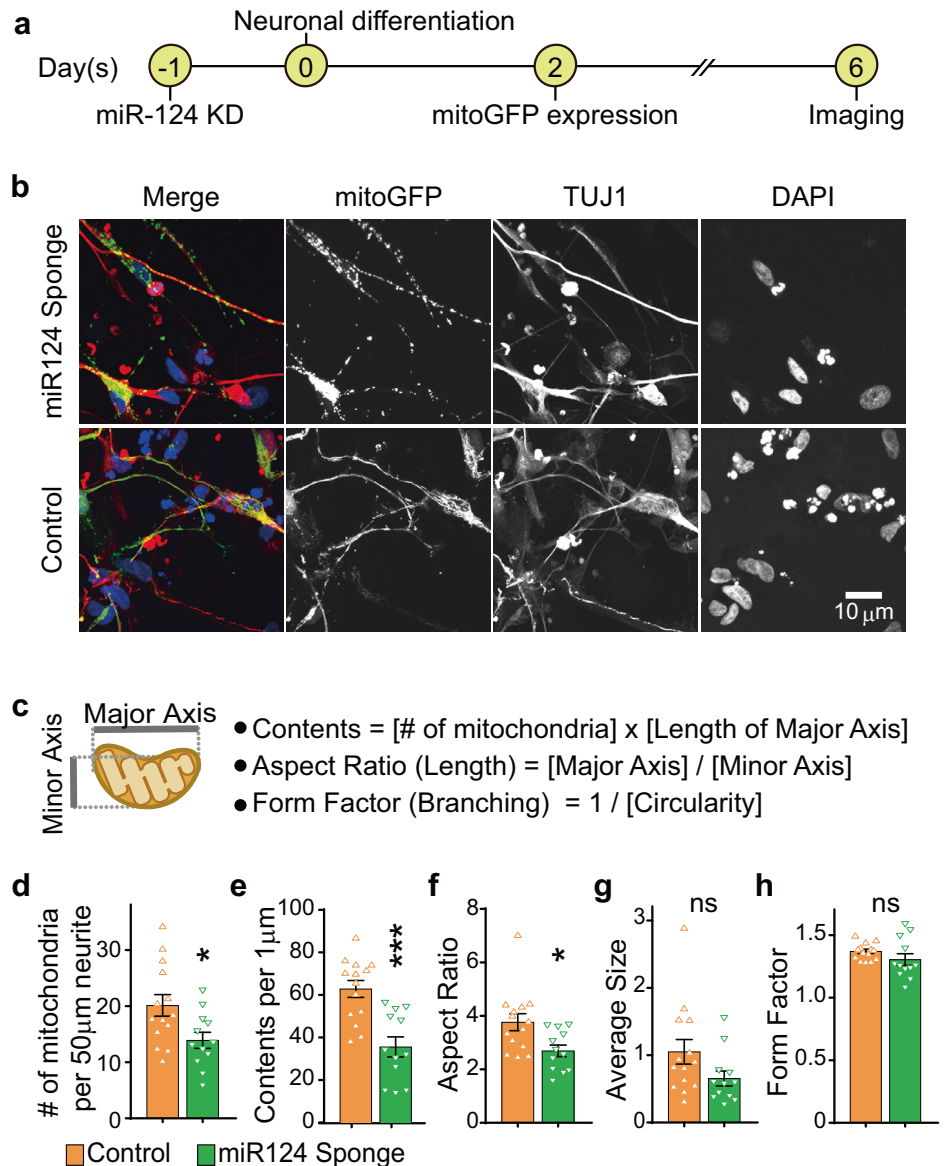
(27 wells for control, 33 wells for miR-124 KD, error bar = stdev). **c** The parameters of the Cell Mito Stress Test are presented as relative values between the control and miR-124 KD, allowing for the integration of results from all three biological experiments ( $n = 3$ , two-tailed unpaired t-test, \* $p$ -value (Basal) = 0.0428, \* $p$ -value (ATP) = 0.0379, \*\* $p$ -value (Maximal) = 0.0022, \*\* $p$ -value (Spare) = 0.0096,  $p$ -value (Proton) = 0.0661, \*\*\* $p$ -value (Non-MT) = 0.0002,  $p$ -value (Coupling) = 0.6320, ns = non-significant, error bar = stdev).

Next, the Cell Mito Stress Test was performed to further assess the functionality of OXPHOS in mitochondria (Fig. 4b). Interestingly, the initial oxygen consumption rate (OCR) measured in miR-124 KD neurons, indicative of a lower level of basal respiration (Fig. 4b, c). Next, following the treatment of mitochondrial inhibitors, an overall decrease in

OCR exhibiting a reduced level of cell respiration was detected for miR-124 KD neurons (Fig. 4b, c). Among the mitochondrial respiration parameters, coupling efficiencies comparing the ratio of the ATP synthase-coupled OCR to the basal OCR were not different between miR-124 KD and control neurons (Fig. 4c). This result, along with the reduced level of ATP synthase-

**Fig. 5 | Effects of miR-124 on the morphological features of mitochondria on day-6 neurons.**

**a** Timeline of experimental steps for analyzing mitochondrial morphology in day-6 neurons from control and miR-124 knockdown groups. **b** Representative images of mitochondria along neurites. **c–g** Details of analyzed parameters and quantification using particle analysis plugin in FIJI (Control = 14, Sponge = 12, two-tailed unpaired *t*-test, error bar = s.e.m.). **d** Number of mitochondria per 50  $\mu$ m neurite (\**p*-value = 0.0188). **e** Contents per 1  $\mu$ m (\*\**p*-value = 0.0002). **f** Aspect Ratio (\**p*-value = 0.0128). **g** Average Size (ns = non-significant, *p*-value = 0.0866). **h** Form Factor (ns = non-significant, *p*-value = 0.1770).



coupled OCR, can be used to deduce that the formation of proton gradient by the electron transport chain is dysregulated with reduced miR-124 during neurogenesis.

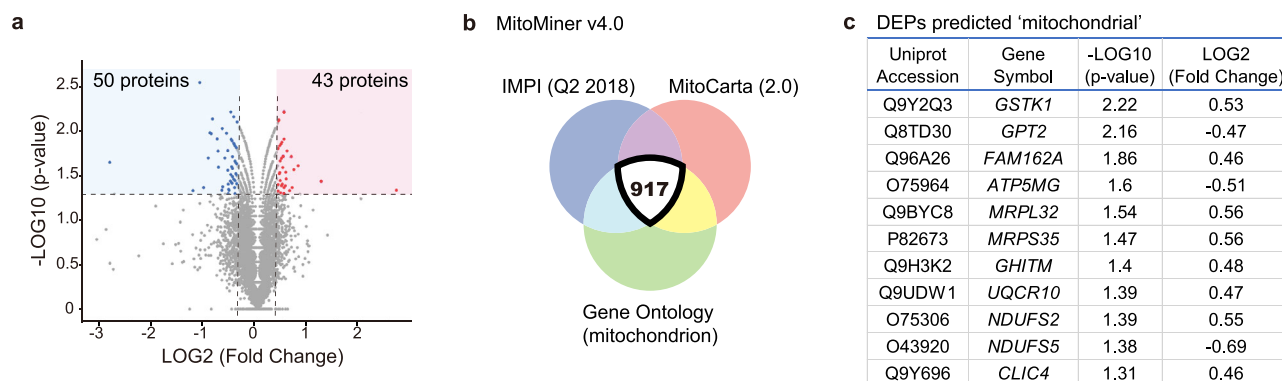
### Morphological changes of mitochondria in miR-124 KD neurons

To further validate the functional defects of mitochondria caused by miR-124 KD, we examined mitochondrial morphologies in TUJ1-positive immature neurons, differentiated for a week, because morphology and function are highly correlated and tightly regulated together<sup>24–29</sup> (Fig. 5). The mitochondrial morphologies were analyzed by expressing mitochondrial targeting sequence fused GFP (mitoGFP) in neurons differentiated for 6 days (Fig. 5a, b). The longer and shorter sides of the imaged mitochondria were first designated as the major and minor axes, respectively, then the lengths of both axes were measured (Fig. 5c). Afterwards, a number of mitochondria per 50  $\mu$ m neurites were counted to analyze the mitochondrial content (Fig. 5d, e). The number and content of mitochondria within the specified unit of neurite were both decreased in miR-124 KD neurons relative to control neurons (Fig. 5d, e). Next, the ratio of the major axis to the minor axis (aspect ratio) and the reciprocal of the value for circularity (form factor) were analyzed to demonstrate the length and branching of mitochondria (Fig. 5c). In miR-124 KD neurons, the aspect ratio of mitochondria

was significantly decreased, indicating shorter and rounder mitochondria (Fig. 5c, f). Although the average size and form factor of mitochondria were only comparable between miR-124 KD and control neurons, some decreasing trends in these parameters could be noted (Fig. 5c, g, and h). All of these data indicate that miR-124 KD impairs mitochondrial functions in developing neurons.

