APOE Expression and Secretion are Modulated by Copper-Dependent and -Independent Mitochondrial Dysfunction

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Abstract

Mitochondria are dynamic organelles that influence cellular function through both cell-autonomous and non-cell autonomous mechanisms, such as production of paracrine and endocrine factors. Here, we demonstrate that mitochondrial regulation of the secretome is more extensive than previously appreciated, as both genetic and pharmacological disruption of the inner mitochondrial membrane caused upregulation of the Alzheimer's disease risk factor apolipoprotein E (APOE) and other secretome components. This upregulation of secretory proteins was of a similar extent as modifications to the mitochondrial annotated proteome. Gene editing of SLC25A family inner mitochondrial membrane transporters, as well as genetic and pharmacological disruption of copper-dependent and independent steps of electron transport chain assembly and function, caused upregulation of APOE transcript, protein, and secretion, up to 16-fold. These APOE phenotypes were robustly expressed in diverse cell types and iPSC-derived human astrocytes as part of an inflammatory gene expression program. Moreover, age-dependent high expression of APOE in the 5xFAD mouse model correlated with low expression of respiratory chain complexes in brain. We propose that mitochondria act as novel upstream regulators of APOE-dependent cellular processes in health and disease.

Introduction

Mitochondria are necessary for maintaining cellular and organismal health and function, by generating energy and serving as hubs for diverse metabolic and signaling pathways (Nunnari and Suomalainen, 2012). The majority of the mitochondrial functions described so far, are cell-autonomous. However, mitochondria are also capable of influencing cellular function from a distance in a non-cell-autonomous manner. These non-cell-autonomous mechanisms are mostly elicited after cellular or mitochondrial damage. These non-cell-autonomous mechanisms, mostly elicited after cellular or mitochondrial damage, encompass intercellular transfer of mitochondria to secretion of endocrine and paracrine factors (D'Acunzo et al., 2021; Durieux et al., 2011; Hayakawa et al., 2016; Liu et al., 2021). These secreted factors include proteins encoded in the nuclear genome, such as alpha-fetoprotein, inflammatory cytokines and type I interferons, and growth factor mitokines (Bar-Ziv et al., 2020; Chung et al., 2017; Dhir et al., 2018; Durieux et al., 2011; Jett et al., 2021; Kim et al., 2013; Riley and Tait, 2020; Shimada et al., 2012; West et al., 2015). A second class of non-cell-autonomous factors are mitochondrially-derived peptides, encoded in the mitochondrial genome (Kim et al., 2017). Mitokines and mitochondrially-derived peptides modulate cell survival, metabolic and lipid homeostasis, body weight, longevity, and aging, a primary risk factor for cognitive decline in humans (Chung et al., 2017; Flippo and Potthoff, 2021; Klaus and Ost, 2020; Mullican et al., 2017; Tsai et al., 2018). In models of Alzheimer's disease, the mitochondrially-derived peptide humanin can reduce apoptosis, inflammation, accumulation of plaque-forming Aβ peptides, and cognitive deficits (Hashimoto et al., 2001; Tajima et al., 2005; Yen et al., 2013). The ability of factors encoded in the nuclear and mitochondrial genomes to regulate inflammation, lipid metabolism, aging, and Alzheimer's disease mechanisms suggests that mitochondrial-dependent modulation of protein secretion could modify neurological disease pathogenesis prior to cell death.

Here, we sought to identify proteins whose expression and secretion is modulated by mitochondrial function through an unbiased interrogation of human transcriptomes and proteomes. We focused our attention on factors whose expression is sensitive to mutations affecting the inner mitochondrial membrane citrate transporter SLC25A1 and the ADP-ATP transporter SLC25A4 (ANT1). We chose these mitochondrial transporters because they have been genetically implicated in neurodevelopment, brain metabolism, psychiatric disease, and neurodegeneration (Balaraju et al., 2020; Chaouch et al., 2014; Edvardson et al., 2013; Gokhale et al., 2019; Kato et al., 2018; Lin-Hendel et al., 2016; Nota et al., 2013; Rigby et al., 2022; Siciliano et al., 2003). For example, SLC25A1 is a causal gene in two genetic diseases, a severe neurometabolic disease (combined D-2- and L-2-hydroxyglutaric aciduria) and a congenital myasthenic syndrome presenting with intellectual disability (Balaraju et al., 2020; Chaouch et al., 2014, Nota et al., 2013) (OMIM 615182- 618197). In addition, SLC25A1 is part of the chromosomal interval deleted in 22q11.2 deletion syndrome, a microdeletion syndrome associated with neurodevelopmental, psychiatric, and neurodegenerative diseases (Butcher et

al., 2013; Schneider et al., 2014; Zinkstok et al., 2019). SLC25A1 has been implicated as a hub factor underlying a mitochondrial protein network, which includes SLC25A4, that is disrupted in 22q11.2 deletion syndrome cells (Gokhale et al., 2019). Since SLC25A1 and SLC25A4 coprecipitate (Gokhale et al., 2019), we hypothesized the existence of common downstream secretory and mitochondrial targets elicited by their mutation. We discovered that loss of SLC25A1 or SLC25A4 affected the secreted proteome to a similar extent as the mitochondrially annotated proteome. Among the secreted factors whose expression was increased in both SLC25A1 and SLC25A4 mutants was apolipoprotein E (APOE). We focused on APOE since it is the main carrier of lipids and cholesterol in the brain (Mahley, 2016), and it is tied to cognitive function, neuroinflammation, and neurological disease risk (Belloy et al., 2019; Lanfranco et al., 2021; O'Donoghue et al., 2018; Parhizkar and Holtzman, 2022). Importantly, the APOE4 allele is known as the strongest genetic risk factor for sporadic Alzheimer’s disease (Belloy et al., 2019). We found that APOE expression was modulated by mutations of multiple mitochondrial SLC25A transporters, as well as copper-dependent and independent pathways required for the integrity of the mitochondrial electron transport chain. While the APOE4 allele is thought to cause mitochondrial dysfunction in Alzheimer's disease (Area-Gomez et al., 2020; Chen et al., 2011; Orr et al., 2019; Tambini et al., 2016; Yin et al., 2020), our study places mitochondria upstream of APOE, uncovering a novel function for these multifaceted organelles.

Results

Systems Analysis Reveals that Genetic Disruption of the Inner Mitochondrial Membrane Alters the Secretome

Our goal was to identify secreted factors whose expression is modulated by genetic defects in nuclear encoded mitochondrial genes. We hypothesized that changes in the secretome would affect the capacity of conditioned media to support cell growth in a genotype-dependent manner. Thus, we applied conditioned media from wild type (*SLC25A1+*) and SLC25A1-null HAP1 cells (*SLC25A1Δ*) to cells from both genotypes and measured cell growth. We used this near haploid human leukemia cell line since it has a short doubling time, and thus rapid protein turnover, making it well-suited to rapidly respond to changes in subproteomes, such as the secretome and mitoproteome. We dialyzed conditioned media from wild type and *SLC25A1Δ* cells to exclude effects of metabolites, pH, and small peptides present in media (Fig. 1A). Dialyzed conditioned media from wild type and *SLC25A1Δ* cells supported wild type cell growth (Fig. 1A). Wild type cells similarly responded to dialyzed media from both genotypes, increasing growth by 50% as compared to non-dialyzed media (Fig. 1A compare columns 1, 3 and 2, 4). In contrast, while *SLC25A1Δ* cells fed with wild type dialyzed conditioned media doubled in number (Fig. 1A, compare columns 5 and 7), dialyzed conditioned media from *SLC25A1Δ* cells fed onto themselves did not support their growth as compared to media from wild type cells (Fig. 1A compare columns 7 and 8 and 6 an 8). These results suggest that *SLC25A1Δ* cells condition media differently than wild type cells.

To identify compositional differences between wild type and *SLC25A1Δ* conditioned media, we analyzed the proteome and transcriptome of *SLC25A1Δ* cells (Fig 1B, D-F, H-I). Fetal bovine serum in media prevented us from a direct analysis of the conditioned media by mass spectrometry. We annotated the *SLC25A1Δ* proteome and transcriptome with the human secretome database (Uhlen et al., 2019) and the Mitocarta 3.0 knowledgebase (Rath et al., 2021) to comprehensively identify differences in secreted factors and the consequences of the SLC25A1 mutation on mitochondria. We simultaneously analyzed the proteome and transcriptome of *SLC25A4Δ* cells to determine whether changes in the *SLC25A1Δ* proteome and transcriptome resulted specifically from loss of SLC25A1, or could be generalized to another form of genetic disruption to the inner mitochondrial membrane (Fig. 1C and G). We selected SLC25A4, as it encodes an ADP-ATP translocator that interacts with SLC25A1 (Gokhale et al., 2019). Tandem mass tagging mass spectrometry and RNAseq revealed that *SLC25A1Δ* cells underwent more extensive changes of their proteome and transcriptome than *SLC25A4Δ* cells (compare Fig. 1B with C and F with G). For example, 668 proteins significantly changed their expression in *SLC25A1Δ* cells as compared to 110 proteins in *SLC25A4Δ* cells (log2 fold of chain of 0.5 and $p<0.05$, Fig. 1B and C). Similarly, the *SLC25A1Δ* transcriptome was represented by 2433 transcripts whose expression was changed in *SLC25A1*-null cells, a 4-fold difference as compared to the 560 transcripts found in *SLC25A4Δ* cells (log2 fold of chain of 1 and $p<0.001$, Fig. 1F and

G). Principal component analysis and 2D-tSNE analysis indicated that the whole measured proteome and transcriptome of *SLC25A1Δ* cells diverged strongly from wild type cells, while *SLC25A4Δ* cells were more closely related to wild type cells than *SLC25A1Δ* cells (Fig 1D and H). The same outcome was obtained by unsupervised clustering when considering proteins and transcripts significantly changed in at least one of these genotypes (Fig 1E and I), as *SLC25A4Δ* clustered with wild type cells rather than *SLC25A1Δ* cells. Despite the abundance of altered gene products in these *SLC25A1Δ* and *SLC25A4Δ* datasets, there was limited overlap in proteomes and transcriptomes, with only 84 proteins and 385 mRNAs shared by both genotypes (Fig 1J). Notably, the congruency of the shared proteomes and transcriptomes reached only 0.9% of all the gene products whose expression was modified. This represents 27 proteins and transcripts similarly modified in *SLC25A1Δ* and *SLC25A4Δ* datasets (Fig 1J). Of these 27 common hits, one was annotated to mitochondria, FASTKD2, and five were annotated to the secreted human proteome, including soluble proteins such as apolipoprotein E (APOE) and cytokine receptor-like factor 1 (CRLF1) (Elson et al., 1998; Wernette-Hammond et al., 1989) (Fig 1J). APOE protein and transcript were among the most upregulated factors in both *SLC25A1Δ* and *SLC25A4Δ* cells (Fig 1B-C and F-G).

Close to 10% of all the *SLC25A1Δ* and *SLC25A4Δ* proteome hits were proteins annotated to mitochondria, with a discrete overlap of 10 mitochondrial proteins between these two mutant genotypes, mostly constituents of complex III of the electron transport chain (Fig. 1K). The enrichment of secreted proteome annotated proteins was modest yet significant in *SLC25A1Δ* and *SLC25A4Δ* cells. Surprisingly, this degree of enrichment in components of the secretome was comparable to the enrichment of Mitocarta 3.0 annotated proteins in both mitochondrial mutants (Fig. 1L, M, and N). These results show that mutations affecting two inner mitochondrial membrane transporters, SLC25A1 and SLC25A4, affect the secreted proteome to a similar extent as the mitochondrial proteome.

We analyzed *SLC25A1Δ* and *SLC25A4Δ* datasets for additional commonalities at the ontological level, using the ClueGO tool to annotate datasets based on genotype and whether a factor was up- or down-regulated. The annotated datasets were used to simultaneously query the KEGG, REACTOME and GO CC databases. The proteome and transcriptome of both mutants identified developmental ontologies as shared terms, irrespective of whether factors were up- or down-regulated ((Fig 1O-P) gray nodes and table 1, (tissue development GO:0009888, Bonferroni corrected p=1.9E-26 and 2.3E-11 for the transcriptome and proteome, respectively). However, there were ontologies that stood out by their genotype- and up-regulation-dependent specificity. For instance, the most prominent ontology annotated to up-regulated *SLC25A1Δ* proteome and transcriptome hits was steroid biosynthesis (KEGG:00100, Bonferroni corrected p= 3.6E-8 and 1.9E-8 for the transcriptome and proteome, respectively. Fig 1 O-P). However, the expression of genes annotated to sterol biosynthesis ontologies was not modified in *SLC25A4Δ* mutants, even though the proteome and transcriptome of *SLC25A4Δ* cells showed increased

expression of APOE, a cholesterol transport lipoprotein. These findings suggest that expression of APOE and other hits common between these two mutant genotypes occurs independently from modifications in cholesterol synthesis pathways.

APOE Expression is Uncoupled from Changes in Cholesterol Synthesis Pathways.

We focused on APOE and the sterol biosynthesis pathways to validate our proteome and transcriptome data. We also sought to identify or exclude changes in cholesterol synthesis pathways as causal mechanisms for increased APOE expression in *SLC25A1Δ* and *SLC25A4Δ* cells. Electrochemical MesoScale ELISA determinations of human APOE revealed increased APOE in cell lysates and conditioned media from *SLC25A1Δ* and *SLC25A4Δ* cells (Fig. 2A) (Chikkaveeraiah et al., 2012; Gaiottino et al., 2013). APOE protein expression and secretion into media were increased ~5-8 times in two CRISPR *SLC25A1Δ* clones and *SLC25A4Δ* cells (Fig. 2A compare column 1 with 2-4). APOE signal in complete media unexposed to cells was undetectable (Fig. 2A compare media columns 1 with 5). We confirmed these findings by immunoblot with a different APOE antibody. We used recombinant human APOE as a standard (Fig. 2B). To exclude that an APOE expression increase was a haploid HAP1 cell peculiarity, we CRISPRed out the SLC25A1 gene in the diploid human neuroblastoma cell line SH-SY5Y (Fig. 2C *SLC25A1Δ/Δ*). Additionally, we knocked out SLC25A4, SLC25A5, and SLC25A6, all isoforms of the ADP-ATP translocator, in HEK293 cells (Fig. 2C *SLC25A1Δ/Δ* and Fig. 2D *SLC25A4,5,6Δ/Δ*). Much like HAP1 cells, *SLC25A1Δ/Δ and SLC25A4,5,6Δ/Δ* cells increased the expression of APOE between 2- to 4.5-fold, in both cells and conditioned media, as compared with wild type cells (Fig. 2C and D, compare lanes 1 and 2). These results reveal a robust upregulation of both cellular and secreted APOE across cell types and mutations affecting SLC25A mitochondrial transporters.

If APOE expression depends on modifications in cholesterol pathways, then the expression of genes annotated to cholesterol metabolism and cholesterol content should be similarly modified in *SLC25A1Δ* and *SLC25A1Δ/Δ* cells. We measured the transcript levels of APOE and genes involved in cholesterol uptake and synthesis, in *SLC25A1Δ, SLC25A4Δ,* and *SLC25A1Δ/Δ* cells. We focused on the LDL receptor (LDLR), as well as cholesterol synthesis enzymes, ACAT2, MSMO1 and HMGCR, the latter the rate limiting enzyme of the cholesterol synthesis pathway (Brown and Goldstein, 1980; Mazein et al., 2013). We chose these genes as upregulated hits from the transcriptome of *SLC25A1Δ* cells. We used VAMP2 and RPS20 as housekeeping gene controls (Fig. 2E-G). APOE mRNA increased ~3 fold in all three mutant cells. In contrast, the expression of cholesterol synthesis pathway genes was increased in *SLC25A1Δ* (Fig. 2E), but not in *SLC25A4Δ* and *SLC25A1Δ/Δ* cells (Fig. 2F and G). The upregulation of cholesterol synthesis pathway genes resulted in a significant increase of cholesterol and all cholesterol-ester species content in *SLC25A1Δ* cells, as determined by mass spectrometry (Fig. 2H). Triglyceride and other measured lipid families were similar in wild type and *SLC25A1Δ* cells (Fig. 2H and K). In contrast, cholesterol and cholesterol-ester species were not modified in *SLC25A4Δ* (Fig. 2I and K) and *SLC25A1Δ/Δ* cells (Fig. 2J and L), even though the expression of APOE was upregulated to the same extent in all

these mutant cells. These results exclude the hypothesis that increased APOE expression is coupled to an upregulation of cholesterol synthesis pathways.

Direct Perturbation of the Electron Transport Chain Complexes I and III Increases APOE Expression.