### Novel metabolic regulators in the initiating stages of neuronal differentiation

To identify novel players regulating mitochondrial metabolic function with miR-124 in the initial stages of neuronal differentiation, the list of DEPs was revisited. Among the 593 DEPs, 50 significantly down-regulated proteins and 43 significantly up-regulated proteins were selected for further investigation (Fig. 6a). These selected 93 proteins were juxtaposed with the list of annotated mitochondrial proteins from all databases (IMPI, MitoCarta 2.0, and GO) on MitoMiner v4.0 (Fig. 6b), and 11 DEPs were identified that may regulate cellular metabolism through mitochondria during neuronal development (Fig. 6c). Of the 11 DEPs, four proteins (ATP5MG for ATP synthase, UQCRI0 for complex III, and NDUF52 and NDUF55 for NADH dehydrogenase) are subunits of the OXPHOS protein complex. Two are mitochondrial ribosomal proteins (MRPL32 and MRPS35), which



**Fig. 6 | Proteomics analysis of miR-124 KD day-6 neurons.** **a** Volcano plot indicating statistically significant 43 upregulated proteins (red) and 50 downregulated proteins (blue) in miR-124 KD day-6 neurons ( $-1.3 < FC < +1.3$  and  $p\text{-value} < 0.05$ ). **b** Proteins

predicted to be 'mitochondrial' in all databases (IMPI, MitoCarta 2.0, and GO) of MitoMiner v4.0. **c** List of DEPs predicted to be 'mitochondrial'.

evidently affect the expression of OXPHOS subunit proteins produced from the mitochondrial genome. Since these six proteins are evidently involved in OXPHOS, we focused on the other five proteins - *GSTK1*, *GPT2*, *FAM162A*, *GHITM*, and *CLIC4*, to explore their function in metabolic shift during neuronal differentiation (Fig. 6c).

*GSTK1* was primarily examined as a potential metabolic regulator of neurogenesis since *GSTK1* was the most significantly altered protein. In addition, *GSTK1* was verified as a target of miR-124 through a luciferase assay (Fig. S3). First, the upregulation of *GSTK1* in miR-124 KD neurons from the proteomic findings was validated by performing a Western blot analysis (Fig. 7a, S5a). Next, we verified whether the increase of *GSTK1* affected cellular metabolism similar to miR-124 depletion during neurogenesis. *GSTK1* is a kappa family member of glutathione S transferase (GST)<sup>30,31</sup>, but the biological roles of *GSTK1* in neuronal differentiation have not yet been investigated.

In human NPCs and neurons, at least two different *GSTK1* isoforms are expressed among four transcript isoforms annotated in the RefSeq database (Fig. S4). Thus, *GSTK1* expression level was upregulated by applying a CRISPR activation (CRISPRa) system rather than introducing individual *GSTK1* cDNA produced from each isoform. Two different sgRNAs targeting different regions of the *GSTK1* promoter successfully increased *GSTK1* protein levels in NPCs (Fig. 7b). The effects of *GSTK1* upregulation by CRISPRa on mitochondrial cellular respiration were examined through the Cell Mito Stress Test in neurons and the decrease in OCR was also statistically significant (Fig. 7c,d). Overexpressing the *GSTK1* coding sequence (CDS) of the major isoform also led to a significant decrease in OCR of developing neurons (Fig. S5b–d). Both *GSTK1* upregulation and *GSTK1* CDS overexpression result in decreased OCR, which are comparable to the cell respirometry results observed with miR-124 KD (Fig. 4b, c).

## Discussion

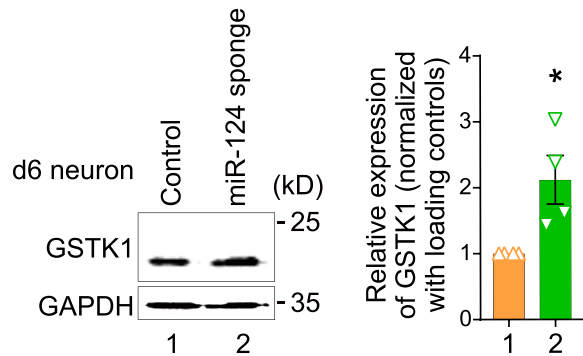
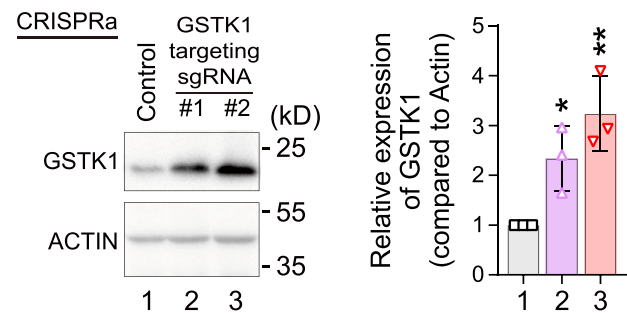
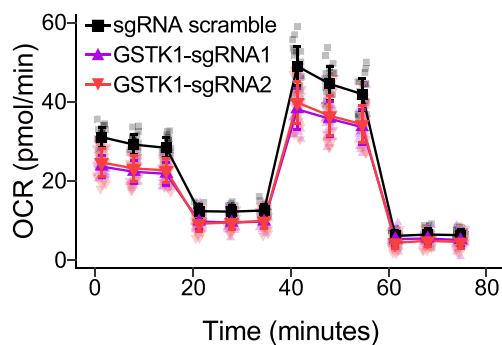
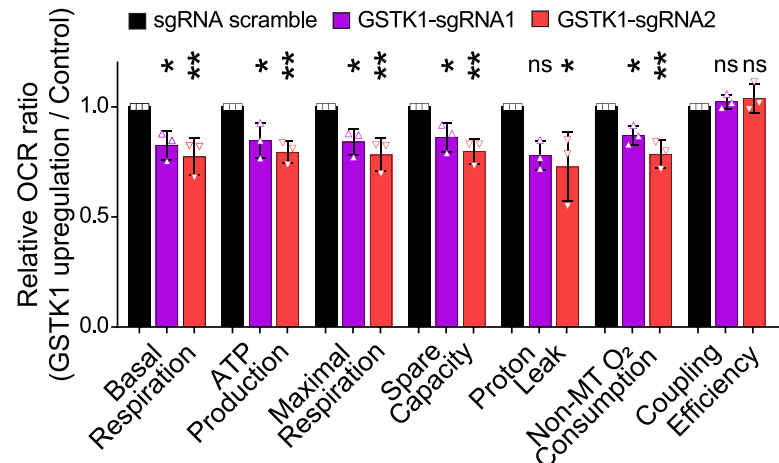
miR-124 expression is gradually increased upon neuronal differentiation of the NPCs. The major pool of mRNAs that miR-124 can target presumably differs depending on the developmental stages of neurons, given the substantial changes in the RNA transcriptome during this period<sup>32,33</sup>. Here, we explored biological processes regulated by miR-124 during early neurogenesis. By performing a proteome analysis after suppressing miR-124 in NPCs followed by an immediate neuronal differentiation, we found that miR-124 alters proteins involved in cellular metabolism, such as OXPHOS, in differentiating neurons (Fig. 3). Similar to these proteome results, depleting miR-124 in early differentiated neurons resulted in decreased cellular respiration and smaller, less mature mitochondria (Figs. 4, 5). Lastly, through regulating *GSTK1*, we showed the metabolism-regulating functions of the DEPs in differentiating neurons (Figs. 6, 7, S3, S5). Our data demonstrate that miR-124 is essential for the cooperating proteins required

for an effective metabolic shift in the early neuronal differentiation of human NPCs.

When neural stem cells differentiate into other neural cell types, energy metabolism is changed to support metabolic states of differentiated cells<sup>1</sup>. A few protein components required for the metabolic shift during neurogenesis have been reported<sup>2,3</sup>, but not all proteins are known. Our proteome results show novel candidates of metabolic regulators in the early stages of neurogenesis. We highlight five potential metabolic proteins that are altered in their expression level by miR-124: *GSTK1*, *GPT2*, *FAM162A*, *GHITM*, and *CLIC4*. Among the five proteins, only *GPT2*, working in gluconeogenesis and amino acid metabolism<sup>34</sup>, is known to be associated with brain developmental defects and neurodegenerative diseases in addition to metabolic dysfunctions<sup>35–41</sup>. *FAM162A*, *GHITM*, and *CLIC4* are proteins involved in apoptosis<sup>42–48</sup>, but further investigations will be needed to understand their metabolic function in brain development and diseases.

Here, we show that *GSTK1* suppresses mitochondrial OCRs for neurons (Fig. 7c, d). Unlike most GST family proteins localized in the cytosol, *GSTK1* is localized in mitochondria and has also been found in other organelles such as peroxisomes and endoplasmic reticulum (ER)<sup>30,31</sup>. Until now, the biological roles of *GSTK1* have been investigated in non-neuronal cells. *GSTK1* regulates adiponectin multimerization, modulates mitochondria-associated membrane integrity, and inhibits the release of mtDNA<sup>49–54</sup>. As neural cells do not express adiponectin, *GSTK1* is assumed to work mainly by regulating mitochondria in human neural cells. Although the precise functions of *GSTK1* and molecular mechanisms relating to mitochondrial respiration have yet to be identified, *GSTK1* deficiency is known to be associated with various pathologic conditions<sup>49–56</sup>. The polymorphisms on *GSTK1* identified in the human genome may be linked to brain disorders related to neural cell metabolism<sup>57</sup>.