We turned our attention to common defects in *SLC25A1Δ* and *SLC25A4Δ* cells that could explain the increased expression of APOE. A shared target of both mutant genotypes was complex III of the electron transport chain (Fig. 1K). We analyzed the expression of all measured electron transport chain subunits in our proteomics dataset and found that, even though complex III was affected in both *SLC25A1Δ* and *SLC25A4Δ* cells (Fig. 1K), the most pronounced defect was in the expression of complex I subunits in *SLC25A1Δ* cells (Fig. 3A). We explored the expression and assembly of respiratory chain complexes in *SLC25A1Δ* cells by SDS-PAGE, blue native electrophoresis, and bidimensional gel electrophoresis (Fig. 3B-D). We performed immunoblot analysis of SDS-PAGE resolved respiratory complex subunits with antibodies against subunits that undergo degradation in misassembled respiratory complexes (Civiletto et al., 2018; Ghazal et al., 2021). These experiments revealed that the most degraded subunits were those from complexes I and III (Fig. 3B). We then analyzed respiratory complexes and supercomplexes by blue native electrophoresis and found decreased expression of high molecular weight complexes containing NDUFS3, UQCRC2, and COX4, which correspond to subunits of the respiratory complexes I, III and IV, respectively (Fig. 3C). Two-dimensional gel electrophoresis showed that high molecular weight respiratory complexes containing subunits of complexes I and III were diminished in *SLC25A1Δ* cells (Fig. 3D). These results demonstrate that the expression and assembly of the respiratory chain is compromised in *SLC25A1Δ* cells.

Respiratory chain defects could be responsible for the change in expression of APOE in inner mitochondrial membrane mutants. We tested this hypothesis by targeting complexes I and III using genetic and pharmacological approaches. We genetically perturbed complex I assembly and function by knocking-out either NDUFS3 or NDUFAF7. NDUFS3 encodes NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, a non-catalytic subunit of complex I necessary for complex I assembly and activity (Benit et al., 2004; D'Angelo et al., 2021) and NDUFAF7 encodes NADH:ubiquinone oxidoreductase complex assembly factor 7, a methylase necessary for the early stages of complex I assembly (Zurita Rendon et al., 2014). We targeted NDUFS3 and NDUFAF7 because these genes localize to genetic loci associated with increased risk of Alzheimer's disease (de Rojas et al., 2021; Kunkle et al., 2019). Moreover, SLC25A1 and NDUFS3 share 91% of their proximity interactions (Antonicka et al., 2020), an observation we corroborated by co-immunoprecipitation of SLC25A1 and NDUFS3 (Fig. S1A-B). NDUFS3 and NDUFAF7 CRISPR mutants compromised mitochondrial respiration, as shown by Seahorse oximetry (Fig. 3E and H). Both mutants increased the expression of APOE mRNA, as compared to reference housekeeping transcripts, (Fig. 3F and I, RER1 and VAMP2) as well as APOE protein in

cells and in conditioned media (Fig. 3G and J). These results demonstrate that APOE expression and secretion are regulated by the integrity and function of complex I.

We inhibited the function of complex III with the specific inhibitor antimycin I (von Jagow and Link, 1986). We inquired whether inhibition of complex III by antimycin would upregulate the expression of APOE in wild type and in *SLC25A1Δ* cells. Since complex III levels were partially reduced in *SLC25A1Δ* cells (Fig 3A, B, and C), we reasoned that inhibition of residual complex III activity in *SLC25A1Δ* cells would reveal additive respiratory chain mechanisms affecting APOE expression. We measured respiration in wild type and *SLC25A1Δ* cells in the absence and presence of increasing concentrations of antimycin (Fig. 3K). *SLC25A1Δ* cells were more sensitive to low doses of antimycin (Fig. 3K), a phenotype predicted for cells with reduced content of complex III. Antimycin addition increased APOE mRNA 3-fold in wild type cells as compared to vehicle (Fig. 3L compare columns 9-10), whereas the expression of two housekeeping mRNAs, RER1 and PCBP1, was minimally affected by complex III inhibition (Fig. 3L compare columns 1-2 and 5-6). *SLC25A1Δ* cells doubled their APOE mRNA when treated with antimycin as compared to vehicle-treated *SLC25A1Δ* cells (Fig. 3L compare columns 11-12). Antimycin also increased APOE secretion in both wild type and *SLC25A1Δ* cells by ~2-3-fold (Fig. 3M). No such increase was detectable in cell lysates in both genotypes (Fig. 3M). These data show that the effect of antimycin on APOE mRNA expression and protein secretion was additive with the *SLC25A1Δ* upregulation phenotype. Taken together, these results demonstrate that the expression of APOE is sensitive to complex III inhibition and adaptable to the activity of the respiratory chain.

Disruption of Copper Delivery to Mitochondria Increases APOE Expression.

We tested whether APOE expression was modulated by additional mitochondrial mechanisms known to directly or indirectly affect the integrity of the electron transport chain. We first focused on complex IV, whose assembly and function requires copper I ions. We CRISPRed out a cytosolic cofactor necessary for copper delivery and assembly of complex IV, COX17, in SH-SY5Y cells (Banci et al., 2008; Cobine et al., 2021; Oswald et al., 2009). Mutagenesis of this complex increased the expression of APOE protein in both cells and conditioned media by ~16-fold in two independent CRISPR mutants (Fig. 4A, *COX17Δ/Δ*). COX17 is part of a pathway that begins with copper uptake at the plasma membrane, a process under homeostatic control of a copper uptake transporter, SLC31A1, and a copper extrusion transporter, ATP7A. Once copper is in the cytoplasm, COX17 bound copper reaches the mitochondrial matrix via the mitochondrial transporter SLC25A3 for its incorporation in complex IV (Boulet et al., 2018) (Fig. 4B). Our model predicted that genetic disruption of this pathway, either upstream or downstream of COX17, should phenocopy the elevated APOE expression observed in COX17 mutants. We decreased cytoplasmic copper availability by either eliminating copper uptake via mutagenesis of the SLC31A1 transporter (Fig. 4C, *SLC31A1Δ/Δ*), or by expressing in ATP7A-null SH-SY5Y cells (*ATP7AΔ/Δ*) an ATP7A mutant that constitutively extrudes copper from cells due to

mutagenesis of its endocytosis sorting signal (Fig. 4D, *ATP7A-LL*). Both forms of cytoplasmic copper reduction increased APOE expression and secretion in two independent mutant or clonal isolates (Fig. 4C and D). Elimination of ATP7A alone, which increases cytoplasmic copper content (Morgan et al., 2019), did not increase APOE expression (Fig. 4E, *ATP7AΔ/Δ*). In addition, mutagenesis of a gene downstream of *COX17*, *SLC25A3*, also increased the expression and secretion of APOE protein in HAP1 cells (Fig. 4F). We confirmed these results by measuring APOE mRNA levels with NanoString technology in two independent mutants of *COX17* and *SLC31A1,* as well as in wild type cells incubated with a cell impermeant copper chelator, bathocuproinedisulfonic acid (Fig. 4G, BCS). These pharmacological and genetic approaches to reduction of cytoplasmic copper robustly increased APOE mRNA as compared to wild type cells (Fig. 4G), while levels of a housekeeping gene, TBP, were unchanged. These results demonstrate that impairing copper delivery to complex IV increases the expression of APOE transcript and protein in diverse cell lines. Along with our experiments targeting complexes I and III, these findings support the concept that diverse mechanisms converging on respiratory chain function and assembly can induce expression or secretion of APOE.

To determine whether there was a relationship between the degree of respiratory chain compromise and the extent of APOE secretion increase, we correlated the normalized secretion of APOE in diverse mitochondrial mutants to their normalized basal oxygen consumption rate (Fig. 4H) and found a correlation of -0.6 (Fig. 4I). This correlation supports the idea that respiratory chain activity regulates APOE expression, while suggesting that other mechanisms are also at play. We confirmed that mitochondrial mechanisms independent from electron transport chain function likely exist through interrogation of mutant cells lacking the carnitine transporter SLC25A20. Although these cells have an intact electron transport chain and respire normally, they still upregulate APOE protein levels and secretion approximately 3-fold (Fig. S3).

We further explored alternative mechanisms from electron transport chain assembly and activity required for increased APOE expression and secretion. We ruled out the possibility that bioenergetic stress was responsible for the APOE increase in *SLC25A1Δ* cells, using activation of the AMPK pathway as a read-out (Fig. S2A). We ruled out accumulation of reactive oxygen species as a causal mechanism for increased APOE, as treatment with the antioxidant N-acetyl cysteine had no effect on APOE mRNA levels in wild type or *SLC25A1Δ* cells (Fig. S2G). We also assessed whether activation of the mitochondrial stress response pathway caused APOE elevation (Fig S2B-F). We found that activation of the mitochondrial stress response with doxycycline (Quiros et al., 2017) modestly increased the secretion of APOE in *SLC25A1Δ* cells (Fig S2D columns 7-8) but not in wild type cells (Fig S2D columns 5-6), even though wild type cells robustly activated the mitochondrial stress response after doxycycline incubation (Fig S2E).

Mitochondrial Dysfunction Induces APOE Expression and Inflammatory Responses in Immortalized Cells and Human Astrocytes.

The APOE4 allele is the strongest genetic risk factor for sporadic Alzheimer's disease and APOE-associated neuroinflammation is thought to play a prominent role in disease pathogenesis (Krasemann et al., 2017; Parhizkar and Holtzman, 2022; Tzioras et al., 2019; Zalocusky et al., 2021). We found that disruption of complex I subunit NDUFS3 and complex I assembly factor NDUFAF7 increases APOE expression (Fig. 3). The NDUFS3 and NDUFAF7 genes are encoded in loci that increase Alzheimer's disease risk (de Rojas et al., 2021; Kunkle et al., 2019). Moreover, the complex IV subunit COX7C is an additional electron transport gene encoded in a novel Alzheimer's risk locus (Bellenguez et al., 2022). These genetic associations between APOE and the respiratory chain with Alzheimer's disease risk prompted us to ask the following questions: 1) Do human brain cells modify the expression of APOE after disruption of the respiratory chain? 2) Is mitochondrially-induced APOE expression an isolated event, or does it co-occur in the context of inflammatory response? 3) Does SLC25A1-sensitive gene expression correlate with human cognitive trajectories during aging?

To address the first question, we treated wild type human iPSC-derived neurons and astrocytes with antimycin for 48 hours. Cells were treated with antimycin concentrations that inhibit mitochondrial respiration in neurons and astrocytes (unpublished data), but do not affect cell viability. Astrocytes robustly increased APOE protein expression two-fold after antimycin treatment (Fig. 5A). These astrocytic APOE increases were detected both in cell lysates and conditioned media (Fig. 5A). In contrast, neuronal cells failed to increase APOE expression after exposure to antimycin (Fig. 5A). We did not detect APOE in neuronal conditioned media (not shown). These findings show that APOE expression and secretion are modulated by intoxication of the electron transport chain in iPSC-derived human astrocytes but not neurons. To determine if APOE expression was coordinated with an inflammatory response, we analyzed the expression of 770 genes using a NanoString mRNA quantification panel. This neuroinflammatory panel reports the activity of 23 neuroinflammation pathways and processes across five brain cell types and 14 cell types of the peripheral immune system. We chose this approach since it is validated in Alzheimer's models, highly sensitive, and measures mRNAs without cDNA amplification (Das et al., 2021; Ramesha et al., 2021). We performed NanoString quantification in wild type, *SLC25A1Δ*, and *SLC25A4Δ* HAP1 cells, as well as in iPSC-derived astrocytes treated for 48 hours with sublethal doses of antimycin (Fig. 5B). We identified upregulation of 3-10% of the genes in the panel across these three cellular models (Fig. 5B). Among these genes, APOE mRNA was upregulated 6-fold in mutant HAP1 cells and 1.5 times in antimycin-treated astrocytes as compared to a housekeeping control, TBP (Fig. 5C). The genes upregulated in antimycin-treated astrocytes were significantly enriched in genes annotated to the secretome, as compared to the content of secretome-annotated genes built into the panel (Fig. 5D, compare enrichment factors of 2.8 and 1.6, respectively). Like in *SLC25A1Δ* and *SLC25A4Δ* HAP1 cells, this enrichment of

altered secretory transcripts induced by mitochondrial damage was of a similar magnitude to changes in mitochondrially-annotated transcripts.

We identified four upregulated mRNAs common to these three experimental conditions (Fig. 5E, APOE, TSPAN18, PTPN6, and LOX). The number of hits increased to 45 upregulated mRNAs when considering shared functional annotations (Fig. 5E). These functional annotations were mostly related to cytokine signaling ontologies (Fig. 5F, R-HSA-1280215 and GO:0034097 Log q-value=-25.19 and -18.18). To explore whether the 85 upregulated genes in antimycin-treated astrocytes were part of a neurotoxic A1 reactive astrocyte phenotype associated with neurodegeneration, we compared the fold of induction of these transcripts with the changes in expression of these mRNAs in astrocytes after inflammatory LPS administration to elicit the A1 transcriptional phenotype (Liddelow et al., 2017). There was no correlation in gene expression among these datasets (data not shown). Furthermore, of 16 genes present in the NanoString panel upregulated in reactive astrocytes, the transcript of the C-C Motif Chemokine Ligand 2 (CCL2) protein was the only significantly modified transcript in antimycin-treated astrocytes, but its expression was decreased (Hasel et al., 2021). These results suggest that increased expression of APOE induced by disruption of mitochondria co-occurs with inflammatory mechanisms, which are distinct from known expression signatures of reactive astrocytes induced by LPS.

The expression of APOE and respiratory chain subunits are inversely correlated in a mouse model of Alzheimer's disease

Increased expression of APOE coinciding with an inflammatory response is reminiscent of Alzheimer's pathology. We hypothesized that the expression of APOE and components of the electron transport chain should be inversely correlated if mitochondrial disruption were to increase APOE expression in Alzheimer's brains. To test this hypothesis, we used the 5xFAD mouse model of Alzheimer's disease that expresses human APP and presenilin 1 encoding five human pathogenic mutations that cause familial Alzheimer's disease (Oakley et al., 2006). Pathology in these mice progresses with aging and these transgenes induce age-dependent expression of APOE and inflammatory gene products (Bai et al., 2020; Oakley et al., 2006). We analyzed a cohort of mice of both genotypes from ages 1.8 to 14.4 months (equally balanced for males and females per group), representing young adults to middle-age adult mice. Neuropathology begins at 2 months of age while cognitive impairment begins after 4 months of age in the 5xFAD mouse model (Girard et al., 2014; Oakley et al., 2006).

We performed TMT mass spectrometry on the cortex from 86 mice and quantified over 8,000 proteins from which we performed an analysis on APOE and 914 Mitocarta 3.0 proteins (Rath et al., 2021), including, all subunits of the five complexes of the electron transport chain, and 33 of the SLC25A transporters present in mitochondria. We analyzed whether the expression of brain APOE correlated with the expression of these mitochondrial proteins and determined the effects of age, the 5xFAD genotype, and the interaction of these factors (Fig. 6A-B). APOE

expression across all ages best correlated with the expression of complex I, III and IV subunits in wild type animals (Fig. 6A-B). Expression of these proteins remained constant after 6 months of age (Fig. 6D). In contrast, APOE expression negatively correlated with the expression of complex I, III and IV subunits in 5xFAD mice (Fig. 6A-B). We further examined the effects of age and genotype on the expression of APOE (Fig. 6C-D), all complex I subunits (Fig. 6C), and representative examples of complex I to IV subunits (Fig. 6D). APOE expression remained constant with age in wild type animals (Fig. 6C-D, gray symbols). Similarly, the expression of complex I subunits, such as NDUFS1 and NDUFS3 plateaued at 6 months of age (Fig. 6C-D, gray symbols). However, APOE expression progressively increased 7-fold in 5xFAD animals (Fig. 6C-D, blue symbols) while most complex I subunits, including NDUFS1 and NDUFS3, as well as the complex III and IV subunits, UQCRC1 and COX5A, decreased their expression after 6 months of age in 5xFAD animals (Fig. 6C-D). Complex II, represented by the SDHC subunit, remained mostly unaffected by the genotype and age factors. These correlative results reveal that the expression of APOE and components of the electron transport chain are modulated by age and Alzheimer's causative mutations.

***SLC25A1*-sensitive gene expression correlates with human cognitive trajectory during aging**

We asked whether the expression of APOE, electron transport chain subunits, and SLC25A transporters correlates with age-dependent cognition in humans. We analyzed the Banner cohort of 106 individuals cognitively normal at the time of enrollment (Beach et al., 2015). These adult brain donors were longitudinally assessed to determine their rate of change of cognitive performance over time (i.e., cognitive trajectory), irrespective of neuropathology. Cognitive trajectory refers to change of performance on the Mini-Mental State Exam (MMSE) over time (Folstein et al., 1975). The donors quantitative brain proteomes were obtained postmortem. In this and other cohorts, the expression of mitochondrial proteins, in particular complex I and III subunits, predicts cognitive preservation while proteins annotated to inflammatory ontologies predict a faster cognitive decline (Johnson et al., 2022; Wingo et al., 2019).

We assessed whether the Banner brain protein expression levels of gene products differentially expressed in SLC25A1-null cells, such as electron transport chain subunits (Fig. 1), correlated with cognitive trajectory in the Banner cohort subjects. We used three differentially expressed gene sets from SLC25A1 null cells. The downregulated *SLC25A1Δ* transcriptome dataset (Fig. 1F), which is enriched in genes associated with neuronal differentiation, axon guidance, and neurotransmission ontologies (Fig. 1P); the *SLC25A1Δ* upregulated mRNAs, that includes APOE and sterol synthesis genes (Fig. 1F and P); and the differentially expressed proteins in the *SLC25A1Δ* TMT proteome (Fig. 1B and O). These differentially expressed gene sets were represented by the first principal component of their protein expression levels in each one of the 106 human subjects in the Banner cohort (Fig. 6E, PC1).

We confirmed the expression of some of the transcripts present in the downregulated *SLC25A1Δ* transcriptome by qRT-PCR, both in HAP1 cells and the human neuroblastoma (Fig. S1C), focusing on genes implicated in neurodevelopment and intellectual disability (Abidi et al., 2002; Akita et al., 2018; Ayalew et al., 2012; Bassani et al., 2012; Becker et al., 2009; Curto et al., 2019; Krocher et al., 2014; Kury et al., 2017; Lee et al., 2015; Leonardo et al., 1997; Picard et al., 2009; Shoukier et al., 2013; Srivastava et al., 2014; Tsuboyama and Iqbal, 2021; Vawter, 2000; Yan et al., 2018; Zemni et al., 2000; Zurek et al., 2016). The brain protein expression representing the downregulated mRNAs associated with a decreased rate of cognitive decline in the Banner cohort proteomes (r=0.472, p<4.45e-07, Fig. 6E). In contrast, the brain protein expression representing the *SLC25A1Δ* upregulated mRNAs correlated with an accelerated cognitive decline (r=0.607, p <1.03e-11, Fig. 6E). Like the *SLC25A1Δ* upregulated mRNAs, increased brain expression of the differentially expressed proteins in the *SLC25A1Δ* TMT proteome (Fig. 1B) also correlated with accelerated cognitive decline (r=0.537, p<4.45e-07, Fig. Fig. 6E). The *SLC25A1Δ* upregulated mRNAs and proteome shared sterol synthesis as a top ontology (Fig. 1O-P). These data suggest that genes and proteins sensitive to SLC25A1 expression regulate cellular processes important for cognition.

We used an orthogonal SLC25A1 dataset obtained from SH-SY5Y neuroblastoma cells to further test the hypothesis that SLC25A1 and proteins it regulates influence cognition. We focused on the SLC25A1 interactome, the proteins that co-precipitate with SLC25A1. We previously identified the SLC25A1 interactome using quantitative mass spectrometry and found that this network of proteins is enriched in respiratory chain subunits, particularly complex I, as well as other SLC25A inner mitochondrial membrane transporters (Gokhale et al., 2019). We reasoned that if the SLC25A1 interactome converges on similar biological processes as the *SLC25A1Δ* proteome or transcriptomes, then the SLC25A1 interactome should also have significant correlations with cognitive trajectory in the Banner cohort. In line with this reasoning, we found that greater expression of proteins belonging to the SLC25A1 interactome strongly correlated with a rapid cognitive decline in the Banner cohort (r=0.527, p <9.92e-08, Fig. 6H). We used an unrelated dataset consisting of the 80 subunits of the mammalian cytoplasmic ribosome (CORUM complex #306), which is not affected in *SLC25A1Δ* cells, as a control for spurious correlations (Fig. 1O-P). The expression of these ribosomal proteins did not correlate with the cognitive trajectory of the Banner subjects (r=-0,11 and p=0.249, data not shown). Taken together, our data suggest that a network of mitochondrial proteins, including the respiratory chain and several members of the SLC25A transporter family, regulate APOE expression and other cellular processes modulating cognition.

Discussion

Here we report our discovery that mutations in nuclear-encoded mitochondrial transporter genes, SLC25A1 and SLC25A4, modify the secretome and mitochondrially-annotated proteome to a similar extent (Fig 1N). Intriguingly, mutations in one of these mitochondrial transporters led to upregulation of expression and secretion of the lipoprotein APOE in diverse cellular systems (Fig. 2A-D). We focused on APOE, as it is a main risk factor for Alzheimer's disease, a disease that compromises mitochondrial function (Wang et al., 2020). We show that this mitochondrial-dependent APOE upregulation phenotype also occurs in response to mutations in other inner mitochondrial membrane SLC25A transporters (Fig. 4F, S3). In addition, we show APOE upregulation results when assembly and function of electron transport chain complexes I, III, and IV are perturbed, genetically or pharmacologically (Fig. 3, 4). This includes disruption of copper supply to mitochondria, a requirement of complex IV function (Fig. 4). The mitochondrial protein network modulating APOE expression includes proteins encoded by prioritized genes within Alzheimer's disease risk loci necessary for electron transport chain complex I assembly and function (de Rojas et al., 2021; Kunkle et al., 2019) (Fig 3). Furthermore, we show that this mitochondrial regulation of APOE expression extends to brain cells, iPSC-derived human astrocytes, and co-occurs with an inflammatory gene expression response (Fig. 5). Together, our data demonstrate that mitochondria robustly regulate APOE expression and secretion, placing them in a novel position upstream of APOE. We propose that this mitochondria-to-APOE mechanism may operate in the pathogenesis of dementia, a proposition supported by our protein expression correlation studies in mouse Alzheimer's brains and aging human brains (Fig. 6).

Our findings expand previous work demonstrating that mitochondrial distress regulates the secretion of inflammatory cytokines and type I interferons (Dhir et al., 2018; Riley and Tait, 2020; Shimada et al., 2012; West et al., 2015), the growth factor mitokines GDF15 and FGF21 (Chung et al., 2017; Kim et al., 2013), the production of mitochondrially-derived peptides encoded in the mitochondrial genome (Kim et al., 2017), and alpha fetoprotein in hepatocytes (Jett et al., 2021). Our study makes two key contributions expanding on these non-cell autonomous mechanisms. First, we identified the first apolipoprotein whose expression and secretion is modulated by mutations affecting mitochondria. Second, while we focus on APOE, we think this apolipoprotein is a harbinger of an extended upregulation of the secreted proteome mediated by mitochondria (Uhlen et al., 2019). The importance of this secreted proteome change can be inferred from its extent and magnitude, which is on par with the changes we observed in the mitochondrially-annotated proteome (Fig 1N, 5D). Our findings add to the growing notion that mitochondrial regulation of the secreted proteome is a more common process than previously appreciated.

Our results support the hypothesis that there are multiple intramitochondrial mechanisms connecting mitochondria to APOE expression and secretion, including copper-

dependent mechanisms. Mitochondrial complex IV depends on copper for its assembly and function. Genetic disruption of copper loading on complex IV with mutants targeting copper flow at the plasma membrane (SLC31A1 and ATP7A), mitochondrial copper transport (SLC25A3), and the chaperone mediating loading of copper onto complex IV (COX17) all produced elevated APOE expression and secretion (Fig. 4). Interestingly, APOE upregulation was heightened with increasing proximity of the copper pathway step to complex IV itself. For instance, APOE protein levels were increased approximately two-fold in SLC31A1 mutants while COX17 mutants displayed a robust 15-fold increase in APOE levels (Fig. 4A, C). Pharmacological targeting of the copper pathway with the copper chelator BCS also increased APOE expression (Fig 4G). In addition to complex IV requiring copper, complexes I to III require iron for electron transport (Stiban et al., 2016)**.** We disrupted complex I either directly by mutagenesis of either NDUFS3 or NDUFAF7, or indirectly by mutating SLC25A1 (Fig. 3). In addition, we disrupted complex III pharmacologically with antimycin in human iPSC-derived astrocytes and SLC25A1-null cells, where residual complex III activity is likely targeted. Mutagenesis of SLC25A1 and SLC25A4 indirectly targeted complex III. All these genetic and pharmacological disruptors increased APOE levels, supporting the idea that copper- and iron-dependent steps of the respiratory chain modulate APOE expression. However, mutagenesis of the mitochondrial carnitine transporter SLC25A20 also upregulated APOE, despite these cells respiring normally and having wild type levels of respiratory chain subunits (Fig S3). Thus, we postulate that multiple mitochondrial mechanisms exist that contribute to APOE expression regulation, spanning metal-dependent electron transport chain disassembly to metal-independent mitochondrial mechanisms not directly related to electron transport chain function.

We asked whether the APOE increase may be part of an inflammatory response by using NanoString mRNA quantification to assess the levels of activity of interleukin and interferon pathways in *SLC25A1Δ* and *SLC25A4Δ* cells and antimycin-treated astrocytes. Although *SLC25A4Δ* cells had a paucity of significant hits compared with *SLC25A1Δ* cells and antimycin-treated astrocytes, we found that all three experimental conditions converged on common hits whose number increased when considering hits belonging to shared ontologies (Fig. 5E). All cells showed increased levels of secreted cytokines and gene ontologies associated with their transcriptional profiles were enriched for cytokine signaling pathways (Fig. 5B, F). Thus, our data suggest that APOE upregulation in response to mitochondrial dysfunction could be part of an inflammatory response initiated by mitochondria. Although mitochondria are often viewed as downstream targets of neuroinflammation, our results with antimycin-treated astrocytes add to growing evidence that mitochondria drive inflammatory signaling in the nervous system (Bader and Winklhofer, 2020; Joshi et al., 2019; Lin et al., 2022).

We tested several additional hypotheses that could account for the link between mitochondrial dysfunction and heightened nuclear expression of APOE. We assessed whether decreased cytoplasmic ATP levels led to APOE upregulation. The AMPK pathway senses drops in

ATP cytoplasmic levels and coordinates a response to increase ATP generation when cellular energy is depleted **(**Herzig and Shaw, 2018). If decreased ATP levels mediate increased APOE protein levels, SLC25A1-null cells should display activation of the AMPK pathway at baseline. We found that, while SLC25A1-null cells are sensitized to respond to an AMPK-activating drug, the pathway is minimally active in the cells at baseline (Fig S2A, compare P-AMPK in lanes 3 and 4). This falls in line with our previous finding that ATP levels between SLC25A1-null and wild type cells do not differ (Gokhale et al., 2019). A second possible mechanism is that APOE expression is a coordinated response with an upregulation of cholesterol synthesis via SREBP transcription factors (Horton et al., 2002). We found that SLC25A1-null HAP1 cells had increased expression of cholesterol synthesis pathway enzymes, accompanied by elevated free cholesterol and cholesterol esters, consistent with SREBP transcription factor activation. However, these phenotypes were not shared by SLC25A1-null neuroblastoma cells or SLC25A4-null cells, even though these cells also displayed increased APOE expression and secretion (Fig. 2, S2C). Thus, increased APOE resulting from mitochondrial dysfunction is not dependent on cholesterol synthesis pathways. A third mitochondria-to-nucleus pathway we investigated is mediated by activation of transcription factors ATF4 and CHOP (Quiros et al., 2017). These factors control the expression of mitokines in response to mitochondrial stress as part of the integrated stress response transcriptional pathway (Chung et al., 2017; Kim et al., 2013). We assessed whether activation of the integrated stress response pathway also causes increased APOE. Administration of doxycycline to trigger the stress response mounted an appropriate transcriptional response (Quiros et al., 2017) in both wild type and SLC25A1-null cells (Fig. S2E-F), although this response was somewhat blunted in the mutant cells, even at baseline (Fig. S2B, F). Despite stress response activation, cellular and secreted levels of APOE protein were unaffected besides a mild increase in secretion in the SLC25A1-null cells (Fig. S2D). Thus, the ATF4-dependent stress response alone cannot account for elevated APOE expression and secretion. A fourth mechanism that could account for the increased APOE expression is dependent on redox imbalance. We think this mechanism is unlikely because the antioxidant N-acetyl cysteine decreased the expression of the mitochondrial glutathione-disulfide reductase in both wild type and SLC25A1-null cells, but it did not change APOE mRNA levels. Finally, we looked for transcriptional signatures in the SLC25A1 and SLC25A4 upregulated transcriptomes and found neither common predicted transcription factors that could account for the changes in gene expression in both genotypes, nor transcription factors known to regulate APOE transcription such as LXRs or C/EBPβ (Fig. S2C) (Laffitte et al., 2001; Xia et al., 2021)**.** We suggest that a screen targeting large swaths of the genome in astrocytes and mutant cell lines used in this study would be an effective method for uncovering further mechanisms by which mitochondria contribute to APOE expression regulation.

What purpose does increased APOE expression and secretion in response to mitochondrial dysfunction serve for the cell? Increased APOE secretion induced by mitochondrial

damage could be adaptive or maladaptive depending on the lipidation and contents of APOE. APOE-mediated lipid exchange between cells can alter the lipid microenvironments of cellular membranes, thereby influencing cell signaling and homeostasis (Martens et al., 2022; Tambini et al., 2016). APOE is a primary lipoprotein in the brain produced mainly by astrocytes, though neurons and other glia also express APOE (Belloy et al., 2019; Martens et al., 2022). APOE particles play necessary roles in handling toxic lipids by shuttling them between cell types, with differential effects depending on the cell type from which the APOE particle originated, the lipid species loaded in the particle, and the fate of the lipid-loaded particle (Guttenplan et al., 2021; Ioannou et al., 2019; Liu et al., 2017). APOE-dependent removal of toxic factors from the cell of origin could explain our finding that dialyzed conditioned media from SLC25A1 mutant cells cannot support their own growth. Astrocytes play a prominent role in carrying and processing toxic lipids in the brain through APOE-dependent mechanisms. Since we observed increased APOE secretion in astrocytes, but not neurons, in response to antimycin, we speculate that APOE released from astrocytes following antimycin administration may not sustain either astrocytes and/or neurons. Profiling the contents of antimycin-induced APOE particles released from astrocytes could further clarify their potential impact on the function and health of neighboring cells.

The APOE E4 allele is the strongest genetic risk factor for sporadic Alzheimer's disease and APOE, along with amyloidogenic processing of the amyloid precursor protein (APP) into Aβ, is thought of as an initiating and driving factor in disease etiology (Frisoni et al., 2022; Huang and Mahley, 2014; Martens et al., 2022). While mitochondrial dysfunction is acknowledged as an important factor in Alzheimer's disease, mitochondria are typically placed downstream of Aβ and APOE, with Aβ or the APOE4 allele perturbing mitochondrial function (Area-Gomez et al., 2020; Chen et al., 2011; Huang et al., 2020; Orr et al., 2019; Tambini et al., 2016; Yin et al., 2020). Some have proposed that mitochondria act as upstream factors in Alzheimer's pathogenesis through metabolic and bioenergetic effects (Rangaraju et al., 2018; Swerdlow, 2018; Wang et al., 2020). We argue that mitochondria could participate in Alzheimer's pathogenesis through modulation of APOE allele-dependent mechanisms. The increased expression of APOE, which is inversely correlated with the expression of respiratory chain subunits in the 5xFAD mouse model, supports this proposition (Fig. 6). For example, increased expression of APOE E4 downstream of mitochondrial dysfunction could either initiate or exacerbate the effects of the APOE4 allele in neurodegenerative processes, since APOE4 is prone to aggregation and poor lipidation (Gong et al., 2002; Hatters et al., 2006; Hubin et al., 2019). Our findings open the possibility that mitochondria could act as initiators or drivers of Alzheimer's pathogenesis through modulation of APOE-dependent disease processes. The correlation of expression levels in the prefrontal cortex of proteins dysregulated in the SLC25A1 proteome and transcriptome with human cognitive trajectory provides evidence for this conception. Together, our work supports the idea

that mitochondria influence brain function and cognition in part through modulation of the secretome, including a novel role in regulation of APOE expression and secretion.

Materials and Methods

Cell lines, gene editing, and culture conditions

Human haploid (HAP1) isogenic cell lines were obtained from Horizon Discovery. In addition to the parental wild type line (C631, RRID: CVCL_Y019), the following CRISPR/Cas9-edited knockout cell lines were used: SLC25A1 (HZGHC001753c010, RRID: CVCL_TM05 and HZGHC001753c003, RRID: CVCL_TM04), SLC25A3 (HZGHC000792c010, RRID:CVCL_TM31), SLC25A4 (HZGHC000778c011, RRID:CVCL_TM45), SLC25A20 (HZGHC000787c00, RRID:CVCL_TM21), SLC25A50/MTCH2 (HZGHC23788), NDUFS3 (HZGHC4722, RRID:CVCL_XQ89), NDUFAF7 (HZGHC55471), PARK2 (HZGHC003208c002, RRID:CVCL_TC07). All HAP1 cells were grown in IMDM (Corning 10-016) with 10% Fetal Bovine Serum (FBS) (VWR, 97068-085) in a 10% CO2 incubator at 37°C, unless otherwise indicated. In experiments where a single clone of SLC25A1-null cells was used, the cell line HZGHC001753c010 (RRID: CVCL_TM05) was used. For each knockout cell line, an individual control line of the HAP1 parental line that was received with the particular knockout line was used. Mutants and their unique control line were cultured and handled as parallel pairs to avoid passage- and culture-induced variation.