Mitochondria are the key organelles regulating cellular metabolism. Perturbing mitochondrial changes affects cell fate determination during development because morphological and molecular features of mitochondria differ among cell types<sup>58,59</sup>. A mouse study showed that expressional change of a single neuron-enriched mitochondrial gene is sufficient to convert glial cells into neurons in vivo<sup>60</sup>. However, we did not observe cell fate changes in miR-124 KD neurons although miR-124 KD resulted in smaller and less mature mitochondria (Figs. S1a, 2d, 5). This outcome may be due to the limited differentiation potentials of human NPCs and mouse adult hippocampal NPCs used in our study, which are committed to differentiate into neuronal cells. Given these characteristics, the metabolic changes induced by miR-124 KD may not be substantial enough to lead to detectable alterations in cell fate. Furthermore, our method of miR-124 KD, utilizing a microRNA sponge in human NPCs and differentiating neurons, may not have been potent enough to significantly alter cellular metabolism to induce a cell fate switch. However, miR-124 KD might influence neuronal subtype differentiation; for instance, induced neurons (iNs) converted directly from induced pluripotent stem cells

**a** GSTK1 Western Blot (124 C vs KD)**b** GSTK1 upregulation by CRISPRa in NPCs**c** Mitochondrial Respiration (Neurons)**d**

**Fig. 7 | Upregulation of GSTK1 suppresses mitochondrial respiration.** **a** Protein expression of GSTK1 increased in miR-124 KD day-6 neurons, confirming proteomics analysis. GSTK1 was used for validation ( $n = 4$ , two-tailed unpaired t-test, normalized by loading controls, \* $p$ -value = 0.0118, error bar = s.e.m.). **b** Using CRISPRa (dCas9-VPR) to upregulate GSTK1 in NPCs. Western blot analysis showing successful upregulation of GSTK1 ( $n = 3$ , two-tailed unpaired t-test, \* $p$ -value = 0.0243, \*\* $p$ -value = 0.0068, error bar = stdev). **c, d** Seahorse Cell Mito Stress test of day-6 neurons after upregulating GSTK1 via CRISPRa. **c** Three biological experiments were performed and one representative experimental result is depicted. Cellular respiration decreased in GSTK1 upregulated neurons (22 wells for sgRNA scramble, 25 wells for GSTK1-sgRNA1, 25 wells for GSTK1-sgRNA2, error bar =

stdev). **d** The parameters of the Cell Mito Stress Test are presented as relative values among control and GSTK1 upregulated neurons, allowing for the integration of results from all three biological experiments ( $n = 3$ , one-way ANOVA, Dunnett's multiple comparisons test, \*adj  $p$ -value (Basal) = 0.0221, \*\*adj  $p$ -value (Basal) = 0.0068, \*adj  $p$ -value (ATP) = 0.0256, \*\*adj  $p$ -value (ATP) = 0.0070, \*adj  $p$ -value (Maximal) = 0.0218, \*\*adj  $p$ -value (Maximal) = 0.0053, \*adj  $p$ -value (Spare) = 0.0257, \*\*adj  $p$ -value (Spare) = 0.0046, adj  $p$ -value (Proton) = 0.0588, \*adj  $p$ -value (Proton) = 0.0266, \*adj  $p$ -value (Non-MT) = 0.0203, \*\*adj  $p$ -value (Non-MT) = 0.0018, adj  $p$ -value (Coupling - sgRNA1) = 0.7291, adj  $p$ -value (Coupling - sgRNA2) = 0.4564, ns = non-significant, error bar = stdev).

(iPSCs) typically express genes of the amygdaloid complex, whereas iNs from miR-124 knockout iPSCs express genes of the striatal neurons along with differential metabolism<sup>15</sup>. Considering the varied metabolic rates across different brain regions, further exploration into the impact of miR-124 on neuronal subtype differentiation could be critical<sup>61–64</sup>.

Our data show that the effects of miR-124 KD on neurites seem to be greater in mouse adult hippocampal neurogenesis than in human neurogenesis in vitro (Fig. 1, 2). Effects of the differentiation factors added to culture hESC-derived neurons in vitro might be very powerful that the effects of miR-124 KD may be counteracted, perturbing neuronal development. Alternatively, different phenotypic outcomes can be caused by species specificity. The biological roles of miR-124 in a metabolic shift during mouse neurogenesis are potentially conserved as miR-124 and metabolic reprogramming in neuronal differentiation are necessary for both mouse and human<sup>2,3,65,66</sup>. However, the temporal regulation of metabolism is thought to regulate the timing of species-specific neuronal development<sup>6–8,67–71</sup>. In addition, the protein composition and abundance of proteins in the brain differ among species<sup>72</sup>. Thus, miR-124 and DEPs

affected by miR-124 KD may have different impacts on metabolic changes in the neurogenesis of human and mouse. Modeling Leigh syndrome using human brain organoids and mice is a good example showing the species specificity<sup>73</sup>. A human brain organoid model with a *SURF1* mutation successfully recapitulated the phenotypes observed in human Leigh syndrome patients, whereas modeling Leigh syndrome using mouse models has not been successful<sup>73</sup>.

Although we focused on the metabolic regulation mediated by miR-124 during neuronal differentiation in this study, it is noteworthy that half of the terms in the KEGG pathway results suggest a link between miR-124 and neurodegeneration (Fig. 3d). Consistent with previous research demonstrating an association between miR-124 and neurodegenerative diseases<sup>74–84</sup>, our proteome data indicate that miR-124 influences DEPs associated with Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease. Intriguingly, 11 out of 15 proteins within the OXPHOS pathway in KEGG that showed significant differential expression are also implicated in all these neurodegenerative diseases. This overlap underscores the potential of miR-124 to modulate key metabolic

functions commonly disrupted in these disorders. As more studies suggest metabolic dysfunction as a primary cause of these conditions, further investigation is necessary to determine whether metabolic proteins regulated by miR-124 during early neurogenesis contribute to neurodegenerative diseases<sup>80,85–89</sup>. A long-term suppression of miR-124 in adult hippocampal neurons of mice led to alterations in OXPHOS gene expression<sup>90</sup>. It would be interesting to compare these OXPHOS genes that we found in the early stages of human neuronal differentiation with those from the mouse study. Analyzing the OXPHOS genes regulated by miR-124 in both early-stage neurogenesis and mature neurons could help identify key molecules involved in neurodegenerative diseases. Notably, modulating miR-124 in animal models of neurodegeneration have shown promising reductions in disease symptoms, reinforcing its potential as a therapeutic molecule. These findings collectively suggest that miR-124 could significantly contribute to the development of new treatments by targeting metabolic dysfunctions integral to neurodegenerative processes.

## Methods

### Cell culture

HEK293T cells were grown in DMEM (Welgene LM001-07) supplemented with 10% fetal bovine serum (FBS; Young-in Frontier US-FBS-500, Gibco 12483-020). HuES6 (Harvard Stem Cell Institute iPS Core) hESCs were maintained on mitotically inactivated mouse embryonic fibroblasts (MEFs) in hESC media: 1% MEM-NEAA (Gibco 11140050), 1.2 mg/ml sodium bicarbonate (Sigma S5761), 100  $\mu$ M 2-mercaptoethanol (Merck M3148), 146.14  $\mu$ g/ml L-glutamine (Sigma G8541), and NaOH (Intron Biotechnology IBS-BS017) to pH 7.4 containing DMEM/F12 (Gibco 12400024) with 20% KnockOut Serum Replacement (Gibco 10828028) and 10 ng/ml FGF2 (R&D systems 4114-TC-01M). hESCs were detached with Collagenase IV (10  $\mu$ g/ml, Gibco 17104019) and transferred to non-attaching 90 mm Petri dishes (SPL 10090) for embryoid body (EB) formations via dual-SMAD inhibition with LDN-193189 (0.1  $\mu$ M, Selleckchem S2618) and SB-431542 (10  $\mu$ M, Cayman CAY-13031) in DMEM/F12+Glutamax (Gibco 10565042) media with N2 (Gibco 17502048) and B27 (Gibco 12587010) supplements. After a week, EBs are attached to 10  $\mu$ g/ml poly-L-ornithine (PLO) (Sigma P3655) and 5  $\mu$ g/ml Natural mouse Laminin (Lam) (Invitrogen 23017-015) coated surfaces for neural rosette formations. Depending on size, attachment, and morphology, parts of neural rosettes are detached by pipetting up and collected together to be dissociated into single cells with Accutase (Innovative Cell Technologies AT104). Dissociated cells are plated onto PLO/Lam coated dishes and grown in FGF2 (20 ng/ml, R&D systems 4114-TC-01M) added DMEM/F12+Glutamax media with N2 and B27 supplements for NPC derivation and proliferation cultures. For neuronal differentiation, BDNF (20 ng/ml, Peprotech 450-02), GDNF (20 ng/ml, Peprotech 450-10), dibutyryl cAMP (500 ng/ml; Tocris 1141, Sigma D0627, Selleckchem S7858), ascorbic acid (200 nM, Sigma A4544), and Lam (1  $\mu$ g/ml) were added to DMEM/F12+Glutamax media supplemented with N2 and B27. All cells were maintained in 37 °C, 5% CO<sub>2</sub> incubator. Protocols describing the use of human ESCs were approved in accordance with the ethical requirements and regulations of the Institutional Review Board of KAIST (IRB # KH2021-069).