Human neuroblastoma SH-SY5Y cells (ATCC, CRL-2266; RRID:CVCL_0019) were grown in DMEM media (Corning, 10-013) containing 10% FBS (VWR, 97068-085) at 37°C in 10% CO_2, unless otherwise indicated. SH-SY5Y cells deficient in SLC31A1 were genome edited using gRNA and Cas9 preassembled complexes by Synthego with a knock-out efficiency of 97%. The gRNAs used were UUGGUGAUCAAUACAGCUGG which targeted transcript ENST00000374212.5 exon 3. Wild-type and mutant cells were cloned by limited dilution and mutagenesis was confirmed by Sanger sequencing with the primer: 5'GGTGGGGGCCTAGTAGAATA. SH-SY5Y cells deficient in ATP7A were genome edited by Synthego using gRNA and Cas9 preassembled complexes with a knock-out efficiency of 80%. The gRNAs used were ACAGACUCCAAAGACCCUAC which targeted transcript ENST00000341514 exon 3. Wild-type and mutant cells were cloned by limited dilution and mutagenesis was confirmed by Sanger sequencing with the primer: 5'TGCCTGATAGGTACCACAGTC. SH-SY5Y cells deficient in Cox17 were genome edited by Synthego using gRNA and Cas9 preassembled complexes with a knock-out efficiency of 94%. The gRNAs used were CCAAGAAGGCGCGCGAUGCG which targeted transcript ENST00000261070 exon 1. Wild-type and mutant cells were cloned by limited dilution and mutagenesis was confirmed by Sanger sequencing with the primer: 5'AGGCCCAATAATTATCTCCAGAGC. The Cox17-deficient cells were supplemented with 50ug/ml uridine (Sigma, U3003) in their growth media. SH-SY5Y cells deficient in SLC25A1 were genome edited by Synthego using gRNA and Cas9 preassembled complexes with a knock-out efficiency of 86%. The gRNAs used were GGGTTCCCGGTCCCTGCAGG which targeted transcript ENST00000215882 exon 2. Wild-type and mutant cells were cloned by limited dilution and mutagenesis was confirmed by Sanger sequencing with the primer: 5'GATGGAACCGTAGAGCAGGG. For each mutant cell line, an

individual control line of the SH-SY5Y parental line that was received with the particular mutant line was used. In APOE protein measurement immunoassays, two separate clones of cells were used to exclude clonal or off-target effects.

ATP7A LL cells contained a di-leucine mutation generated by introducing alanine in L1750 and 1751 by the Emory Genomics Core into the SH-SY5Y ATP7A CRISPR KO cells. HA-tagged ATP7A cDNA in pQCXIP backbone vector was provided by Dr. Michael Petris. SH-SY5Y ATP7A LL and empty vector expressing cells were generated by transfecting ATP7A KO10 cells with lipofectamine 3000 (Invitrogen). Selection was started after 24 h with 0.5ug/ml Puromycin (Gibco). Growth of clones was boosted by supplementing the media with 1uM Copper until sufficient cells grew to freeze down and passage well. All ATP7A and SLC31A1 KO cells were validated by measuring copper content using ICP mass spectrometry (manuscript in preparation).

HEK293 cells were grown in DMEM containing 10% FBS, supplemented with 2 mM L-glutamine (HyClone, SH30034.01), 100 µg/ml zeocin (Gibco, R25001), and 15 µg/ml Blasticidin S (Sigma, SBR00022). The ANT1, ANT2, ANT3 triple knockout cells were additionally supplemented with 1 mM sodium pyruvate (Sigma, S8636) and 50 µg/ml uridine (Sigma, U3003). gRNAs that targeted ANT1, ANT2, and ANT3 were separately cloned into PX459, a gift from Feng Zhang (Addgene). gRNA sequences are GATGGGCGCTACCGCCGTCT (for ANT1), GGGGAAGTAACGGATCACGT (for ANT2), CGGCCGTGGCTCCGATCGAG (for ANT3), respectively. T-REx™ 293 cells (Invitrogen R71007) were transfected with the PX459 constructs individually in order of ANT3, ANT2, and ANT1. Following each transfection, cells were selected with puromycin (2 µg/ml) for 72 hours, single clones were isolated by ring cloning and the absence of the protein expression was analyzed by immunoblotting before proceeding to the next transfection.

Antibodies

Antibody	Dilution	Cat.No	RRID
SDHA	1:1000	11998	AB_2750900
SLC25A1	1:500	15235-1-AP	AB_2297856
HSP90	1:1000	610418	AB_397798
MT-ATP6	1:500	55313-1-AP	AB_2881305
MTCO2	1:1000	AB110258	AB_10887758
TFRC	1:1000	13-6800	AB_86623
ACTIN	1:5000	A5441	AB_476744
COX4	1:1000	4850	AB_2085424
OXPHOS mix	1:250	ab110412	AB_2847807
NDUFS3	1:200	15066-1-AP	AB_2151109
UQCRC2	1:500	ab 14745	AB_2213640
HSP90	1:1000	610418	AB_397798

APOE	1:250	60531	AB_920623
AMPK	1:1000	2532S	AB_330331
P-AMPK	1:1000	2535T	AB_331250
FLAG	1:1000	A190-102A	AB_67407
mouse HRP	1:5000	A10668	AB_2534058
rabbit HRP	1:5000	G21234	AB_2536530

Primers

Primer	Forward Sequence	Reverse Sequence
HMGCR	ACAGATACTTGGGAATGCAGAG	CTGTCGGCGAATAGATACACC
APOE	TGGGTCGCTTTTGGGATTAC	TTCAACTCCTTCATGGTCTCG
ATF3	GGAGCCTGGAGCAAAATGATG	AGGGCGTCAGGTTAGCAAAA
ATF4	CAGCAAGGAGGATGCCTTCT	CCAACAGGGCATCCAAGTC
ATF5	GAGCCCCTGGCAGGTGAT	CAGAGGGAGGAGAGCTGTGAA
CHOP	AGCCAAAATCAGAGCTGGAA	TGGATCAGTCTGGAAAAGCA
ASNS	ATCACTGTCGGGATGTACCC	TGATAAAAGGCAGCCAATCC
CHAC1	GTGGTGACGCTCCTTGAAGA	TTCAGGGCCTTGCTTACCTG
PCK2	CATCCGAAAGCTCCCCAAGT	GCTCTCTACTCGTGCCACAT
WARS	TCAGCAACTCATTCCCACAG	GCAGGGCTGGTTTAGGATAG
RER1	CTTTCTTCGACGCTTTCAACG	CTGTACCTTCTCTTCCCATGTG
PCBP1	AAGACTTGACCACGTAACGAG	ATGCTTCCTACTTCCTTTCCG
GSR	TTCCAGATGTTGACTGCCTG	GCCTTTGACGTTGGTATTCTG
ACAT2	CCCAGAACAGGACAGAGAATG	AGCTTGGACATGGCTTCTATG
MSMO1	TGAACTTCATTGGAAACTATGCTTC	TCTTTCAGGAAGGTTTACGTGAG
LDLR	TTCACTCCATCTCAAGCATCG	ACTGAAAATGGCTTCGTTGATG
RPS20	TGCTGACTTGATAAGAGGCG	GATCCCACGTCTTAGAACCTTC

VAMP2	TCATCTTGGGAGTGATTTGCG	GGGCTGAAAGATATGGCTGAG
TBP	GAGAGTTCTGGGATTGTACCG	ATCCTCATGATTACCGCAGC
TSPAN7	CCTTATTGCCGAGAACTCCAC	ACACCAGGGACAGAAACATG
GABRA5	CCTCCATATTCACCTGCTTCA	CTGGTTGGCATCTGTGAAAAG
GABRB2	ACTCAGAATCACAACCACAGC	CCACGCCAGTAAAACTCAATG
NRG2	CACTCCTGTTCTCCTTCTCAC	TTTGCTGGTACCCACTGATG
CHL1	ATGGCTCCCCAGTTGACA	TGATTTGGTTGAAGGTTGGTAA
NCAM1	TTGTTTTTCCTGGGAACTGC	ACTCTCCAACGCTGATCTCC
ST8SIA4	CATTAGGAAGAGGTGGACGATC	AGAGCTATTGACAAGTGACCG
TRPC3	CAAATGCAGAAGGAGAAGGC	CGTGTTGGCTGATTGAGAATG
UNC5A	CTGTACCAGTGACCTCTGTG	AAACGAGGATGAGGACAAGC
CAMK2B	CAGCCAGAGATCACCAGAAG	CACCAGTGACCAGATCGAAG

Generation of iNeurons from Human iPSCs

Accutase (Gibco, A11105) was used to disassociate iPSC cells. On day -2, the cells were plated on a 12-well plate coated in matrigel (Corning, 354230) in mTeSR medium (StemCell Technologies, 85850) at a density of at 380,000 cells per well. On day −1, hNGN2 lentivirus (TetO-hNGN2-P2A-PuroR (RRID: Addgene_79049) or TetO-hNGN2-P2A-eGFP-T2A-PuroR (RRID: Addgene_79823)) together with FUdeltaGW-rtTA (RRID: Addgene_19780) lentivirus were added in fresh mTeSR medium containing 4µg/µl polybrene (Sigma, TR-1003). The lentiviruses were added at 1×106 pfu/ml per plasmid (multiplicity of infection (MOI) of 2). On day 0, the culture medium was replaced with fresh KSR, consisting of KnockOut DMEM (Gibco, 10829018), Knockout Replacement Serum (Gibco, 10828028), 1X Glutamax (Gibco, 35050061), 1 X MEM Non-essential Amino Acids (NEAA, Gibco, 11140050) and 100µM β-Mercaptoethanol (Gibco, 21985023). In addition, 2µg/ml doxycycline (Sigma, D9891) was added to the media, inducing TetO gene expression. Doxycycline was retained in the medium until the end of the experiment. Puromycin selection (5µg/ml) was started on day 1. On day 4, the culture medium was replaced with Neurobasal medium (Gibco, 21103049), supplemented with B27 supplement (Gibco, 17504044), 1 X Glutamax, 20% Dextrose (Sigma, D9434), 10ng/ml BDNF (Peprotech, 45002), 10ng/ml GDNF (PeproTech, 45010), 2µg/ml doxycycline (Sigma, D9891) and 5µg/mL puromycin

(InvivoGen, ant-pr-1). Beginning on day 7, half of the medium in each well was replaced every week. On day 24, hNGN2-induced neurons were assayed (Zhang et al., 2013).

Differentiation of iPSCs into forebrain specific neural progenitors and astrocytes

One hour of 1 mg/ml collagenase (Therm Fisher Scientific, 17104019) treatment was used to detach iPSC colonies. Following collagenase treatment, cells were suspended in embryoid body (EB) medium in non-treated polystyrene plates for 7 days. During this time, the medium was changed daily. EB medium consisted of DMEM/F12 (Gibco, 11330032), 20% Knockout Serum Replacement (Gibo, 10828028), 1 X Glutamax (Gibco, 35050061), 1 X MEM Non-essential Amino Acids (NEAA, Gibco, 11140050), 100µM β-Mercaptoethanol (Gibco, 21985023), 2 µM dorsomorphin (Tocris, 3093) and 2 µM A-83 (Tocris, 692). After 7 days, EB medium was replaced by neural induction medium (hNPC medium), consisting of DMEM/F12, 1 X N2 supplement (Gibco, 17502048), B27 supplement, 1X NEAA, 1 X Glutamax, 2 µg/ml heparin (Sigma) and 2 µM cyclopamine (Tocris, 1623). On day 7, the floating EBs were then transferred to Matrigel-coated 6-well plates to form neural tube-like rosettes. The attached rosettes were kept for 15 days. During this time, the hNPC media of the rosettes was changed every other day. On day 22, the rosettes were picked mechanically and transferred to low attachment plates (Corning) in hNPC medium containing B27 supplement.

For astrocyte differentiation (Tcw et al., 2017), resuspended neural progenitor spheres were disassociated with accutase at 37°C for 10 min. After disassociation, they were placed on Matrigel coated 6 well plates. Forebrain NPCs were maintained at high density in hNPC medium. To differentiate NPCs to astrocytes, disassociated single cells were seeded at a density of 15,000 cells/cm^2 on Matrigel-coated plates and grown in astrocyte medium (ScienCell: 1801, astrocyte medium (1801-b), 2% fetal bovine serum (0010), astrocyte growth supplement (1852) and 10U/ml penicillin/streptomycin solution (0503)). From day 2, cells were fed every 48 hours for 20-30 days. Astrocytes were split when the cells reached 90-95% confluency (approximately every 6-7 days) and seeded again at their initial seeding density (15,000 cells/cm^2) as single cells in astrocyte medium.

Seahorse metabolic oximetry

Extracellular flux analysis was performed on the Seahorse XFe96 Analyzer (Seahorse Bioscience) following manufacturer recommendations. HAP1 cells and SH-SY5Y cells were seeded at a density of 40,000 cells/well on Seahorse XF96 V3-PS Microplates (Agilent Technologies, 101085-004) after being trypsinized and counted (Bio-Rad TC20 automated Cell Counter) All Hap1 cells were grown in normal growth media (IMDM) with 10% FBS except for Ndufaf7-null cells and their control line, which were also supplemented with uridine at 50ug/ml (Sigma, U3003). SH-SY5Y cells were grown in normal growth media (DMEM) with 10% FBS, except for COX17-deficient cells and their control line, which were also supplemented with uridine at

50ug/ml (Sigma, U3003). XFe96 extracellular flux assay kit probes (Agilent Technologies, 102416-100) incubated with the included manufacturer calibration solution overnight at 37°C without CO_2 injection. The following day, wells were washed twice in Seahorse stress test media. The stress test media consisted of Seahorse XF base media (Agilent Technologies, 102353-100) with the addition of 2 mM L-glutamine (HyClone ,SH30034.01), 1 mM sodium pyruvate (Sigma, S8636), and 10 mM D-glucose (Sigma, G8769). After washes, cells incubated at 37°C without CO_2 injection for approximately 1 hour prior to the stress test. During this time, flux plate probes were loaded and calibrated. After calibration, the flux plate containing calibrant solution was exchanged for the Seahorse cell culture plate and equilibrated. Seahorse injection ports were filled with 10-fold concentrated solution of oligomycin A (Sigma, 75351), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; Sigma, C2920), and rotenone (Sigma , R8875) mixed with antimycin A (Sigma, A8674) for final testing conditions of oligomycin (1.0 µM), FCCP (0.125 µM for Hap1, 0.25 µM for SH-SY5Y), rotenone (0.5 µM), and antimycin A (0.5 µM). In antimycin A sensitivity experiments, a separate solution of antimycin A was made at 100nm and 50nm concentrations to give a concentration of 10nm and 5nm in the well. These Seahorse drugs were dissolved in DMSO and diluted in Seahorse stress test media for the Seahorse protocol. The flux analyzer protocol included three basal read cycles and three reads following injection of oligomycin A, FCCP, and rotenone plus antimycin A. In antimycin sensitivity experiments, antimycin A was injected following the basal read cycles and ten reads were taken before the protocol proceeded as usual with injections of oligomycin A, FCCP, and rotenone plus antimycin A. Each read cycle included a 3 minute mix cycle followed by a 3 minute read cycle where oxygen consumption rate was determined over time. In all experiments, oxygen consumption rate readings in each well were normalized by protein concentration in the well. Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23227) according to manufacturer protocol. The BCA assay absorbance was read by a BioTek Synergy HT microplate reader using Gen5 software. For data analysis of oxygen consumption rates, the Seahorse Wave Software version 2.2.0.276 was used. Experiments were repeated at least in triplicate. Non-mitochondrial respiration was determined as the lowest oxygen consumption rate following injection of rotenone plus antimycin A. Basal respiration was calculated from the oxygen consumption rate just before oligomycin injection minus the non-mitochondrial respiration. Non-mitochondrial respiration was determined as the lowest oxygen consumption rate following injection of rotenone plus antimycin A. ATP-dependent respiration was calculated as the difference in oxygen consumption rates just before oligomycin injection to the minimum oxygen consumption rate following oligomycin injection but before FCCP injection. Maximal respiration was calculated as the maximum oxygen consumption rate of the three readings following FCCP injection minus non-mitochondrial respiration.

Electrochemiluminescent immunoassays for APOE protein measurement

HAP1, SH-SY5Y, and HEK293 cells were plated in 6 well dishes (Falcon, 353046). Each experimental condition was plated in four replicate wells. iPSC neurons and astrocytes were plated in 24 well dishes and grown to approximately 80% confluence. Eight wells were used for each experimental condition. Material from two wells was combined to generate an experimental replicate. For experiments where there was no drug treatment, cells were left overnight and samples were collected 22 – 26 hours later. In these experiments, Hap1 cells were plated at a density of 750,000 cells per well and SHYSY5Y cells were plated at a density of 1,000,000 cells per well. In experiments with doxycycline applied at 9.75µm (Sigma, D9891), cells were left incubating for 46-50 hours in drug or vehicle (cell culture grade water) before sample collection, with fresh drug and vehicle media applied after approximately 24 hours. In these experiments, Hap1 cells were plated at a density of 300,000 cells per well and SH-SY5Y cells were plated at a density of 500,000 cells per well. To collect samples of cell lysate and conditioned media, cells were washed twice with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$, and then lysed on a rocker at 4°C in Buffer A (10mM HEPES, 0.15M NaCl, 1mM EGTA, and 100µM $MgCl_2$) + 0.5% Triton X-100 containing 10% protease inhibitor cocktail cOmplete (Roche, 118614500, prepared at 50x in distilled water). The lysis was spun down at 4°C at 13,000 rpm for 20 mins. The supernatant was collected and flash frozen for APOE protein measurement. Conditioned media was spun down at 4°C at 13,000 rpm for 15 mins to clarify before collecting and flash freezing for APOE protein measurement. Growth media unexposed to cells was incubated and collected in the same manner to serve as a control for APOE present in the media that might be detected by the antibody. Cell lysis and media samples were stored at -80°C for up to a month before APOE was measured. Media was spun down again at 4°C, 2000rpm for 5 mins to get rid of any precipitate after thawing. APOE measurements were conducted by the Emory Immunoassay Core facility using electrochemiluminescent detection of the APOE antibody (Meso Scale Diagnostics, F212I) according to manufacturer protocols with the suggested reagents (Diluent 37 (R50A), MSD GOLD Read Buffer A (R92TG), and MSD GOLD 96-well Small Spot Streptavidin SECTOR Plates (L45SA-1)). Samples were not diluted for the electrochemiluminescence protocol (Chikkaveeraiah et al., 2012; Gaiottino et al., 2013).

Conditioned media dialysis

HAP1 SLC25A1-null cells and their control cell line were grown for approximately 48 hours to confluency before collecting their media. The conditioned media was spun down for 5 minutes at 800g and 4°C and then filtered with a syringe and 0.22 µm filter (Millipore, SLGV033RS) and stored at 4°C for no more than three days before being applied to cells. Conditioned media was dialyzed using 10K molecular weight cutoff cassettes (Thermo Scientific, 88252) according to manufacturer instructions. Cassettes were dialyzed 3 times consecutively with the volume in the cassette to the naïve media (IMDM, Corning 10-016) being 1:1000. The media was exchanged

after 3 hours of dialysis for each cycle, with the final dialysis cycle occurring overnight. The dialyzed media was collected the next morning and stored at 4°C for no more than three days before being applied to cells. Viability of cells exposed to the conditioned media was assessed using an Alamar blue assay. For Alamar blue cell viability, HAP1 cells were plated at a density of 2000 cells/well in a 96 well plate and allowed to adhere for 30 mins in normal growth media, IMDM (Corning 10-016) with 10% FBS, before this media was swapped out with conditioned media (dialyzed or undialyzed) collected previously. Cells grew in conditioned media for approximately 48 hours, with fresh conditioned media being put on after approximately 24 hours. After 48 hours, viability was measured with the BioTek Synergy HT plate reader using Gen5 software after a 2 hour incubation in Alamar blue (R&D Systems, AR002). Wells without cells and incubated in Alamar blue were used as a background reading.

RNA extraction, cDNA preparation, and qPCR

In all experiments, cell growth media was changed approximately 24 hours before RNA extraction occurred. At least three replicate plates per experimental condition were used in each experiment. For experiments with doxycycline (Sigma, D9891) and N-Acetyl-L-cysteine (Sigma, A9165), cells incubated in the drug or vehicle (cell culture grade water, Corning 25-055) for 48 hours and fresh drug or vehicle was applied in new media 24 hours before RNA extraction. Doxycycline was applied at 9.75µM and N-Acetyl-L-cysteine was applied at 2mM. RNA was extracted from cells using Trizol reagent (Invitrogen, 15596026). Cells were washed twice in ice-cold PBS containing 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$ and then 1 ml of Trizol was added to the samples. A cell scraper was used before collecting the sample into a tube. The samples in Trizol incubated for 10 min at room temperature on an end-to-end rotator and then 200 µl of chloroform was added to each tube. After vigorous vortexing and a brief incubation, the chloroform mixture was centrifuged at 13,000 rpm at 4°C for 15 min. The aqueous layer was collected and 500 µl of isopropanol was added to it. The isopropanol mixture then rotated for 10 min at room temperature, followed by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant was discarded and the remaining pellet was washed with freshly prepared 75% ethanol in Milli-Q purified water using a brief vortex to lift the pellet. After washing, the pellet in ethanol was centrifuged again for 5 minutes at 13,000 rpm at 4°C. The ethanol was aspirated and the pellet was allowed to air dry. Lastly, the pellet was dissolved in 20-50 µl of molecular grade RNAase-free water and stored overnight at -20°C before the concentration and purity were checked using the Nanodrop One^{C} (Thermo Fisher Scientific). cDNA was synthesized using 5 µg RNA as a template per reaction with the Superscript III First Strand Synthesis System Kit (Invitrogen, 18080-051) and its provided random hexamers. RNA was incubated with the random hexamers and dNTPs at 65°C for 5 min. The samples were then placed on ice while a cDNA synthesis mix was prepared according to the kit instructions. The cDNA synthesis mix was added to each of the tubes and the samples were then incubated at 25°C for 10 min, followed by 50 min

at 50°C. The reaction was terminated at 85°C for 5 min. Finally, the samples were treated with kit-provided RNase H at 37°C for 20 min. A BioRad T100 thermal cycler was used to carry out this synthesis protocol.

For qPCR, the Real-Time qPCR Assay Entry on the IDT website was used to design primers. Primers were synthesized by Sigma-Aldrich Custom DNA Oligo service. Primer annealing and melting curves were used to confirm primer quality and specificity for single transcripts. qRT-PCR was performed using LightCycler 480 SYBR Green I Master (Roche, 04707516001) with 1 µl of the newly synthesized cDNA on the QuantStudio 6 Flex instrument (Applied Biosystems) in a 96 well format. The qRT-PCR protocol carried out by the QuantStudio 6 Flex consisted of initial denaturation at 95°C for 5 min, followed by 45 cycles of amplification with a 5 s hold at 95°C ramped at 4.4°C/s to 55°C. Temperature was maintained for 10 s at 55°C, then ramped up to 72°C at 2.2°C/s. Temperature was held at 72°C for 20 s and a single acquisition point was collected before ramping at 4.4°C/s to begin the cycle anew. The temperature was then held at 65°C for 1 min and ramped to 97°C at a rate of 0.11°C/s. Five acquisition points were collected per °C. Standard curves collected for each individual primer set were used to quantify data using QuantStudio RT-PCR Software version 1.2.

Lipidomics

HAP1 or SH-SY5Y cells were grown to 80 – 90% confluency on 15cm sterile dishes in normal growth media. Material from 2-4 plates was combined for a single replicate and four replicates were used for an experiment. The cells were washed three times in ice-cold PBS containing 10 mM EDTA and then incubated for approximately 30 minutes at 4°C. After incubation, the cells were lifted from the plate on ice and spun down at 800 rpm for 5 minutes at 4°C. Since SH-SY5Y cells lifted easily, the incubation step was skipped in these experiments. Cells were resuspended in ice-cold PBS and a cell count was then taken (Bio-Rad TC20 automated Cell Counter) for normalization and to ensure cells were at least 90% alive and at least 20 million cells would be present in each replicate sample. After counting, cells were again spun down at 800 rpm for 5 minutes at 4°C and the resulting cell pellets were flash-frozen and stored at -80°C for a few weeks before lipidomic analysis was performed by the Emory Integrated Metabolomics and Lipidomics Core.

Briefly, for lipidomics HPLC grade water, chloroform, methanol, and acetonitrile were purchased from Fisher Scientific (Hampton, NH, USA). Formic acid and ammonium acetate were purchased from Sigma Aldrich (St. Louis, MO, USA). Lipid standards SPLASH® LIPIDOMIX® Mass Spec (cat# 330707) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

Prior to the lipid extraction samples were thawed on ice and spiked with SPLASH LIPIDOMIX deuterium-labeled internal standards (Avanti Polar Lipids Inc.). The lipids were extracted from samples using a modified Bligh and Dyer total lipid extraction method (Bligh and Dyer, 1959). Briefly, 2ml of the ice-cold mixture of methanol:chloroform (2:1, v/v) and 0.1%

butylated hydroxytoluene (BHT) to prevent the auto-oxidation of polyunsaturated lipid species was added to the samples. The resulting monophasic mixtures were incubated at 4°C for 30 minutes. Then samples were centrifuged at 4000 rpm for 10 minutes and the supernatant was separated from the pellet by transferring to another tube. For the organic and aqueous phase separation 1ml of 0.5M sodium chloride solution was added and samples were vortexed for 10 minutes and centrifuged at 4000 rpm for 10 minutes. The organic fractions were separated and collected in glass vials. LC-MS/MS analysis was performed on Q Exactive HF mass spectrometer system coupled to the Ultimate 3000 liquid chromatography system (Thermo Scientific, USA) and reverse phase C18 column (2.1*50mm, 17mm particle size) (Waters, USA). The mobile phase A and B consisted of 40:60 water : acetonitrile (v/v) and 90:10 isopropanol : acetonitrile (v/v) respectively and both phases contain 10mM ammonium acetate and 0.1 % formic acid. The gradient flow parameters of mobile phase B were as follows: 0–1 min 40%–45%, 1.0–1.1 min 45%–50%, 1.1–5.0 min 50%–55%, 5.0–5.1 min 55%–70%, 5.1–8.0 min 70%–99%, 8.0–8.1 min 99%–40%, 8.1–9.5 min 40%. The flow rate was 0.4 mL/min during total 9.5 minutes run. The temperature for the autosampler and column was set for 4°C and 50°C respectively. Data acquisition was performed in positive electrospray ionization (ESI) mode and full and data-dependent acquisition (DDA) scans data were collected. LC-MS data processing and lipid species identification were performed using LipidSearch software version 4.2 (Thermo Scientific).

Preparing cell lysates for immunoblot

HAP1 cells were grown to 80-90% confluency in normal growth media (IMDM + 10%FBS). In experiments with the OXPHOS mix antibody (RRID: AB_2085424), cells were enriched for mitochondrial membranes as detailed below. The cells were washed 3 times with ice-cold PBS containing ice-cold PBS containing 10mM EDTA and then incubated for approximately 30 minutes at 4°C. After incubation, the cells were lifted from the plate on ice and spun down at 800 rpm for 5 minutes at 4°C. The resulting cell pellet was resuspended in a lysis buffer of Buffer A + 0.5% Triton X-100 containing 5% protease inhibitor cocktail cOmplete (Roche, 118614500, prepared at 50x in distilled water). Protein concentration was determined using the Bradford Assay (Bio-Rad, 5000006) and samples were diluted to 2ug/ul. Equal volumes of these cell lysate samples were combined with Laemmli buffer (SDS and 2-mercaptoethanol) reduced and denatured and heated for 5 min at 75°C. Samples were flash frozen and stored at -80°C until immunoblotting. In immunoblot experiments with the OXPHOS mix antibody and blue native gel electrophoresis experiments, cell lysates were enriched for mitochondrial membranes as follows. For each experimental condition, two 150mm dishes with HAP1 cells at 80-90% confluency were used. Trypsin was used to release the cells were and the cell pellet was washed by centrifugation with PBS. Crude mitochondria were enriched according to an established protocol (Wieckowski et al., 2009). Briefly, cell homogenization took place in isolation buffer (225-mM mannitol (Sigma, M9647), 75-mM sucrose (Fisher S5-500), 0.1-mM EGTA, and 30-mM Tris–HCl pH 7.4) using 20

strokes in a Potter-Elvehjem homogenizer at 6,000rpm at 4ºC. Centrifugation at 600g for 5min was used to collect unbroken cells and nuclei and mitochondria were recovered from this supernatant by centrifugation at 7,000g for 10 min. After 1 wash of this pellet, membrane solubilization took place in 1.5 M 6-aminocaproic acid (Sigma A2504), 50 mM Bis-Tris pH 7.0 buffer with cOmplete antiprotease (Roche) and freshly prepared 4 g/g digitonin (Miillipore, 300410).

Immunoblots

HAP1 or SH-SY5Y cell lysates or one nanogram of recombinant APOE (Novus Biologicals, 99158) were suspended in Buffer A + 0.5% Tx-100 and Laemmli sample buffer. Along with protein ladder (BioRad, 161-0373), the cell lysates or recombinant protein were loaded on a 4-20% Criterion gel (BioRad, 3450032) for SDS-PAGE and transferred to PVDF membrane (Millipore, IPFL00010) using the semidry transfer method. In experiments with AICAR (Sigma, A9978), cells were exposed to the drug at 0.4mM concentration for 72 hours before lysate preparation. Membranes were incubated in a blocking solution of TBS containing 5% nonfat milk and 0.05% Triton X-100. The membranes then incubated overnight with primary antibody solutions diluted in a buffer containing PBS with 3% BSA and 0.2% sodium azide. The following day, membranes were washed three times in TBS containing 0.05% Triton X-100 (TBST) and then incubated in secondary antibody (mouse HRP or rabbit HRP) diluted in blocking solution at room temperature for 30 minutes. The membranes were then rinsed in TBST 3 times and treated with Western Lightning Plus ECL reagent (PerkinElmer, NEL105001EA). Membranes were exposed to GE Healthcare Hyperfilm ECL (28906839) for visualization.

Blue native gel electrophoresis

Procedures were performed according to established protocols (Diaz et al., 2009; Timon-Gomez et al., 2020). 3-12% gradient native gels (Invitrogen, BN2011BX10) were used to separate proteins by blue native gel electrophoresis. Molecular weight standards used were 10mg/ml Ferritin (404 and 880 kDa, Sigma F4503) and BSA (66 and 132kDa) (Roche, 03116956001). The first dimension was run at 150V for 30 min at room temperature with Coomassie blue (Serva, 17524), then at 250V for 150min at 4ºC in cathode buffer without Coomassie. For immunoblot, proteins were transferred to PVDF membranes and probed with the indicated antibodies, as detailed above. For separation in a second dimension, the lanes from the native gels were cut and loaded on a 10% denaturing SDS-PAGE gel with a single broad lane.

Co-Immunoprecipitation

Stable cell lines expressing FLAG-tagged SLC25A1 were prepared by transfecting human neuroblastoma SH-SY5Y cells (ATCC, CRL-2266; RRID:CVCL_0019) with ORF expression clone containing N terminally tagged FLAG-SLC25A1 (GeneCopoeia, EX-A1932-Lv1020GS) (Gokhale et

al., 2019). These cell lines were grown in DMEM containing 10% FBS, 100 µg/ml penicillin and streptomycin, and puromycin 2 µg/ml (Invitrogen, A1113803). For co-imunoprecipitation experiments, cells were grown in 10 cm dishes. On the day of the experiment, the plated cells were placed on ice and rinsed twice with cold PBS (Corning, 21-040) containing 0.1 mM $CaCl_2$ and 1.0 mm $MgCl_2$. Lysis buffer containing 150 mm NaCl, 10 mM HEPES, 1 mM EGTA, and 0.1 mM $MgCl_2$, pH 7.4 (Buffer A) with 0.5% Triton X-100 and Complete anti-protease (Roche, 11245200) was added to the plates and cells were scraped and put into Eppendorf tubes. Cell lysates were incubated on ice for 30 min and centrifuged at 16,100 × g for 10 min. Bradford Assay (Bio-Rad, 5000006) was used to determine protein concentration of the recovered clarified supernatant. 500µg of the soluble protein extract was incubated with 30 µl Dynal magnetic beads (Invitrogen, 110.31) coated with 1 µg of mouse monoclonal FLAG antibody (RRID: AB_259529). The beads combined with the cell lysates were incubated on an end-to-end rotator for 2 h at 4°C. In some cases, as controls, beads were incubated with the lysis buffer, without any antibodies or the immunoprecipitation was outcompeted with the 3XFLAG peptide (340 µm; Sigma, F4799). Beads were then washed 6 times with Buffer A with 0.1% Triton X-100 followed by elution with Laemmli buffer. Samples were then analyzed by SDS-PAGE, immunoblot using polyclonal antibodies against FLAG (RRID: AB_67407) and NDUFS3 (AB_2151109).

NanoString mRNA Quantification

HAP1 cells or SH-SY5Y cells were grown to confluency in a 10 cm sterile dish in normal growth media. SH-SY5Y SLC31A1 knockout cells were treated with vehicle (cell culture grade water) or BCS (Sigma, B1125) at 200µm for 24 hours. Three replicate plates were used for each experimental condition in experiments with HAP1 or SH-SY5Y cells. Astrocytes were seeded at a density of 15,000 cells/cm^2 on 6 well tissue culture dishes and grown until they reached 80% confluency. At least 500,000 cells per replicate were used. Astrocytes were treated either with a DMSO vehicle control or 80nM Antimycin A (Sigma, A8674) for 48 hours, with media being replaced every 24 hours. For preparation of samples, all cells were washed twice in ice-cold PBS containing 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$. 1 ml of Trizol was added to the samples and the Trizol mixture was flash frozen and stored at -80°C for a few weeks until RNA Extraction and NanoString processing was completed by the Emory Integrated Genomics Core. The Core assessed RNA quality before proceeding with the NanoString protocol. The NanoString Neuroinflammation gene panel kit (115000230) or Metabolic Pathways Panel (115000367) was used for mRNA quantification. mRNA counts were normalized by the expression of the housekeeping gene TADA2B. Data were analyzed using Qlucore Omics Explorer Version 3.7(24) as indicated below.