### MEF isolation and mitotic inactivation for use as a feeder cell layer

To isolate MEFs, a pregnant female E13.5 CF-1 (CrI: CF1) mouse was obtained from LARRC of KRIBB and euthanized before dissecting the uterus out to disengage embryos. Head and red tissues of the embryo were removed and the remaining tissue was fully dissociated into single cells with 0.25% Trypsin/EDTA (Welgene LS015-10). Dissociated MEFs are grown MEF media: 1% MEM-NEAA, 100  $\mu$ M 2-mercaptoethanol, and 15% FBS added DMEM. MEF are mitotically inactivated with 10  $\mu$ g/ml mitomycin C (AG Scientific M-1108) for 2 hours and cryopreserved for use as a feeder cell layer. Mitotically inactive MEFs are plated on 0.1% gelatin (Sigma G1890) coated surfaces a day before plating hESCs. Isolation and use of MEFs were

performed in accordance with guidelines and protocols approved by the Institutional Animal Care and Use Committees of KAIST (KA2020-37).

### Plasmids and oligonucleotides

To generate the control and miR-124 sponge, oligonucleotides including miR-124 binding sites were annealed as dsDNA and cloned to lenti- or retroviral vectors containing sequences of RFP or *Renilla* luciferase. For overexpression of miR-124, rat pri-miR-124 was amplified using forward primer 5'-TCCCATCCTTCCTTCTCCGATC-3' and reverse primer 5'-GTTGTGCTGAGTCGATCCTTCCG-3' and subcloned into pcDNA3.1. For mitoGFP expression in neurons using lentivirus (LV), mitoGFP from pAcGFP-1 Mito (Clontech 632432) was subcloned into a lentiviral construct. For CRISPRa of GSK1, two single guide RNA (sgRNA) sequences were designed using CRISPIK (BROAD institute): sgRNA1 - 5'-CCGGAGGTGGACCTCTACCA-3' and sgRNA2 - 5'-GAGGTCCACCTCCGGCGCAG-3'. For the control, previously reported scrambled sgRNA sequences were used<sup>91</sup>. Annealed sgRNA oligonucleotides were ligated into BsmBI digested Lentiguide Puro (Addgene 52963) vector, resulting in the production of Lentiguide puro-scrambled sgRNA, -GSK1 sgRNA1, and -GSK1 sgRNA2. To produce a lentiviral construct expressing dCas9-VPR and a blasticidin resistance gene (BSD), dCas9-VPR (Addgene 63798) was subcloned first into the lentiviral construct having a CAG promoter; then P2A-BSD (Addgene 52962) was subcloned, resulting in the production of pBOB-CAG-dCas9-VPR-P2A-BSD. For the luciferase assay, 3' UTR of *GSK1* was amplified by PCR using cDNA from human neurons and ligated into a pGEM T easy vector (Promega A1360) first, then subcloned to a firefly luciferase construct expressed by a CAG promoter. To amplify the 3' UTR of *GSK1*, the following primers were used: 5'-AATAATTCTA-GACTCGAGCCGTGAATGCCAGACTTTAA-3' and 5'-TGGAATTCTGAGATATCTACAGACACAGAAATGTTTT-3'. To generate a firefly luciferase plasmid with a mutated miR-124 binding site in the 3' UTR of *GSK1*, synthesized oligonucleotides (Bionics) were subcloned into the same backbone, which expresses firefly luciferase under a CAG promoter. For the detection of expressed *GSK1* transcript variants in human NPCs and neurons, the coding sequence (CDS) of *GSK1* was amplified by PCR using the following primers: GSK1-CDS-F 5'-ACTGCTCTTCCGGAGCCT-3' and GSK1-CDS-R 5'-TTAAAGTCTGGCATTAC-3'. To overexpress *GSK1* CDS, the major isoform was PCR-amplified using GSK1-CDS-F and -R primers, then subcloned into a lentiviral vector expressing the transgene and an IRES-puromycin resistance gene (PuroR) under a CAG promoter, resulting in the production of pBOB-CAG-GSK1 CDS-IRES-PuroR.

### PEI transfection for transient protein expression and virus production

Polyethylenimine (PEI; Polysciences 23966) was used to deliver plasmids into HEK293T. Cells were plated a day before transfection, then the media was changed four hours post-transfection. For transient transfections, 2  $\mu$ g of plasmid DNA were delivered to  $5 \times 10^5$  cells of HEK293T. Cells were harvested two to three days after transfection depending on experimental purposes. For retrovirus (RV) production, RV plasmid of interest and RV packaging plasmids (VSVG, Gag-Pol) were transfected. For LV production, LV plasmid of interest and LV packaging plasmids (MDL, RSV-Rev, and VSVG) were transfected. Culture media for RV production were harvested on days two and three post-transfection, and for LV production were harvested on day three. Both RV and LV were concentrated by ultracentrifugation, with RV undergoing an additional ultracentrifugation step before animal brain injections.

### Preparation of NPCs expressing dCas9-VPR

To upregulate *GSK1* using CRISPRa, NPCs expressing dCas9-VPR were produced. For this, NPCs were transduced with LVs produced using the plasmid pBOB-CAG-dCas9-VPR-P2A-BSD (MOI = ~1) and selected with 10  $\mu$ g/ml blasticidin (invivogen ant-bl-05) for five days. NPCs that survived

after blasticidin selection were either used directly for experiments or cryopreserved for future uses.

### Animals and stereotaxic injection

Retrovirus was delivered to the brain of six- to seven-week-old female C57BL/6 mice as described before<sup>21</sup>. Briefly, 1–1.5 µl of virus at  $1-5 \times 10^8$ /ml was infused into the hemisphere using the following coordinates in reference from Bregma:  $-2$  a/p,  $\pm 1.5$  m/l, and  $-2.3$  d/v for the dentate gyrus. Mice were sacrificed three weeks post-injection of retrovirus for immunohistochemistry. All animal procedures were performed in accordance with guidelines and protocols approved by the Institutional Animal Care and Use Committees of KAIST (KA2023-144-v1) and the Salk Institute for Biological Studies. We have complied with all relevant ethical regulations for animal use.

### Immunocytochemistry

hESC-derived NPCs and neurons were fixed for 10–30 minutes at room temperature (RT) with 4% paraformaldehyde (Pierce 28906). After a single rinse and three washes with PBS-T (0.1% Tween-20 in PBS), cells were permeabilized with 0.1% Triton X-100 (Promega H5141) in PBS for 10–30 minutes at RT. Then, the permeabilized cells were blocked for one hour at RT using 5% normal donkey serum (Abcam AB7475) in PBS-T. After blocking, cells were incubated overnight at 4 °C with primary antibodies diluted 1:1000 in 5% normal donkey serum. Next, cells were rinsed once and washed three times with PBS-T (0.1% Tween-20 in PBS), then incubated with 1:1000 diluted secondary antibodies for 1 hour at RT. Finally, cells were incubated with DAPI (1 µg/ml, Sigma D9542) for 5 minutes at RT and mounted on slides with fluorescence mounting medium (DAKO S3023). Primary antibodies anti-NESTIN (Millipore MAB5326) and anti-SOX2 (Cell signaling 3579S) were used to validate human NPCs, when anti-TUJ1 (Abcam ab52623) and anti-MAP2 (Abcam ab11267) were used to validate human neurons.

### Immunohistochemistry

Mouse brains were dissected after fixation by cardiac perfusion using 4% paraformaldehyde (PFA) at 4 °C, followed by overnight post-fixation with 4% PFA at 4 °C. Post-fixed brains were transferred to 30% sucrose solution and stored at 4 °C until brains sank. Then, brains were embedded in O.C.T. compound (Sakura 4583) and frozen at  $-80$  °C. Once frozen, the embedded brains were cryo-sectioned into 40 µm slices, immediately immersed in tissue-collecting solution (50% glycerol in PBS), and stored at  $-20$  °C until used for immunostaining. Free-floating slices were washed with PBS to remove residual tissue-collecting solution and incubated for an hour at RT with a blocking buffer (3% Normal Donkey Serum or Bovine Serum Albumin with 0.3% Triton X-100 in PBS) for permeabilization and blocking. Primary antibodies were incubated overnight at 4 °C in the blocking buffer. Primary antibodies against GFP (1:1000, Aves labs GFP-1020), TUJ1 (1:100, Abcam ab52623), PROX1 (1:200, ReliaTech 102-PA32), SOX2 (1:400, SCBT sc-365823), OLIG2 (1:100, Abcam ab109186), GFAP (1:4000, Dako Z0334), and IBA1 (1:200, Wako 019-19741) were used. Subsequently, secondary antibodies (1:1000; Abcam ab150169, ab150107, ab150075) and DAPI (1 µg/ml, Sigma D9542) were applied for staining before mounting onto glass slides with coverslips for imaging.