Preparation of cell pellets for proteomics and RNAseq

Wild type or gene-edited HAP1 cells (SLC25A1 and SLC25A4) were grown in IMDM supplemented with 10% FBS and 100 µg/ml penicillin and streptomycin at 37°C in a 10% CO_2 incubator. Cells were grown on 10cm tissue culture dishes to 85% confluency. Cells were placed on ice, washed 3 times with ice cold PBS (Corning, 21-040), and then incubated with PBS with 10mM EDTA for 30 minutes at 4°C. Mechanical agitation with a 10ml pipette was used to remove cells from plates. The collected cells were then centrifuged at 800xg for 5 minutes at 4°C. Following centrifugation, the supernatant was removed and the pellet was washed with ice cold PBS. The resuspended pellet was then spun at 16,100 x g for 5 minutes. The supernatant was aspirated away and the remaining pellet was flash frozen on dry ice for at least 5 minutes and stored at -80°C until further use. For proteomic and transcriptomic analysis, the experiment was conducted at least in triplicate.

TMT mass spectrometry for proteomics

Cell pellets were lysed in 200 µL of urea lysis buffer (8M urea, 100 mM NaH2PO4, pH 8.5), supplemented with 2 µL (100x stock) HALT protease and phosphatase inhibitor cocktail (Pierce). Lysates were then subjected to 3 rounds of probe sonication. Each round consisted of 5 seconds of activation at 30% amplitude and 15 of seconds of rest on ice. Protein concentration was determined by bicinchoninic acid (BCA) analysis and 100 ug of each lysate was aliquoted and volumes were equilibrated with additional lysis buffer. Aliquots were diluted with 50mM and was treated with 1mM DTT and 5mM IAA in sequential steps. Both steps were performed in room temperature with end to end rotation for 30 minutes. The alkylation step with IAA was performed in the dark. Lysyl endopeptidase (Wako) was added at a 1:50 (w/w) enzyme to protein ratio and the samples were digested for overnight. Samples were then diluted with 50mM triethylammonium bicarbonate (TEAB) to a urea concentration of 1M. Trypsin (Promega) was added at a 1:50 (w/w) enzyme to protein ratio and digestion proceeded overnight. Resulting peptides were desalted with a Sep-Pak C18 column (Waters). An aliquot equivalent to 20 ug of total protein was taken out of each sample and combined to obtain a global internal standard (GIS) use later for TMT labeling. All samples (16 individual and 4 GIS) were then dried under vacuum.

TMT labeling was performed according to the manufacturer's protocol. Briefly (Ping et al., 2018), the reagents were allowed to equilibrate to room temperature. Dried peptide samples (90 µg each) were resuspended in 100 µl of 100 mm TEAB buffer (supplied with the kit). Anhydrous acetonitrile (41 µl) was added to each labeling reagent tube and the peptide solutions were transferred into their respective channel tubes. The reaction was incubated for 1 h and quenched for 15 min afterward with 8 µl of 5% hydroxylamine. All samples were combined and dried down. Peptides were resuspended in 100 µl of 90% acetonitrile and 0.01% acetic acid. The entire sample was loaded onto an offline electrostatic repulsion–hydrophilic interaction chromatography

fractionation HPLC system and 96 fractions were collected. The fractions were combined into 24 fractions and dried down. Dried peptide fractions were resuspended in 100 µl of peptide loading buffer (0.1% formic acid, 0.03% trifluoroacetic acid, 1% acetonitrile). Peptide mixtures (2 µl) were separated on a self-packed C18 (1.9 µm Dr. Maisch, Germany) fused silica column (25 cm × 75 µm internal diameter; New Objective) by a Easy-nLC 1200 and monitored on a Fusion Lumos mass spectrometer (ThermoFisher Scientific). Elution was performed over a 140 min gradient at a rate of 350 nl/min with buffer B ranging from 3% to 90% (buffer A: 0.1% formic acid in water, buffer B: 0.1% formic in 80% acetonitrile). The mass spectrometer cycle was programmed to collect at the top speed for 3 s cycles. The MS scans (375-1500 m/z range, 400,000 AGC, 50 ms maximum ion time) were collected at a resolution of 120,000 at m/z 200 in profile mode. HCD MS/MS spectra (1.2 m/z isolation width, 36% collision energy, 50,000 AGC target, 86 ms maximum ion time) were collected in the Orbitrap at a resolution of 50000. Dynamic exclusion was set to exclude previous sequenced precursor ions for 15 s within a 10 ppm window. Precursor ions with +1 and +8 or higher charge states were excluded from sequencing.

MS/MS spectra were searched against human database from Uniprot (downloaded on 04/2015) with Proteome Discoverer 2.1.1.21 (ThermoFisher Scientific). Methionine oxidation (+15.9949 Da), asparagine, and glutamine deamidation (+0.9840 Da) and protein N-terminal acetylation (+42.0106 Da) were variable modifications (up to 3 allowed per peptide); static modifications included cysteine carbamidomethyl (+57.0215 Da), peptide n terminus TMT (+229.16293 Da), and lysine TMT (+229.16293 Da). Only fully tryptic peptides were considered with up to two miscleavages in the database search. A precursor mass tolerance of ±20 ppm and a fragment mass tolerance of 0.6 Da were applied. Spectra matches were filtered by Percolator to a peptide-spectrum matches false discovery rate of <1%. Only razor and unique peptides were used for abundance calculations. Ratio of sample over the GIS of normalized channel abundances were used for comparison across all samples. Data files have been uploaded to ProteomeExchange with PXD XXX**.**

For mouse brain proteomic studies, we performed TMT mass spectrometry on whole cortical brain homogenates from 43 WT and 43 5xFAD mice (MMRC#034848-JAX) and sampled age groups from 1.8 mo to 14.4 months. All groups contained equal numbers of males and females, with n=4 males and 4 females per age group. The methods used for these studies have been previously published (Johnson et al., 2022). Briefly, brain tissues were homogenized using a bullet blender along with sonication in 8M Urea lysis buffer containing HALT protease and phosphatase inhibitor (ThermoFisher). Proteins were reduced, alkylated and then digested with Lysyl endopeptidase and Trypsin, followed by peptide cleanup. TMT (16-plex kit) peptide labeling was performed as per manufacturer's instructions, with inclusion of one global internal standard (GIS) in each batch. All samples in a given batch were randomized across six TMT batches, while maintaining nearly-equal representation of age, sex and genotype across all six batches. A complete description of the TMT mass spectrometry study, including methods for sample

preparation, mass spectrometry methodology and data processing, are available online (https://www.synapse.org/#!Synapse:syn27023828) and a comprehensive analysis of these data will be published separately. Mass spectrometry raw data were processed in Proteome Discover (Ver 2.1) and then searched against Uniprot mouse database (version 2020), and then processed downstream as described for human brain TMT mass spectrometry studies above. Batch effect was adjusted using bootstrap regression which modelled genotype, age, sex and batch, but covariance with batch only was removed (Wingo et al., 2020). From the 8,535 proteins identified in this mouse brain proteome, we analyzed data related to APOE and 914 proteins that were also found in the Mitocarta database (Rath et al., 2021).

RNAseq and data analysis

RNA isolation, library construction, and sequencing were performed by the Beijing Genomics Institute. Total RNA concentration was measured with Agilent 2100 Bio analyzer (Agilent RNA 6000 Nano Kit), QC metrics: RIN values, 28S/18S ratio and fragment length distribution. mRNA purification was achieved by poly-T oligo immobilized to magnetic beads. the mRNA was fragmented using divalent cations plus elevated temperature. RNA fragments were copied into first strand cDNA by reverse transcriptase and random primers. Second strand cDNA synthesis was performed with DNA Polymerase I and RNase H. A single 'A' base and subsequent ligation of adapter was done on cDNA fragments. Products were purified and enriched with PCR amplification. The PCR yield was quantified using a Qubit and samples were pooled together to make a single strand DNA circle (ssDNA circle) producing the final library. DNA nanoballs were generated with the ssDNA circle by rolling circle replication to enlarge fluorescent signals during the sequencing process. DNA nanoballs were loaded into the patterned nanoarrays and pair-end reads of 100 bp were read through on the DNBseq platform. The DNBseq platform combined the DNA nanoball-based nanoarrays and stepwise sequencing using Combinational Probe-Anchor Synthesis Sequencing Method. On average, we produced ~4.41 Gb bases per sample. The average mapping ratio with reference genome was 92.56%, the average mapping ratio with gene was 72.22%; 17,240 genes were identified. 16,875 novel transcripts were identified. Read quality metrics: 4.56% of the total amount of reads contained more than 5% unknown N base; 5.92% of the total amount of reads which contained adaptors; 1.38% of the total reads were considered low quality meaning more than 20% of bases in the total read have quality score lower than 15. 88.14% of total amount of reads were considered clean reads and used for further analysis.

Sequencing reads were uploaded to the Galaxy web platform. We used the public server usegalaxy.eu to analyze the data (Afgan et al., 2018). FastQC was used to remove samples of suboptimal quality (Andrews, 2010). All mapping was performed using Galaxy server (v. 19.09) running Hisat2 (Galaxy Version 2.1.0+galaxy5), HTseq-Count (Galaxy Version 1.6.2), and DeSeq2 (Galaxy Version 2.11.40.2) (Anders et al., 2015; Kim et al., 2015; Love et al., 2014) . The Genome Reference Consortium build of the reference sequence (GRCh38/hg38) and the GTF files (NCBI)

were used and can be acquired from iGenome (Illumina). Hisat2 was run with the following parameters: paired-end, unstranded, default settings were used except for a GTF file was used for transcript assembly. Alignments were visualized using IGV viewer (IGV-Web app version 1.7.0, igv.js version 2.10.5) with Ensembl v90 annotation file and Human (GRCh38/hg38) genome (Robinson et al., 2020; Robinson et al., 2011).

Aligned SAM/BAM files were processed using HTseq-count (Default settings except used GRCh38 GTF file and output for DESeq2 and gene length file). HTseq-count output files and raw read files are publicly available (GEO with accession GSE201889). The HTseq-count compiled file is GSE201889_ RawHTseqCounts_ALL. Gene counts were normalized using DESeq2 (Love et al., 2014) followed by a regularized log transformation. Differential Expression was determined using DESeq2 with the following settings: Factors were cell type, pairwise comparisons between mutant cell lines versus control line was done, output all normalized tables, size estimation was the standard median ratio, fit type was parametric, outliers were filtered using a Cook's distance cutoff.

Bioinformatic analyses and statistical analyses

Data from proteomes, RNAseq, NanoString, and lipidomics were processed with Qlucore Omics Explorer Version 3.6(33) normalizing log2 data to a mean of 0 and a variance of 1. Qlucore Omics was used to generate volcano plots, Euclidean hierarchical clustering, PCI and 2D- tSNE. 2D-tSNE was calculated with a perplexity of 5. All other statistical analyses were performed with Prism v9.2.0(283) using two tailed statistics and Alpha of 0.05 using test specified in each figure legend. No outlier identification and exclusion were applied. Asterisks denoting significance followed Prism specifications. Estimation statistics in Fig. 5A was performed as described (Ho et al., 2019)**.**

Gene ontology analyses were carried out with Cluego. ClueGo v2.58 run on Cytoscape v3.8.2 (Bindea et al., 2009; Shannon et al., 2003). ClueGo was run querying GO BP, REACTOME, and KEGG considering all evidence, Medium Level of Network Specificity, and selecting pathways with a Bonferroni corrected p value <0.001. ClueGo was run with Go Term Fusion. Analysis of Nanostring Ontologies was performed with Metascape (Zhou et al., 2019). All statistically enriched terms based on the default choices under Express Analysis, accumulative hypergeometric p-values and enrichment factors were calculated and used for filtering. Remaining significant terms were then hierarchically clustered into a tree based on Kappa-statistical similarities among their gene memberships. Kappa score of 0.3 was applied as the threshold to cast the tree into term clusters (Zhou et al., 2019).

The interactome of NDUFS3 and SLC25A1 was generated from the proximity interaction datasets by Antonicka et al. (Antonicka et al., 2020). Data were imported into Cytoscape v3.8.2.

Enrichment (Representation Factor) and exact hypergeometric probability of gene set enrichments with hits from the Human Secretome (Uhlen et al., 2019) and Human Mitocarta 3.0

(Rath et al., 2021) in Figs. 1N and 5D were calculated with the engine nemates.org using the 20,577 gene count of the Uniprot Human Proteome Reference

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Deutsch et al., 2020) partner repository with dataset identifier: XXXX Project DOI: XXXX

RNAseq data were deposited in GEO with accession GSE201889

Figure Legends

Figure 1. The Secreted and Mitochondrial Proteomes are Modified by Inner Mitochondrial Membrane Transporter Mutants.

A. Cell number determinations of wild type (columns 1-4) and *SLC25A1*-null HAP1 cells (*SLC25A1Δ*, columns 5-8) grown in the presence of conditioned media from each genotype. Conditioned media was applied to cells for 48 hours before (columns 1, 2, 5 and 6) or after dialysis (columns 3, 4, 7, and 8). Cell number was determined by Alamar blue cell viability assay. Mean± SEM, n=5, Two-Way ANOVA followed by Benjamini, Krieger and Yekutieli corrections. **B-C**. Volcano plots of TMT proteomic data from wild type HAP1 cells (n=3), *SLC25A1Δ* (B, n=3 for two independent CRISPR clones), and *SLC25A4Δ* mutants (C, n=3), depicted are $\log_{10}$ p values and $\log_2$ fold of change. **D**. Principal component analysis and 2D-tSNE analyses of datasets in B-C. **E**. Hierarchical clustering of all proteome hits where differential expression is significant with an α<0.001 in at least one mutant genotype. **F-G**. Volcano plots of RNAseq data from wild type HAP1 cells (n=4), *SLC25A1Δ* (B, n=4 for one independent CRISPR clone), and *SLC25A4Δ* mutants (C, n=4), depicted are $\log_{10}$ p values and $\log_2$ fold of change. **H**. PCA and 2D-tSNE analyses of datasets in F-G. Subject grouping was determined by k-means clustering. **I**. Hierarchical clustering of all RNAseq hits where differential expression is significant with an α=0.001 in at least one mutant genotype. **J**. Venn diagram of protein and transcript hits shared by *SLC25A1Δ* and *SLC25A4Δ* mutants. 27 shared protein and RNA hits are annotated to either the human secretome (orange font) or annotated to Mitocarta 3.0 (green font). **K.** Venn diagram of protein hits in *SLC25A1Δ* and *SLC25A4Δ* mutants annotated in Mitocarta 3.0. **L.** Venn diagram of protein hits in *SLC25A1Δ* and *SLC25A4Δ* mutants annotated in the Human Secretome (Uhlen et al., 2019). **M.** Hierarchical clustering of all proteins annotated to the human secretome across genotypes. **N.** Magnitude of compromise in secreted and mitochondrial proteomes in *SLC25A1Δ* and *SLC25A4Δ* mutants. p value was calculated with exact hypergeometric probability. **O-P.** Gene ontology analysis of proteome (N) and transcriptome (O) in *SLC25A1Δ* and *SLC25A4Δ* mutants. Overlapping and mutant-specific ontologies are color-coded by percent of contribution >50% to an ontological category. Gray represents ontologies where all three mutants similarly contribute hits.

Figure 2. APOE Transcripts and Protein Are Upregulated Independent from Cholesterol Levels in *SLC25A1* and *SLC25A4* Mutants.

A. MesoScale electrochemiluminescence solid phase ELISA determinations of human APOE in wild type (column 1), *SLC25A1Δ* (columns 2 and 3), and *SLC25A4Δ* (column 4) HAP1 mutant cell lysates and conditioned media. Two independent *SLC25A1Δ* clones were tested (columns 2-3). Column 5 depicts complete media not exposed to cells. n=4. **B**. APOE immunoblot of cellular extracts from wild type, *SLC25A1Δ,* and *SLC25A4Δ* HAP1 mutant cells. HSP90 was used as a loading control. Lane 4 presents recombinant human APOE. **C**. MesoScale ELISA measurements of human APOE in wild type and *SLC25A1Δ/Δ* SH-SY5Y mutant cell lysates and

conditioned media. **D**. MesoScale ELISA determinations of human APOE in wild type and *SLC25A4,5,6Δ/Δ* triple knock out HEK293 cell lysates and conditioned media. **E-G**. qRT-PCR quantification of APOE, sterol metabolism annotated genes, and housekeeping controls (VAMP2 and RPS20) in wild type and diverse mutant cell lines. **E** and **F** show transcript levels in *SLC25A1Δ* and *SLC25A4Δ* HAP1 mutant cells, respectively. **G** depicts transcript levels in *SLC25A1Δ/Δ* SH-SY5Y mutant cells. All data are expressed as transcript ratio between mutant and wild type. n=3 for E-G. **H-J**. Volcano plots of positive mode untargeted lipidomics performed in *SLC25A1Δ*, *SLC25A4Δ,* and *SLC25A1Δ/Δ* mutant HAP1 and SH-SY5Y cells and their controls. Upper inserts present the distribution of cholesterol ester and triglyceride species marked by triangles. Depicted are $\log_{10}$ p values and $\log_2$ fold of change. n=4 per clone for the two *SLC25A1Δ* clones, n=4 for *SLC25A4Δ,* and n=4 for *SLC25A1Δ/Δ.* **K**. Total cellular levels of free cholesterol (Ch), cholesterol ester (ChE), and triglycerides (TG) in wild type, *SLC25A1Δ*, and *SLC25A4Δ* HAP1 cells. **L**. Total cellular levels of free cholesterol (Ch), cholesterol ester (ChE), and triglycerides (TG) in wild type and *SLC25A1Δ/Δ* SH-SY5Y cells. Average ± SEM, One-Way ANOVA followed by Bonferroni or Holm-Šydák's (E-G) multiple corrections, or unpaired t-test (L).

Figure 3. The Integrity of Respiratory Chain Complex I is Required to Control APOE Expression.

A. Expression of respiratory complex subunits in wild type, *SLC25A1Δ*, and *SLC25A4Δ* HAP1 cells quantified by TMT mass spectrometry. Kendal Tau hierarchical clustering analysis. **B**. Immunoblots with OxPhos antibody mix in mitochondrial fractions from wild type and *SLC25A1Δ* cells. **C**. Blue native electrophoresis of mitochondrial fractions from wild type and *SLC25A1Δ* cells. Shown are Coomassie stained native gel and immunoblots probed with antibodies against complex, I, III, IV, and SLC25A1. **D**. Blue native electrophoresis followed by SDS-PAGE then immunoblot with antibodies against complex, I, II, III, and V in mitochondrial fractions from wild type and *SLC25A1Δ* cells. **E-G**. Seahorse stress test, APOE qRT-PCR, and APOE MesoScale ELISA analysis respectively in wild type and *NDUFS3Δ* HAP1 cells. In F, APOE was measured with two primer sets. **H-J**. Seahorse stress test, APOE qRT-PCR, and APOE MesoScale ELISA analysis respectively in wild type and *NDUFAF7Δ* HAP1 cells. VAMP2 or RER1 transcripts were used as controls in F and J. All qRT-PCR data are expressed as ratio between mutant and wild type. E to M average ± SEM. One-Way ANOVA followed by Šydák's multiple correction (F), or unpaired t-test with Welch's correction (G, I, and J). Arrows in E (n=4) and H (n=3) show sequential addition of oligomycin, FCCP, and rotenone-antimycin during the Seahorse stress test. **K**. *SLC25A1Δ* cells are more sensitive to antimycin than wild type HAP1 cells. Wild type and *SLC25A1Δ* cells were exposed to vehicle or increasing concentrations of antimycin. Basal cellular respiration was measured for 90 min after additions (arrow) using Seahorse. Data are presented normalized to basal respiration in the absence of drug. Average ± SEM, n=3, Gray square shows significant differences between wild type and *SLC25A1Δ* drug-treated cells as determined by multiple

unpaired t-tests followed by corrections with the Benjamini-Krieger-Yekuiteli method (FDR=5%). **L-M**. APOE qRT-PCR and APOE MesoScale ELISA in wild type and *SLC25A1Δ* HAP1 cells, respectively, treated with vehicle or antimycin. 20 nM antimycin was used in qPCR experiments. 20–80 nM was used in MesoScale ELISA experiments. RER1 (columns 1-4) and PCBP1 transcripts (columns 5-8) were used as housekeeping controls. All qRT-PCR data are expressed as ratio between mutant and wild type. Average ± SEM, One-Way ANOVA followed by Benjamini-Krieger-Yekuiteli multiple comparison corrections (FDR=5%).

Figure 4. Disruption of Copper Delivery to Mitochondria and Respiratory Chain Increases APOE Expression.

A. MesoScale ELISA determinations of human APOE in wild type and two independent *COX17Δ/Δ* mutant SH-SY5Y cell clones. Shown are APOE content in lysates and conditioned media. **B**. Diagram of copper transport (ATP7A, SLC31A1 and SLC25A3) and chaperoning (COX17) from the plasma membrane (ATP7A, SLC31A1) to the mitochondrial inner membrane (SLC25A3). **C**. MesoScale ELISA determinations of human APOE as in A except that wild type and two independent *SLC31A1Δ/Δ* mutant SH-SY5Y cell clones were studied. **D**. MesoScale ELISA determinations of human APOE as in A except that wild type and two independent *ATP7AΔ/Δ* mutant SH-SY5Y cell clones transfected with the endocytosis-deficient ATP7A-LL construct were studied. **E**. MesoScale ELISA determinations of human APOE as in A. Wild type and two independent *ATP7AΔ/Δ* mutant SH-SY5Y cell clones were analyzed. **F**. MesoScale ELISA determinations of human APOE as in A. Wild type and *SLC25A3Δ* mutant HAP1 cells were studied. n=4 for all genotypes analyzed by MesoScale. **G**. NanoString mRNA quantification of human APOE and TBP transcripts in wild type, and two independent mutant clones of either *COX17Δ/Δ* or *SLC31A1Δ/Δ* mutant SH-SY5Y cells. Wild type and *SLC31A1Δ/Δ* cells were treated with vehicle or 200 micromolar of the copper chelator bathocuproinedisulfonic acid (BCS). TBP was used as a housekeeping control transcript. n=3. **H-I**. MesoScale ELISA determinations of human APOE as in A. Wild type and *PARK2Δ* mutant HAP1 cells were studied n=4. **J**. Seahorse basal cellular respiration across different genotypes normalized to the corresponding wild type cell. **K.** Correlation between APOE in conditioned media and basal cellular respiration (n=3-9). Simple linear regression fit and 95% confidence interval is shown. All data are presented as average ± SEM. For A, C-E, G, and J One-Way ANOVA followed by Benjamini-Krieger-Yekuiteli multiple comparison corrections (FDR=5%). F, H, I and J unpaired t-test with Welch's correction.

Fig. 5. APOE Expression is Increased in Human Astrocytes after Complex III Inhibition.

A. MesoScale ELISA determinations of human APOE in wild type iPSC-derived human neurons and astrocytes treated with vehicle or 40-80 nM antimycin for 48h. APOE determinations were performed in cell lysates and conditioned media and expressed normalized to a control value. n=4. *P* was obtained with two-sided estimation statistics. **B-G**. Present analyses of changes

in mRNA expression measured with NanoString Neuroinflammation panel. **B**. Volcano plots of wild type, *SLC25A1Δ*, and *SLC25A4Δ* HAP1 cells (n=3 per genotype) and iPSC-derived astrocytes treated with vehicle or 80nM antimycin for 48h (n=3). Yellow symbols represent upregulated genes in mutant or drug treated cells. **C**. mRNA expression APOE and TBP was expressed as APOE /TADA2B or TBP/TADA2B ratios. TBP and TADA2B are both housekeeping control transcripts One-Way ANOVA followed by Benjamini-Krieger-Yekuiteli multiple comparison corrections (FDR=5%). **D.** Magnitude of compromise of significantly upregulated mRNAs annotated to secreted and mitochondrial proteomes in antimycin-treated astrocytes and compared to all genes present in the Neuroinflammation NanoString panel. p value was calculated with exact hypergeometric probability. **E.** Circos plot of shared upregulated hits in *SLC25A1Δ*, and *SLC25A4Δ* HAP1 cells and iPSC-derived astrocytes treated with 80nM antimycin. Outside arc represents the identity of each gene list. Inside arc represents a gene list, where each gene member of that list is assigned a spot on the arc. Dark orange color represents genes that are shared by multiple lists and light orange color represents genes that are unique to that gene list. Shared genes are presented by purple lines and different genes that belong to the same functional ontologies are connected by light blue lines. **F**. Metascape ontology analysis and clustering of genes upregulated in *SLC25A1Δ*, and *SLC25A4Δ* HAP1 cells and iPSC-derived astrocytes treated with 80nM antimycin. Accumulative hypergeometric p-values. **G**. Correlation of fold of expression between the antimycin-upregulated genes in astrocytes and the same genes in A1 reactive astrocytes induced by a neurotoxic stimulus (Liddelow et al., 2017).

Fig. 6. Correlative Studies of APOE Expression and Electron Transport Chain Subunits in an Alzheimer's mouse model and aging humans.

A. Protein expression similarity matrix of APOE, complexes I to V (CI-CV) of the electron transport chain, and transporters of the SLC25A family in wild type and 5xFAD mouse models of increasing age (1.8 to 14.4 months). Data were obtained by TMT mass spectrometry from mouse cortices. Similarity was calculated with Spearman Rank correlation. **B.** Shows inserts from A highlighting complex I (CI) as the respiratory complex best correlated with APOE expression in wild type. The 5xFAD genotype reverses the correlation between APOE and complex I expression. **C.** Kendall Tau Hierarchical clustering of complex I subunits and APOE across ages and genotypes. **D.** Quantification of human A beta 42 peptide, APP, APOE, complex I subunits (NDUFAS1, 3 and 7), complex II subunit SDHC, complex III subunit UQCRC1, and complex IV subunit COX5A in wild type and 5xFAD mice (grey and blue symbols, respectively). Two-way ANOVA followed by Šídák's multiple comparisons tests. Factors are age (*A*), genotype (*G*), and their interaction (*I*). Asterixs denote significant differences between genotypes at a defined age. See supplementary table 1 for all Mitocarta hits in the 5xFAD mouse study. **E.** The SLC25A1 RNAseq, proteome, and interactome correlate with the cognitive trajectory of human subjects. Graphs depict the correlation between the cognitive trajectory (Mini-Mental State Examination (MMSE)) in subjects

belonging to the Banner collection that were longitudinally followed for an average of 14 years (n=106) (Beach et al., 2015; Wingo et al., 2019). The *SLC25A1Δ* RNAseq, up and downregulated hits, proteome hits, as well as the SLC25A1 interactome hits principal components were derived by estimating eigenvectors of the expression matrix of protein abundance data. Linear regression was used to test for an association between cognitive decline and the first principal component. Best-fit regression line drawn in blue and the 99.9% confidence interval for the regression line shaded in gray. No covariate was applied because sex, age at enrollment, and education have been regressed out of cognitive trajectory. Sex, age at death, and postmortem interval have been regressed out of the Banner proteomic profile. Circles represent males, crossed circles represent females (48.1%). APOE E4 gene dosage is depicted by color (gray-zero APOE4 copies, blue-one APOE4 copy, purple-two APOE E4 copies).

Fig. S1. Interactions between SLC25A1 and NDUFS3 and Neuronal Ontology Annotated Genes Downregulated in *SLC25A1* Mutant Cells.

A. Proximity ligation map of interactions shared between SLC25A1 and NDUFS3. **B.** SLC25A1-FLAG expressing SH-SY5Y cell lysates were immunoprecipitated with magnetic beads alone (lane 1) orFLAG antibodies (lanes 2-3), either in the absence or presence of an excess FLAG peptide for out-competition (Lanes 2 and 3, respectively). Immunoprecipitated complexes were probed by immunoblot with NDUSF3 antibody. **C.** qRT-PCR quantification of neuronal ontology annotated genes and housekeeping controls (TBP and RPS20) in wild type and *SLC25A1* mutant HAP1 and SH-SY5Y cells. Average ± SEM, One-Way ANOVA followed by Holm-Šydák's multiple correction test. n=3.

Fig. S2. AMPK and Mitochondrial Stress Response in Wild Type and *SLC25A1Δ* HAP1 cells.

A. Activity of the AMPK in wild type (lanes 1-2) and *SLC25A1Δ* (lanes 3-4) HAP1 cells was assessed with antibodies against AMPK, phospho-AMPK, and SLC25A1. Cells were treated with AICAR 0.4 mM for 72 hours to induce the activation of AMPK (lanes 2 and 4). **B.** RNAseq determinations of FGF21 and GDF21 as well ATF transcription factors in wild type and mutant cells. **C.** Cis-regulatory sequence analysis with iRegulon to infer transcriptional responses activated in the *SLC25A1Δ* and *SLC25A4Δ* upregulated transcriptome (1429 transcripts $p<0.001$ and log2 fold of change=1) (Janky et al., 2014). Significance threshold is set at 3. There are no ATF3, 4, or 5 target genes in the *SLC25A1Δ* upregulated transcriptome. **D**. Activation of the mitochondrial stress response with doxycycline modestly increases APOE in conditioned media

from *SLC25A1Δ* cells but not in wild type cells. MesoScale ELISA determinations of human APOE in wild type and *SLC25A1Δ* cells treated in the absence or presence of doxycycline at a concentration of 9.75 micromolar for 48 hours. APOE was measured in cell lysates and media. **E-F**. Activation of the mitochondrial stress response with doxycycline in in wild type (D) and *SLC25A1Δ* (E) HAP1 cells. Doxycycline treatment was performed as in C. Gene expression was determined by qRT-PCR measuring the transcription factors ATF3-5 and their selected target genes ASNS, CHAC1, CHOP, PCK2 and WARS. RER1 and PCBP1 were used as housekeeping control transcripts. n=3. **G.** APOE mRNA expression is not responsive to the antioxidant n-acetyl cysteine (NAC) in wild type and *SLC25A1Δ* cells. RER1 was used as housekeeping transcript and GSR to assess the effects of NAC incubation. Cells were treated with 2 mM NAC for 48 hours, n=3. E to G show data as a ratio between drug/vehicle ratio. All figures depict average ± SEM. For C and F, One-Way ANOVA followed by Šydák's multiple correction. D and E One-Way ANOVA followed by Benjamini-Krieger-Yekuiteli multiple comparison corrections (FDR=5%).

Fig. S3. Upregulation of APOE Expression and Secretion in *SLC25A20* Mutant Cells.

A. Seahorse stress test in wild type and *SLC25A20Δ* HAP1 cells. Arrows show sequential addition of oligomycin, FCCP, and rotenone-antimycin during the Seahorse stress test. **B**. Depicts basal, ATP-dependent and maximal oxygen consumption rates. p value, Mann-Whitney U test, n=4. **C**. Immunoblots with OxPhos antibody mix in mitochondrial fractions from wild type and *SLC25A20Δ* cells. **D**. MesoScale ELISA determinations of human APOE in wild type and *SLC25A20Δ* mutant cells. Shown are APOE content in lysates and conditioned media. p value, Mann-Whitney U test, n=4. All graphs depict average ± SEM.

Table S1. Raw Data for Proteomics, Transcriptomics and Gene Ontologies.

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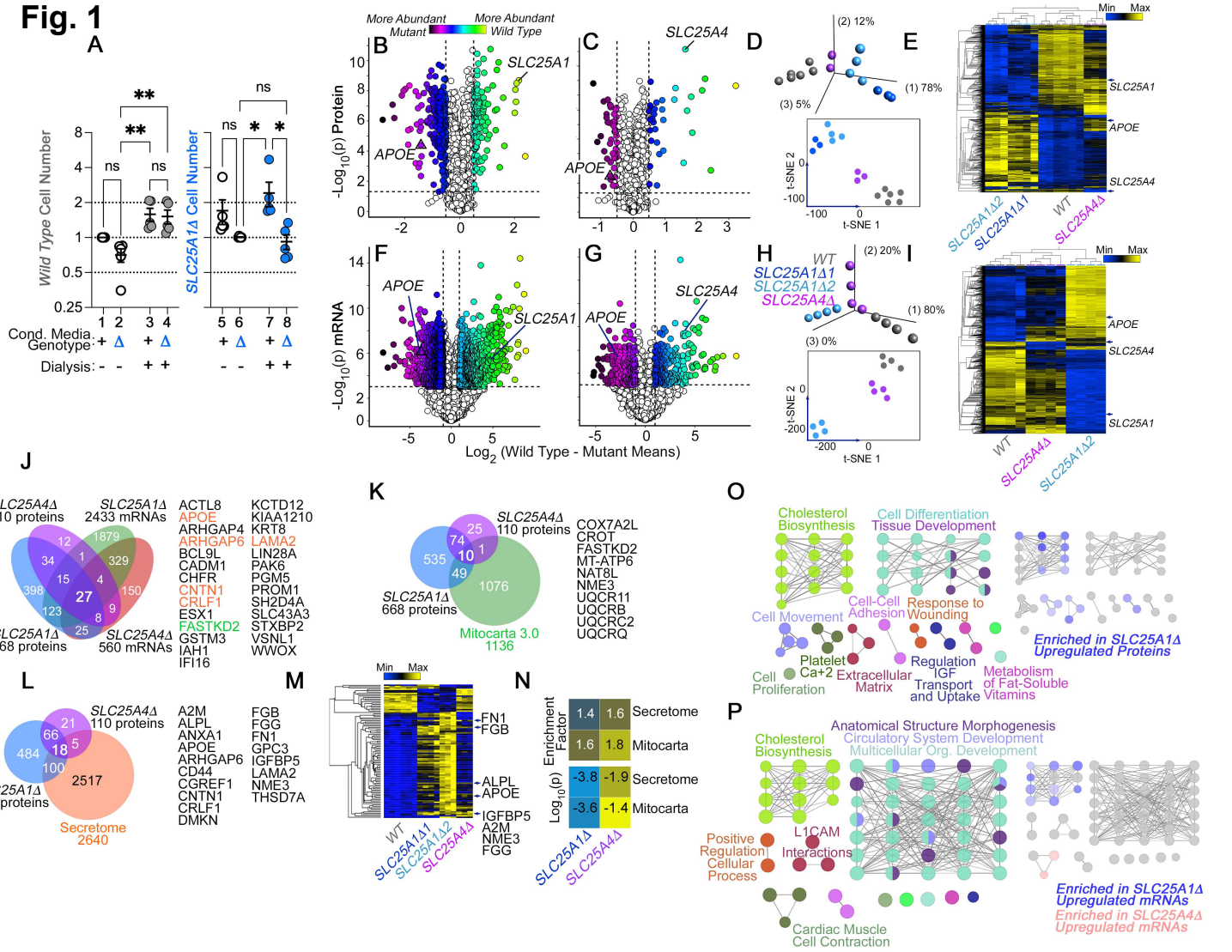
Fig. 1