### Neurite analysis

For analyzing dendrites of adult mouse-born neurons, images were acquired with the 20× objective using confocal microscopes (Zeiss LSM780 and LSM980) and reconstructed using the Neurolucida software (MBF Bioscience). A two-tailed unpaired t-test and two-way ANOVA with Bonferroni's multiple comparisons test were used to compare the quantification results between the two groups. For analyzing neurites of human neurons cultured in vitro, NPCs infected with either control or miR-124 sponge LVs were co-infected with GFP-expressing LVs and differentiated into neurons. Neurites were imaged with a confocal microscope (Zeiss LSM800) and

processed with Zen Blue and Black (Zeiss) software. Neurite tracing and quantification were conducted using a FIJI plugin – NeuronJ. Traced neurons were analyzed and visualized with R<sup>92</sup> and GraphPad Prism.

### Microelectrode array (MEA)

A CytoView MEA 96-White plate (Axion Biosystems M768-tMEA-96W-5) with or without pre-coating of 0.1% PEI was coated with 50 µg/ml Poly L-ornithine and 5 µg/ml Laminin before plating 13,000 NPCs per well. On the following day, control and miR-124 sponge LVs were infected. The next day, LV-infected NPCs were differentiated into neurons. Maestro Pro (Axion Biosystems) was utilized for MEA measurements. A neural metric tool (Axion Biosystems) and Excel (Microsoft) were utilized for quantification and obtaining the raster plot. GraphPad Prism was used for bar graphs.

### RNA extraction, cDNA synthesis, and Real-Time PCR

Total RNA was extracted with TRIzol (Invitrogen 15596018), chloroform (Junsei 28560-0350), isopropanol (Merck 109634.1011), and ethanol (Merck 100983.1011) under RNase-free conditions. cDNA was synthesized with RevertAid Reverse Transcriptase (Thermo Scientific EP0442) using Oligo d(T)20 primers. Real-Time PCR was performed with a CFX96 Real-Time PCR Detection System with Power SYBR Green PCR Master Mix (Invitrogen 4368706). The following primers were used for the analysis: human *DCX* (5'-CGAAGCAAGTCTCCAGCTGACTC-3' and 5'-AGGACTGGTGGGCGTAGAGATG-3'), human *MAP2* (5'-TGCTGAGGCTGTAGCAGTCCT-3' and 5'-TGGAGAAGGAGGCA-GATTAGCTG-3'), human *ACTB* (5'-GCCAACC GCGAGAAGATGAC-3' and 5'-GAGGCGTACAGGGATAGCACAG-3'), and human *GAPDH* (5'-CCACTCCTCCACCTTTGAC-3' and 5'-CACCCTGTTGCTGTAGCCA-3'). For mature miRNA quantification, Taqman microRNA assay was performed following the manufacturer's instructions (Applied Biosystems 4427975, 4366597, 4440040).

### Dual luciferase assay

Cells were plated one day before transfection. Luciferase activity was measured using a Dual-Luciferase Reporter System (Promega E1980) two days after transfection, following the manufacturer's instructions. For miR-124 sponge test, *Renilla* luciferase plasmid containing the miR-124 sponge or control was transfected with firefly luciferase plasmid in 9:1 (w/w) ratio to HEK293T. Firefly activity was used for normalization for the miR-124 sponge test. For the test of a miR-124 binding motif on *GSTK1* 3' UTR, firefly luciferase plasmid containing the wildtype *GSTK1* 3' UTR, *GSTK1* 3' UTR with a mutated miR-124 binding site, or a null control was transfected with *Renilla* luciferase plasmid in 3:1 (w/w) ratio to NPCs. Here, *Renilla* luciferase activity was used for normalization. Luciferase activities of firefly with or without miR-124 were quantified compared to the control.

### Western blot analysis

Cells were rinsed once with Dulbecco's phosphate-buffered saline (DPBS), detached, and centrifuged at 6000 rpm for 5 minutes at 4 °C. The supernatant was discarded, and the cell pellet was lysed using RIPA buffer (Biosesang R2002). After centrifuging the lysates at 20,000 × g for 15 minutes at 4 °C, protein concentrations were measured using a BCA protein assay kit (Pierce 23225) following the manufacturer's instructions. Proteins separated via SDS-PAGE were transferred to nitrocellulose (NC) membranes (Amersham 10600002). SDS-PAGE and membrane transfer were performed with a Surecast gel electrophoresis kit (Invitrogen HC1000SR). *GSTK1* was detected using anti-*GSTK1* antibody (1:1000, SCBT sc-515580) and HRP-linked anti-mouse IgG (1:5000, Cell signaling 7076S). Actin-HRP (1:1000, SCBT sc-1615) or *GAPDH*-HRP (1:1000, SCBT sc-47724) were used to detect loading controls. Then, bands for each protein were imaged with enhanced chemiluminescence (WBKLS0500) using Chemidoc XRS+ (Biorad) and iBright (Invitrogen) imaging systems.

## Preparation of samples for a quantitative mass spectrometry (MS) analysis

To analyze the total proteome of day-6 neurons after either control or miR-124 sponge LVs, one PLO/Lam-coated 35 mm cell culture dish was plated with  $5 \times 10^5$  NPCs per experiment, a day before infection. A day after infection, infected NPCs were differentiated into neurons for six days. Cell pellets were obtained by rinsing once with DPBS, then carefully detached in DPBS before being centrifuged at 6000 rpm for 5 minutes, when the supernatant was discarded and only the cell pellet was frozen until preparation of samples for LC-MS/MS. Cell pellets were then resuspended with 8 M urea (Sigma U5378) in 50 mM ammonium bicarbonate (ABC) (Sigma 09830) buffer (pH 8.5). Protein quantities were determined using a BCA protein assay kit (Thermo Scientific 23227). Samples were first reduced by 10 mM DTT (Sigma 43815) for 1 hour at 37 °C followed by alkylation with 40 mM iodoacetamide (Sigma I1149) for 1 hour at 37 °C in the dark. The samples were diluted with 50 mM ABC to make the final concentration of urea to be less than 1 M. Then, trypsin protease (Thermo Scientific 90057) was added to 2% (w/w) and incubated at 37 °C overnight. The samples were acidified by 0.4% TFA (Sigma 80457) and desalted by a C18 SPE cartridge (Sigma 52601-U). After desalting, peptide samples were reconstituted with 50 mM HEPES (pH 7.5) buffer (Sigma 54457), and peptide amount was obtained based on BCA protein assay and equal amount of peptides were labeled with TMTpro 16plex label reagents (Thermo Scientific A44521) according to the manufacturer's protocol. TMT labeled samples were combined and desalted by a C18 SPE cartridge (Sigma 52601-U). The cleaned-up sample was dried and reconstituted in 10 mM ABC buffer. Fractionation was done with the micro-scale fractionation system as previously described<sup>93</sup>. High-pH reverse phase fractionation was performed using a capillary column, 320  $\mu$ m inner diameter (i.d.)  $\times$  55 cm in-house packed with Jupiter 3  $\mu$ m C18 beads (Phenomenex 04A-4263). A linear gradient of solvent A (10 mM ABC in water) and solvent B (10 mM ABC in 90% acetonitrile (ACN; Sigma 100029)) was used for the fractionation; 2% solvent B isocratic for initial 14 minutes, 2–10% solvent B for following 2 minutes, 10–40% solvent B for next 100 minutes. The fractionated samples were automatically concatenated into 24 fractions which were dried and reconstituted with 25 mM ABC buffer for LC-MS/MS analysis.

## LC-MS/MS analysis

Orbitrap Fusion Eclipse (Thermo Scientific) coupled to the nanoAcquity UPLC system (Waters) was utilized for peptide separation and mass spectrometry analysis. In-house packed, with Jupiter 3  $\mu$ m C18 beads (Phenomenex 04A-4263), analytical capillary column (100 cm  $\times$  75  $\mu$ m i.d.) and the trap column (3 cm  $\times$  150  $\mu$ m i.d.) were utilized for liquid chromatography. The analytical capillary column was heated at 45 °C with the column heater (Analytical Sales and Services H-2004C) during the peptide separation. UPLC system was operated at 300 nL/min flow rate. A linear gradient of solvent A (H<sub>2</sub>O (Sigma 1153334000) with 0.1% formic acid (Thermo Scientific 28905)) and solvent B (100% ACN with 0.1% formic acid); solvent B was raised from 5–10% for the initial 10 minutes, 10–40% solvent B for the next 200 minutes. Total runtime of SPS-MS3 was 220 minutes and following setup was used for MS acquisition; MS1 was acquired with 60 K resolution, 400 K AGC target value, 30% RF and maximum injection time was set at auto; MS2 was acquired with 15 K resolution, 50 K AGC target value, HCD collision energy of 30% and maximum injection time set at auto; MS3 was acquired with 30 K orbitrap resolution, 250 K AGC target value, HCD collision energy of 55% and 54 ms maximum injection time. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>94</sup> partner repository with the dataset identifier PXD042371.

## Proteomics data analysis

Proteome Discoverer (PD) platform, version 2.1 (Thermo Fisher Scientific), was used for the protein identification and quantitative analysis using UniProt human reference proteome UP000005640 (last modified on

November 25, 2020) based on the default processing workflow for TMTpro SPS MS3 analysis and consensus workflow for MS3 reporter quantification. Protein identification and TMT reporter ion intensity values obtained from the PD search were utilized for all following analyses. The TMT reporter ion intensity values of the control and miR-124 sponge neurons were first each used to cluster with Pearson correlation distance using a plot\_heatmap in R. Then, the fold change of the identified proteins in the same biological group was calculated by dividing the TMT reporter ion intensity values of miR-124 sponge neurons by those of control neurons using Excel (Microsoft). The list of proteins with Log2(FC) > 0.4 and Log2(FC) < -0.4 were used to depict a heatmap with flaski (<https://flaski.age.mpg.de/>) that clustered DEPs depending on the experimental variable – miR-124 function. This list was used to perform the Database for Annotation, Visualization and Integrated Discovery (DAVID) gene ontology (GO) analysis with DAVID Knowledgebase v2022q2. Identified GO terms under KEGG pathway with statistical significance of  $p$ -value < 0.05 were obtained. To further examine OXPHOS- or metabolism-related proteins, the fold change of DEPs used for DAVID GO analysis was imported into Perseus software to narrow down the list into DEPs with statistical significance of  $p$ -value < 0.05 and to draw a volcano plot showing the statistically significant up(red)- and down(blue)-regulated DEPs. The volcano plot was further processed with Adobe Illustrator. The 93 identified statistically significant DEPs were juxtaposed to commonly predicted mitochondrial proteins in MitoMiner v4.0 (<http://mitominer.mrc-mbu.cam.ac.uk/>) - a database that summarizes IMPI (Q2 2018), MitoCarta (2.0), and Gene Ontology (mitochondrion), resulting in 11 proteins.

## Mitochondrial membrane potential (MMP) analysis

MMPs of miR-124-depleted neurons were measured through the JC-1 assay. Neurons were incubated with JC-1 dyes (Molecular Probes) at 37 °C for 15–30 minutes. The cells dissociated using Accutase were resuspended in warm PBS. Red and green fluorescence of JC-1 dyes were measured with flow cytometry (BD FACS CantoII). Histogram plots of red and green fluorescence were generated using FlowJo 10 (TreeStar) and the red-to-green ratio was then calculated in Microsoft Excel. Bar graph drawn with GraphPad Prism. Gating strategy is provided in Supplementary Information.

## Cell respirometry–Seahorse Mitostress Test

For measuring cell respirometry of neurons, 6000 NPCs (for miR-124 experiments) and 9000 NPCs (for GSKT1 experiments) were seeded onto each well of the Seahorse XFe 96 well plate (Agilent 101085-004) coated with PLO/Lam. For GSKT1 upregulation via CRISPRa, NPCs expressing dCas9-VPR were plated. A day after seeding NPCs, LVs (miR-124 sponge and relative control, GSKT1 CDS and relative control, and sgRNAs-GSKT1 1,2 and scrambled) were added to the culture media to manipulate gene expression (MOI ~1.2–1.5). On the following day, NPCs were differentiated into neurons for six days. For GSKT1 experiments, LV-infected cells were selected by treating 1  $\mu$ g/ml of puromycin (Gibco A1113803) a day after lentivirus infection. The OCR was measured by following the default Mito Stress Test protocol provided by the manufacturer (Agilent 102416-100). For the assay medium, 0.7 ml of 1 M D-glucose, 0.2 ml of 100 mM sodium pyruvate, and 0.5 ml of 200 mM L-glutamine were added to 40 ml of customized DMEM (Wegene LM001-228) (pH 7.4, 5 mM HEPES, no D-glucose, no sodium bicarbonate, 143 mM NaCl, 5.4 mM KCl) for final concentrations of 17.5 mM D-glucose, 0.5 mM sodium pyruvate, and 2.5 mM L-glutamine (same concentration with DMEM/F-12+Glutamax media). For the OCR measurement, 1.5  $\mu$ M oligomycin (Sigma 75351), 1  $\mu$ M FCCP (Sigma C2920), 0.5  $\mu$ M rotenone (Millipore 557368), and 0.5  $\mu$ M antimycin A (Sigma A8674) were treated. Depending on the experiment, 15–46 wells of XF96 Cell Culture Microplate were used for measurement. The Wave software was used to analyze the results. Experiments were performed three times independently. Assay results were analyzed and visualized with R and GraphPad Prism.

## Mitochondrial morphology

Control and miR-124 sponge LVs were used to infect NPCs one day after plating. Then, NPCs were differentiated into neurons for 6 days. Each group was infected with mitoGFP LVs on neuronal differentiation day 2. Neurons were fixed and stained with anti-TUJ1 (1:500, Covance PRB-435P) and DAPI (1 µg/ml, Sigma D9542). Neurons were imaged with a confocal microscope (Zeiss LSM 780) at 63X. Images were analyzed and exported using Zen Blue (Zeiss). The particle analysis feature in ImageJ was used to quantify the content, number, aspect ratio, average size, and form factor. Bar graphs and statistic results were obtained from GraphPad Prism.

## Statistics and reproducibility

Information on the number of experimental replicates and which statistical analyses were used for computing *p*-values are described in the main text, figure legends, and/or Methods. All data presented were reproducible with biological replicates referring to fully repeated experiments. As described in figure legends, data are shown with error bars representing mean ± s.e.m. or mean ± stdev. *p*-values less than 0.05 (*p* < 0.05) were determined statistically significant. Statistical analyses were performed with R or GraphPad Prism 7.04.

## Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

## Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD042371. All other data that support the findings of this study are included within this manuscript and its Supplementary Information files. Supplementary Tables are provided as Supplementary Data 1. The source data for all figures of this study are provided in Supplementary Data 2.

Received: 28 July 2023; Accepted: 16 October 2024;

Published online: 25 October 2024

## References

- Son, G. & Han, J. Roles of mitochondria in neuronal development. *BMB Rep.* **51**, 549–556 (2018).
- Agostini, M. et al. Metabolic reprogramming during neuronal differentiation. *Cell Death Differ.* **23**, 1502–1514 (2016).
- Zheng, X. et al. Metabolic reprogramming during neuronal differentiation from aerobic glycolysis to neuronal oxidative phosphorylation. *eLife* **5**, 1–25 (2016).
- Zhu, X.-H. et al. Quantitative imaging of energy expenditure in human brain. *NeuroImage* **60**, 2107–2117 (2012).
- Iwata, R., Casimir, P. & Vanderhaeghen, P. Mitochondrial dynamics in postmitotic cells regulate neurogenesis. *Science* **369**, 858–862 (2020).
- Lindhout, F. W. et al. Quantitative mapping of transcriptome and proteome dynamics during polarization of human iPSC-derived neurons. *eLife* **9**, (2020). <https://doi.org/10.7554/eLife.58124>.
- Frese, C. K. et al. Quantitative map of proteome dynamics during neuronal differentiation. *Cell Rep.* **18**, 1527–1542 (2017).
- Blair, J. D., Hockemeyer, D., Doudna, J. A., Bateup, H. S. & Floor, S. N. Widespread translational remodeling during human neuronal differentiation. *Cell Rep.* **21**, 2005–2016 (2017).
- Hong, D. & Jeong, S. 3'UTR diversity: Expanding Repertoire of RNA alterations in human mRNAs. *Mol. Cells* **46**, 48–56 (2023).
- Conaco, C., Otto, S., Han, J. J. & Mandel, G. Reciprocal actions of REST and a microRNA promote neuronal identity. *Proc. Natl Acad. Sci. USA* **103**, (2006). <https://doi.org/10.1073/pnas.0511041103>.
- Jiao, S., Liu, Y., Yao, Y. & Teng, J. miR-124 promotes proliferation and differentiation of neuronal stem cells through inactivating Notch pathway. *Cell Biosci.* **7**, 68–68 (2017).
- Cheng, L.-C., Pastrana, E., Tavazoie, M. & Doetsch, F. miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. *Nat. Neurosci.* **12**, 399–408 (2009).
- Sanuki, R. et al. MiR-124a is required for hippocampal axogenesis and retinal cone survival through Lhx2 suppression. *Nat. Neurosci.* **14**, (2011). <https://doi.org/10.1038/nn.2897>.
- Åkerblom, M. et al. MicroRNA-124 is a subventricular zone neuronal fate determinant. *J. Neurosci.* **32**, (2012). <https://doi.org/10.1523/JNEUROSCI.0558-12.2012>.
- Kutsche, L. K. et al. Combined experimental and system-level analyses reveal the complex regulatory network of miR-124 during human neurogenesis. *Cell Syst.* **7**, (2018). <https://doi.org/10.1016/j.cels.2018.08.011>.
- Yoo, A. S., Staahl, B. T., Chen, L. & Crabtree, G. R. MicroRNA-mediated switching of chromatin-remodelling complexes in neural development. *Nature* **460**, (2009). <https://doi.org/10.1038/nature08139>.
- Visvanathan, J., Lee, S., Lee, B., Lee, J. W. & Lee, S. K. The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. *Genes Dev.* **21**, (2007). <https://doi.org/10.1101/gad.1519107>.
- Arvanitis, D. N., Jungas, T., Behar, A. & Davy, A. Ephrin-B1 Reverse signaling controls a posttranscriptional feedback mechanism via miR-124. *Mol. Cell. Biol.* **30**, (2010). <https://doi.org/10.1128/mcb.01620-09>.
- Makeyev, E. V., Zhang, J., Carrasco, M. A. & Maniatis, T. The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative Pre-mRNA splicing. *Mol. Cell* **27**, (2007). <https://doi.org/10.1016/j.molcel.2007.07.015>.
- Ebert, M. S., Neilson, J. R. & Sharp, P. A. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat. Methods* **4**, 721–726 (2007).
- Han, J. et al. Functional Implications of miR-19 in the migration of newborn neurons in the adult brain. *Neuron* **91**, 79–89 (2016).
- Huang, D. W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44–57 (2009).
- Sherman, B. T. et al. DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). *Nucl. Acids Res.* **50**, (2022). <https://doi.org/10.1093/nar/gkac194>.
- Koehl, M. A. R. When does morphology matter? *Annu. Rev. Ecol. Syst.* **27**, 501–542 (1996).
- Van Zutphen, T. & Van Der Klei, I. J. Quantitative analysis of organelle abundance, morphology and dynamics. *Curr. Opin. Biotechnol.* **22**, 127–132 (2011).
- Heald, R. & Cohen-Fix, O. Morphology and function of membrane-bound organelles. *Curr. Opin. Cell Biol.* **26**, 79–86 (2014).
- Garcia, G. C. et al. Mitochondrial morphology provides a mechanism for energy buffering at synapses. *Sci. Rep.* **9**, 18306 (2019).
- Faitg, J., Davey, T., Turnbull, D. M., White, K. & Vincent, A. E. Mitochondrial morphology and function: two for the price of one! *J. Microsc.-Oxf.* **278**, 89–106 (2020).
- Jerabkova-Roda, K., Marwaha, R., Das, T. & Goetz, J. G. Organelle morphology and positioning orchestrate physiological and disease-associated processes. *Curr. Opin. Cell Biol.* **86**, 102293 (2024).
- Morel, F. et al. Gene and protein characterization of the human Glutathione S-Transferase Kappa and evidence for a peroxisomal localization. *J. Biol. Chem.* **279**, 16246–16253 (2004).
- Thomson, R. E. et al. Tissue-specific expression and subcellular distribution of murine Glutathione S-transferase Class Kappa. *J. Histochem. Cytochem.* **52**, 653–662 (2004).
- Kuruş, M. et al. Transcriptome Dynamics of Human Neuronal Differentiation From iPSC. *Front. Cell Dev. Biol.* **9**, (2021). <https://doi.org/10.3389/fcell.2021.727747>.

33. Wilkens, R. et al. Diverse maturity-dependent and complementary anti-apoptotic brakes safeguard human iPSC-derived neurons from cell death. *Cell Death Dis.* **13**, (2022). <https://doi.org/10.1038/s41419-022-05340-4>.
34. Kim, M., Gwak, J., Hwang, S., Yang, S. & Jeong, S. M. Mitochondrial GPT2 plays a pivotal role in metabolic adaptation to the perturbation of mitochondrial glutamine metabolism. *Oncogene* **38**, 4729–4738 (2019).
35. Baytas, O., Kauer, J. A. & Morrow, E. M. Loss of mitochondrial enzyme GPT2 causes early neurodegeneration in locus coeruleus. *Neurobiol. Dis.* **173**, 105831–105831 (2022).
36. Binaafar, S. et al. A novel missense variant in GPT2 causes non-syndromic autosomal recessive intellectual disability in a consanguineous Iranian family. *Eur. J. Med. Genet.* **63**, 103853–103853 (2020).
37. Breschi, A., Gingeras, T. R. & Guigo, R. Comparative transcriptomics in human and mouse. *Nat. Rev. Genet.* **18**, 425–440 (2017).
38. Celis, K. et al. Loss of function mutation in glutamic pyruvate transaminase 2 (GPT2) causes developmental encephalopathy. *J. Inherit. Metab. Dis.* **38**, 941–948 (2015).
39. Kaymakcalan, H. et al. Novel compound heterozygous mutations in GPT2 linked to microcephaly, and intellectual developmental disability with or without spastic paraplegia. *Am. J. Med. Genet. Part A* **176**, 421–425 (2018).
40. Ouyang, Q. et al. Mutations in mitochondrial enzyme GPT2 cause metabolic dysfunction and neurological disease with developmental and progressive features. *Proc. Natl Acad. Sci. USA* **113**, E5598–E5607 (2016).
41. Ouyang, Q. et al. GPT2 mutations in autosomal recessive developmental disability: extending the clinical phenotype and population prevalence estimates. *Hum. Genet.* **138**, 1183–1200 (2019).
42. Cho, Y. E. et al. mHGTD-P mediates hypoxic neuronal cell death via the release of apoptosis-inducing factor. *Neurosci. Lett.* **416**, 144–149 (2007).
43. Nuebel, E., Manganas, P. & Tokatlidis, K. Orphan proteins of unknown function in the mitochondrial intermembrane space proteome: New pathways and metabolic cross-talk. *Biochim. Biophys. Acta (BBA) - Mol. Cell Res.* **1863**, 2613–2623 (2016).
44. Oka, T. et al. Identification of a novel protein MICS1 that is involved in maintenance of mitochondrial morphology and apoptotic release of Cytochrome c. *Mol. Biol. Cell* **19**, 2597–2608 (2008).
45. Lisak, D. A. et al. The transmembrane Bax inhibitor motif (TMBIM) containing protein family: Tissue expression, intracellular localization and effects on the ER CA2+-filling state. *Biochim. Biophys. Acta (BBA) - Mol. Cell Res.* **1853**, 2104–2114 (2015).
46. Suh, K. S. et al. The organellar chloride channel protein CLIC4/mtCLIC translocates to the nucleus in response to cellular stress and accelerates apoptosis. *J. Biol. Chem.* **279**, 4632–4641 (2004).
47. He, G. et al. Role of CLIC4 in the host innate responses to bacterial lipopolysaccharide. *Eur. J. Immunol.* **41**, 1221–1230 (2011).
48. Li, B. et al. Role of c-Myc/chloride intracellular channel 4 pathway in lipopolysaccharide-induced neurodegenerative diseases. *Toxicology* **429**, 152312–152312 (2020).
49. Bai, J. et al. DsbA-L prevents obesity-induced inflammation and insulin resistance by suppressing the mtDNA release-activated cGAS-cGAMP-STING pathway. *Proc. Natl Acad. Sci.* **114**, 12196–12201 (2017).
50. Deng, X. et al. Plasma mtDNA copy numbers are associated with GSTK1 expression and inflammation in type 2 diabetes. *Diabet. Med.* **37**, 1874–1878 (2020).
51. Li, X. et al. DsbA-L interacts with VDAC1 in mitochondrion-mediated tubular cell apoptosis and contributes to the progression of acute kidney disease. *eBioMedicine* **76**, 103859–103859 (2022).
52. Liu, M. et al. A disulfide-bond A oxidoreductase-like protein (DsbA-L) regulates adiponectin multimerization. *Proc. Natl Acad. Sci.* **105**, 18302–18307 (2008).
53. Yang, M. et al. DsbA-L ameliorates high glucose-induced tubular damage by maintaining MAM integrity. *EBioMedicine* **43**, 607–619 (2019).
54. Zhou, H. et al. DsbA-L deficiency in T cells promotes diet-induced thermogenesis by suppressing IFN- $\gamma$  production. *Nat. Commun.* **12**, 1–14 (2021).
55. Blackburn, A. C. et al. Glutathione transferase kappa deficiency causes glomerular nephropathy without overt oxidative stress. *Lab. Invest.* **91**, 1572–1583 (2011).
56. Chandrasekaran, A., Avci, H. X., Leist, M., Kobolak, J. & Dinnyes, A. Astrocyte differentiation of human pluripotent stem cells: new tools for neurological disorder research. *Front Cell Neurosci.* **10**, 215 (2016).
57. Shield, A. J., Murray, T. P., Cappello, J. Y., Coggan, M. & Board, P. G. Polymorphisms in the human glutathione transferase Kappa (GSTK1) promoter alter gene expression. *Genomics* **95**, 299–305 (2010).
58. Pagliarini, D. J. et al. A mitochondrial protein compendium elucidates complex I disease biology. *Cell* **134**, 112–123 (2008).
59. Fecher, C. et al. Cell-type-specific profiling of brain mitochondria reveals functional and molecular diversity. *Nat. Neurosci.* **22**, 1731–1742 (2019).
60. Russo, G. L. et al. CRISPR-mediated induction of neuron-enriched mitochondrial proteins boosts direct glia-to-neuron conversion. *Cell Stem Cell* **28**, 524–534 e527 (2021).
61. Cheng, X. et al. Variability of mitochondrial energy balance across brain regions. *J. Neurochem.* **157**, 1234–1243 (2021).
62. Wynne, M. E. et al. Heterogeneous expression of nuclear-encoded mitochondrial genes distinguishes inhibitory and excitatory neurons. *eneuro* **8**, ENEURO.0232–0221.2021 (2021).
63. Anusha-Kiran, Y. et al. Regional heterogeneity in mitochondrial function underlies region-specific vulnerability in human brain ageing: Implications for neurodegeneration. *Free Radic. Biol. Med.* **193**, 34–57 (2022).
64. Mosharov, E. V. et al. A human brain map of mitochondrial respiratory capacity and diversity. *bioRxiv* (2024). <https://doi.org/10.1101/2024.03.05.583623>.
65. Lagos-Quintana, M. et al. Identification of tissue-specific MicroRNAs from mouse. *Curr. Biol.* **12**, 735–739 (2002).
66. Sempere, L. F. et al. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol.* **5**, R13 (2004).
67. Iwata, R. et al. Mitochondria metabolism sets the species-specific tempo of neuronal development. *Science* **379**, eabn4705 (2023).
68. Diaz-Cuadros, M. et al. Metabolic regulation of species-specific developmental rates. *Nature* **613**, 550–557 (2023).
69. Matsuda, M. et al. Species-specific segmentation clock periods are due to differential biochemical reaction speeds. *Science* **369**, 1450–1455 (2020).
70. Rayon, T. et al. Species-specific pace of development is associated with differences in protein stability. *Science* **369**, (2020). <https://doi.org/10.1126/science.aba7667>.
71. Goyal, M. S., Hawrylycz, M., Miller, J. A., Snyder, A. Z. & Raichle, M. E. Aerobic glycolysis in the human brain is associated with development and neotenuous gene expression. *Cell Metab.* **19**, 49–57 (2014).
72. Sjostedt, E. et al. An atlas of the protein-coding genes in the human, pig, and mouse brain. *Science* **367**, (2020). <https://doi.org/10.1126/science.aay5947>.
73. Inak, G. et al. Defective metabolic programming impairs early neuronal morphogenesis in neural cultures and an organoid model of Leigh syndrome. *Nat. Commun.* **12**, 1929 (2021).
74. Johnson, R. et al. A microRNA-based gene dysregulation pathway in Huntington's disease. *Neurobiol. Dis.* **29**, 438–445 (2008).
75. Packer, A. N., Xing, Y., Harper, S. Q., Jones, L. & Davidson, B. L. The Bifunctional microRNA miR-9/miR-9\* Regulates REST and CoREST

- and Is Downregulated in Huntington's Disease. *J. Neurosci.* **28**, 14341–14346 (2008).
76. Fang, M. et al. The miR-124 regulates the expression of BACE1/ $\beta$ -secretase correlated with cell death in Alzheimer's disease. *Toxicol. Lett.* **209**, 94–105 (2012).
  77. Gascon, E. et al. Alterations in microRNA-124 and AMPA receptors contribute to social behavioral deficits in frontotemporal dementia. *Nat. Med.* **20**, 1444–1451 (2014).
  78. Liu, T., Im, W., Mook-Jung, I. & Kim, M. MicroRNA-124 slows down the progression of Huntington's disease by promoting neurogenesis in the striatum. *Neural Regen. Res.* **10**, (2015). <https://doi.org/10.4103/1673-5374.156978>.
  79. Li, N. et al. Plasma levels of miR-137 and miR-124 are associated with Parkinson's disease but not with Parkinson's disease with depression. *Neurol. Sci.* **38**, 761–767 (2017).
  80. Angelopoulou, E., Paudel, Y. N. & Piperi, C. miR-124 and Parkinson's disease: A biomarker with therapeutic potential. *Pharmacol. Res.* **150**, 104515 (2019).
  81. Yelick, J. et al. Elevated exosomal secretion of miR-124-3p from spinal neurons positively associates with disease severity in ALS. *Exp. Neurol.* **333**, 113414 (2020).
  82. Vaz, A. R. et al. Overexpression of miR-124 in motor neurons plays a key role in ALS pathological processes. *Int. J. Mol. Sci.* **22**, 6128 (2021).
  83. Zhang, W.-H., Jiang, L., Li, M. & Liu, J. MicroRNA-124: an emerging therapeutic target in central nervous system disorders. *Exp. Brain Res.* **241**, 1215–1226 (2023).
  84. Kim, K.-H. et al. Posttranscriptional regulation of FAN1 by miR-124-3p at rs3512 underlies onset-delaying genetic modification in Huntington's disease. *Proc. Natl Acad. Sci.* **121**, e2322924121 (2024).
  85. Lin, M. T. & Beal, M. F. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* **443**, 787–795 (2006).
  86. Dragicevic, N. et al. Mitochondrial Amyloid- $\beta$  levels are associated with the extent of mitochondrial dysfunction in different brain regions and the degree of cognitive impairment in Alzheimer's transgenic mice. *J. Alzheimer's Dis.* **20**, S535–S550 (2010).
  87. Burman, J. L., Yu, S., Poole, A. C., Decal, R. B. & Pallanck, L. Analysis of neural subtypes reveals selective mitochondrial dysfunction in dopaminergic neurons from parkin mutants. *Proc. Natl Acad. Sci.* **109**, 10438–10443 (2012).
  88. Bartscher, J., Di Pardo, A., Maglione, V., Schwarzer, C. & Squitieri, F. Mitochondrial respiration changes in R6/2 Huntington's disease model mice during aging in a brain region specific manner. *Int. J. Mol. Sci.* **21**, 5412 (2020).
  89. Motori, E. et al. Neuronal metabolic rewiring promotes resilience to neurodegeneration caused by mitochondrial dysfunction. *Sci. Adv.* **6**, eaba8271 (2020).
  90. Malmvik, J. et al. Distinct cognitive effects and underlying transcriptome changes upon inhibition of individual miRNAs in hippocampal neurons. *Sci. Rep.* **6**, 19879 (2016).
  91. Nielsen, A. A. & Voigt, C. A. Multi-input CRISPR/Cas genetic circuits that interface host regulatory networks. *Mol. Syst. Biol.* **10**, 763 (2014).
  92. Xu, S. et al. Use ggbreak to effectively utilize plotting space to deal with large datasets and outliers. *Front. Genet.* **12**, (2021). <https://doi.org/10.3389/fgene.2021.774846>.
  93. Choi, Y. et al. MS1-level proteome quantification platform allowing maximally increased multiplexity for SILAC and in vitro chemical labeling. *Anal. Chem.* **92**, 4980–4989 (2020).
  94. Perez-Riverol, Y. et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* **47**, D442–D450 (2019).

## Acknowledgements

We thank M.L. Gage for his editorial comments. This work was supported by the National Research Foundation of Korea (NRF-2022R1C1C2003822 to Y.N.; NRF-2022R1A2C3008991 to S.-B.P.; NRF-2019R1C1C1010482, RS-

2024-00398786, and RS-2024-00335144 to J.H.), the Korean Fund for Regenerative Medicine (21A0402L1-12 to Y.-M.H.), and the Institute for Basic Science (IBS-R008-D1 to J.-S.K. and IBS-R002-A1 to J.H.) from the Ministry of Science and ICT of Korea. F.H.G. was supported by the National Institutes of Health of USA (R01 AG056306, UCSD - R01 AG057706, and UCSD - R01 AG056511), the American Heart Association and the Paul G. Allen Frontiers Group (Grant #19PABH134610000/TEAM LEADER: Fred H. Gage/2019), and the Milky Way Research Foundation. We also acknowledge the support by the NGS Core Facility of the Salk Institute with funding from NIH-NCI CCSG: P30 014195.

## Author contributions

J.H. and F.H.G. conceived the project. G.S. and J.H. designed experiments and analyzed all data. Y.N. and J.-S.K. designed and performed quantitative mass spectrometry and analyzed the data. Y.K. performed mitochondrial morphology analyses. G.S., J.-H.S., H.D., D.K., I.A., M.J., C.S.K., and J.H. performed molecular biology and cell biology experiments. J.-H.S. A.P. and D.K. participated in bioinformatics data analysis and visualization. G.S., G.D.C., S.T.S., J.Y.Y., S.L.P., A.H.M., G.K., S.-H.L., and J.H. were involved in animal data generation and analysis. Y.J. and Y.-M.H. contributed to hESC culture optimization. D.K., E.J., and J.K. contributed to the preparation of MEFs. Y.P. and S.-B.P. contributed to MEA data analysis. J.Hur contributed to the design of CRISPRa experiment. G.S. and J.H. wrote the manuscript. G.S., J.H., and F.H.G. revised the manuscript with contributions from all authors.

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s42003-024-07089-2>.

**Correspondence** and requests for materials should be addressed to Jinju Han.

**Peer review information** *Communications Biology* thanks the anonymous reviewers for their contribution to the peer review of this work. Primary Handling Editors: Pavithra Chavali and Joao Valente.

**Reprints and permissions information** is available at <http://www.nature.com/reprints>

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Open Access** This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2024