A
Wild Type Cell Number
SLC25A1Δ Cell Number
Cond. Media.
Genotype
Dialysis
B
C
D
E
More Abundant Mutant
More Abundant Wild Type
SLC25A1
SLC25A4
APOE
-Log10(p) Protein
(2) 12%
(1) 78%
(3) 5%
t-SNE 2
t-SNE 1
Min
Max
SLC25A1Δ2
SLC25A1Δ1
WT
SLC25A4Δ
F
G
H
I
-Log10(p) mRNA
Log2 (Wild Type - Mutant Means)
(2) 20%
(1) 80%
(3) 0%
J
SLC25A4Δ 110 proteins
SLC25A1Δ 2433 mRNAs
SLC25A1Δ 668 proteins
SLC25A4Δ 560 mRNAs
ACTL8
APOE
ARHGAP4
ARHGAP6
BCL9L
CADM1
CHFR
CNTN1
CRLF1
ESX1
FASTKD2
GSTM3
IAH1
IFI16
KCTD12
KIAA1210
KRT8
LAMA2
LIN28A
PAK6
PGM5
PROM1
SH2D4A
SLC43A3
STXBP2
VSNL1
WWOX
K
SLC25A4Δ 110 proteins
SLC25A1Δ 668 proteins
Mitocarta 3.0 1136
COX7A2L
CROT
FASTKD2
MT-ATP6
NAT8L
NME3
UQCR11
UQCRB
UQCRC2
UQCRQ
O
Cholesterol Biosynthesis
Cell Differentiation
Tissue Development
Cell Movement
Cell-Cell Adhesion
Response to Wounding
Platelet
Ca+2
Extracellular Matrix
Regulation
IGF
Transport and Uptake
Metabolism of Fat-Soluble Vitamins
Cell Proliferation
Enriched in SLC25A1Δ Upregulated Proteins
L
SLC25A4Δ 110 proteins
SLC25A1Δ 668 proteins
Secretome 2640
A2M
ALPL
ANXA1
APOE
ARHGAP6
CD44
CGREF1
CNTN1
CRLF1
DMKN
FGB
FGG
FN1
GPC3
IGFBP5
LAMA2
NME3
THSD7A
M
FN1
FGB
ALPL
APOE
IGFBP5
A2M
NME3
FGG
N
Enrichment Factor
Log10(p)
Secretome
Mitocarta
P
Anatomical Structure Morphogenesis
Cholesterol Biosynthesis
Circulatory System Development
Multicellular Org. Development
Positive Regulation Cellular Process
L1CAM Interactions
Cardiac Muscle Cell Contraction
Enriched in SLC25A1Δ Upregulated mRNAs
Enriched in SLC25A4Δ Upregulated mRNAs

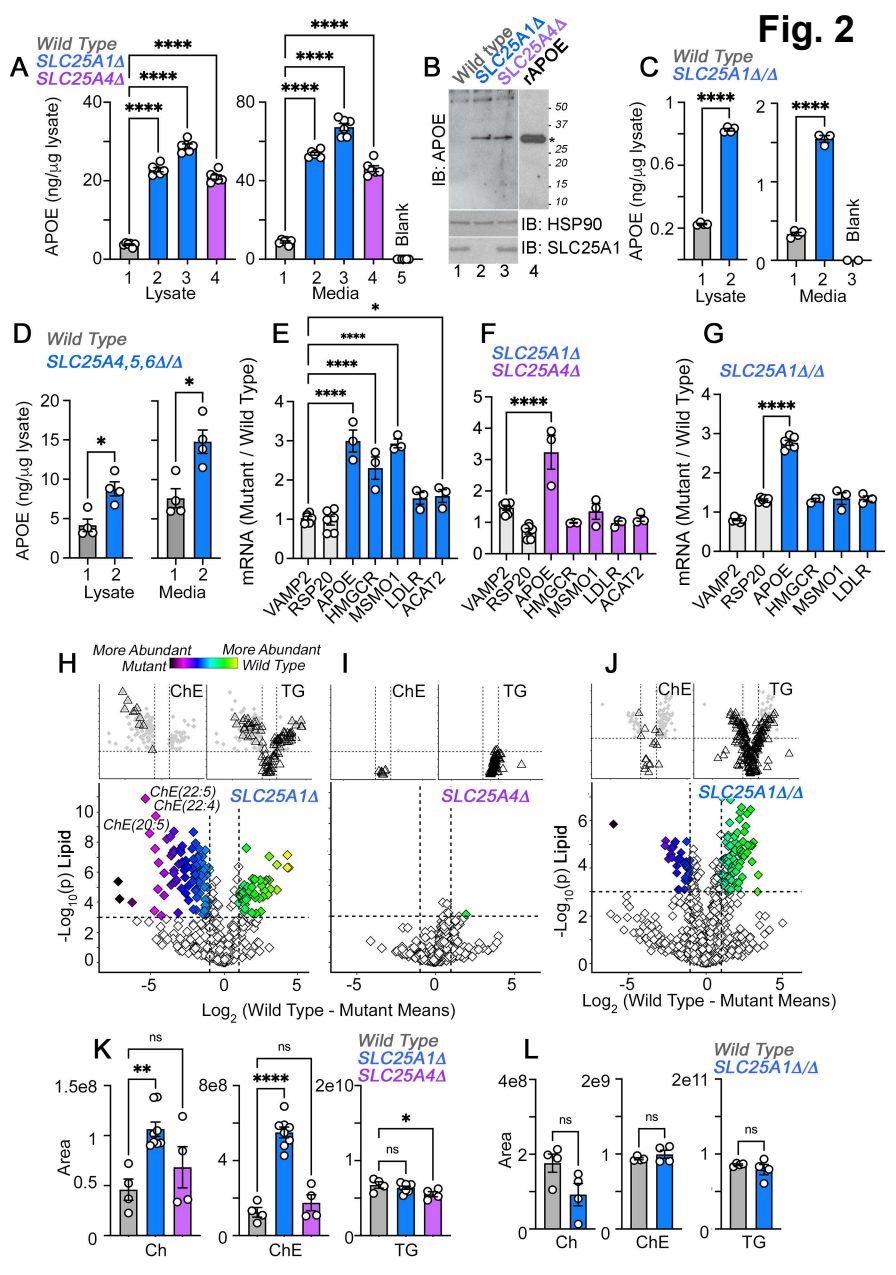


Fig. 2
A
Wild Type
SLC25A1Δ
SLC25A4Δ
APOE (ng/μg lysate)
Lysate
Media
Blank
B
Wild type
SLC25A1Δ
SLC25A4Δ
rAPOE
IB: APOE
IB: HSP90
IB: SLC25A1
C
Wild Type
SLC25A1Δ/Δ
D
Wild Type
SLC25A4,5,6Δ/Δ
E
mRNA (Mutant / Wild Type)
VAMP2
RSP20
APOE
HMGCR
MSMO1
LDLR
ACAT2
F
SLC25A1Δ
SLC25A4Δ
G
SLC25A1Δ/Δ
H
More Abundant Mutant
More Abundant Wild Type
ChE
TG
ChE(22:5)
ChE(22:4)
ChE(20:5)
SLC25A1Δ
-Log10(p) Lipid
Log2 (Wild Type - Mutant Means)
I
SLC25A4Δ
J
SLC25A1Δ/Δ
K
Area
Ch
ns
L

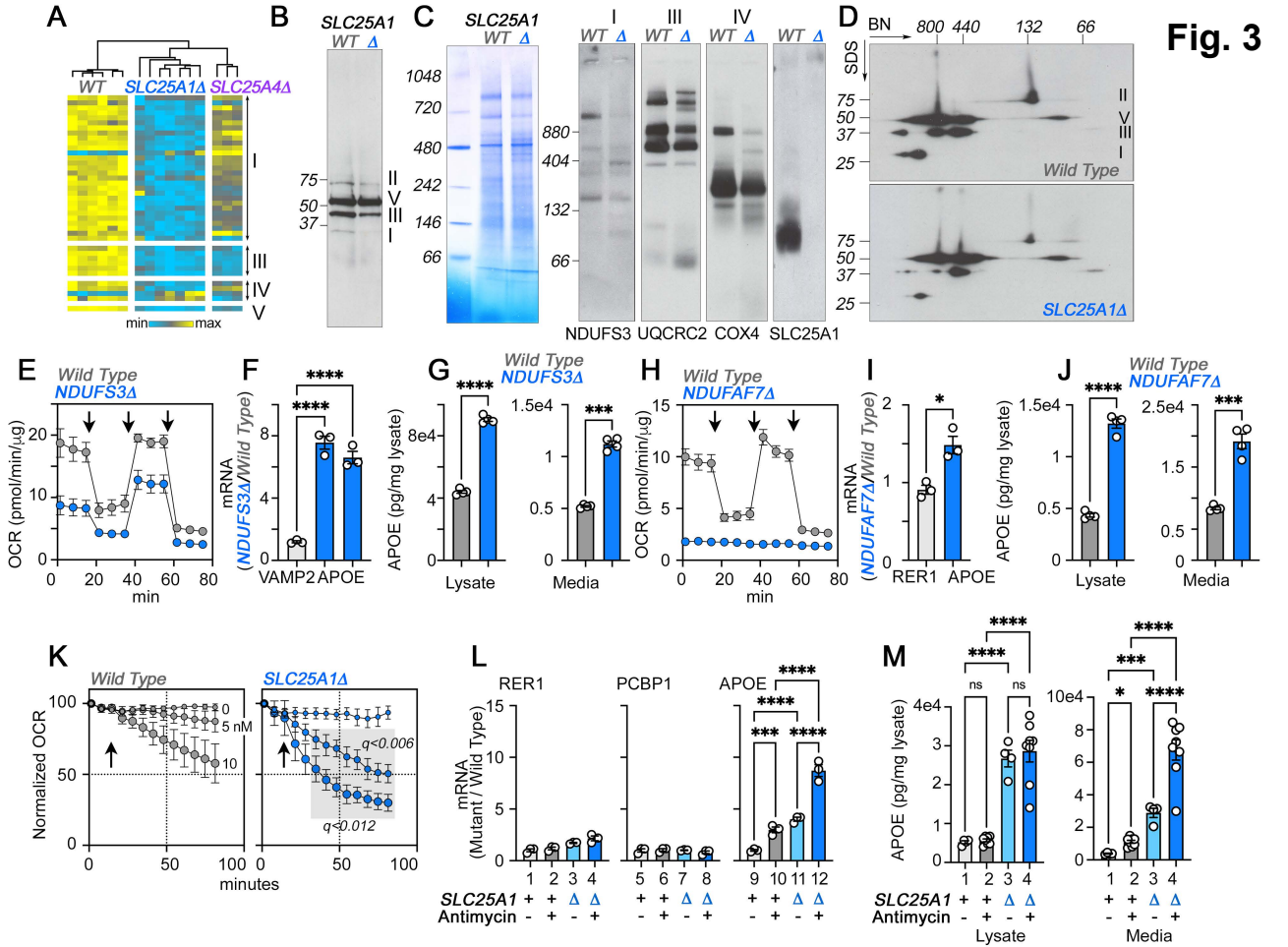
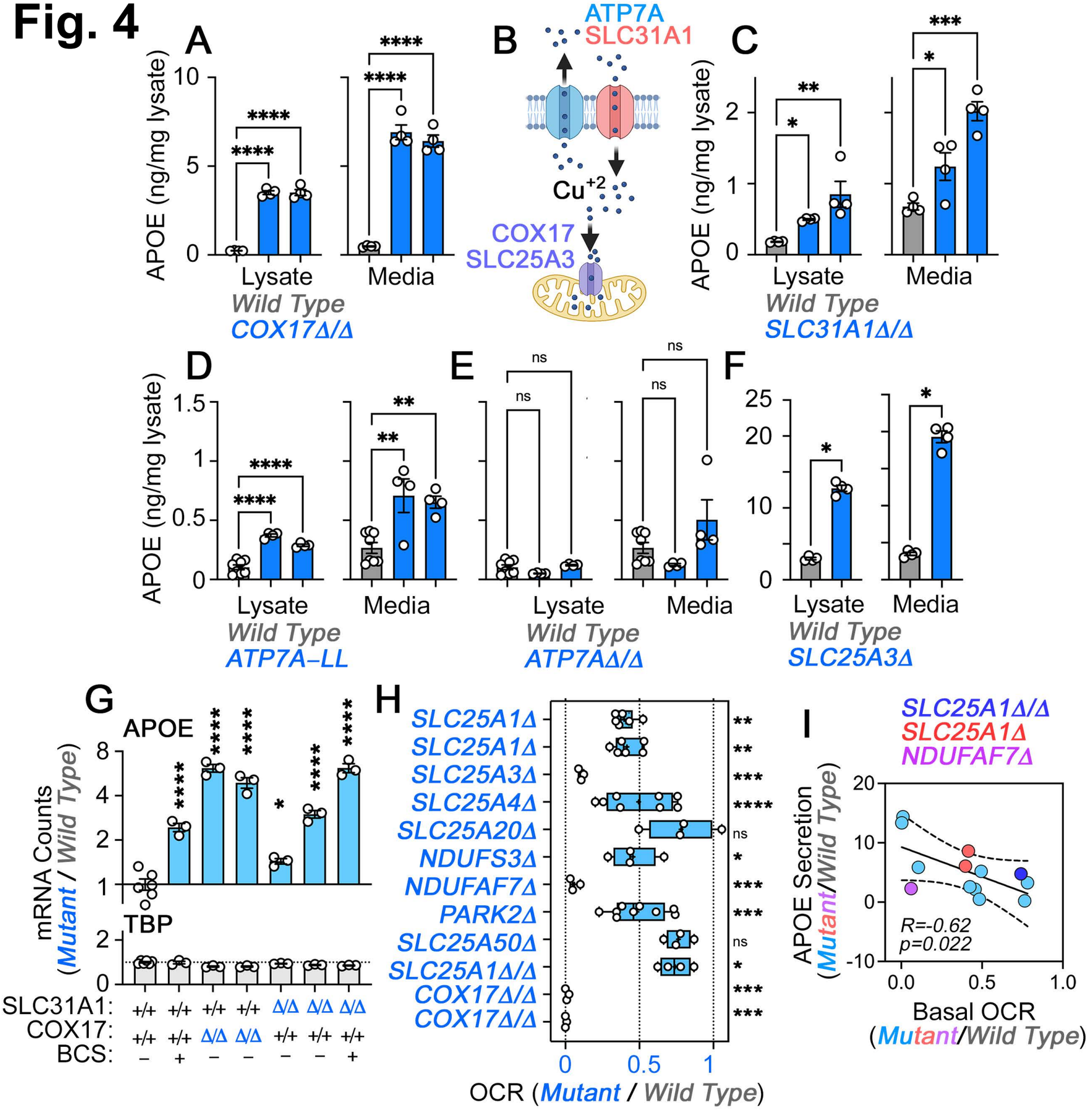


Fig. 3
A
WT
SLC25A1Δ
SLC25A4Δ
I
III
IV
V
min
max
B
SLC25A1
WT Δ
75
50
37
II
V
III
I
C
SLC25A1
WT Δ
1048
720
480
242
146
66
I
III
IV
WT Δ
WT Δ
WT Δ
WT Δ
880
404
132
66
NDUFS3
UQCRC2
COX4
SLC25A1
D
BN
800
440
132
66
SDS
75
50
37
25
II
V
III
I
Wild Type
SLC25A1Δ
E
Wild Type
NDUFS3Δ
OCR (pmol/min/μg)
min
F
****
****
mRNA (NDUFS3Δ/Wild Type)
VAMP2
APOE
G
Wild Type
NDUFS3Δ
****
***
APOE (pg/mg lysate)
Lysate
Media
H
Wild Type
NDUFAF7Δ
OCR (pmol/min/μg)
min
I
*
mRNA (NDUFAF7Δ/Wild Type)
RER1
APOE
J
Wild Type
NDUFAF7Δ
****
***
APOE (pg/mg lysate)
Lysate
Media
K
Wild Type
SLC25A1Δ
Normalized OCR
0
5 nM
10
q<0.006
q<0.012
minutes
L
RER1
PCBP1
APOE
****
****
***
****
mRNA (Mutant / Wild Type)
SLC25A1
Antimycin
M
****
****
ns
ns
****
***
*
****
APOE (pg/mg lysate)
SLC25A1
Antimycin
Lysate
Media

Fig. 4


A
APOE (ng/mg lysate)
Lysate
Media
Wild Type
COX17Δ/Δ
B
ATP7A
SLC31A1
Cu+2
COX17
SLC25A3
C
SLC31A1Δ/Δ
D
ATP7A–LL
E
ATP7AΔ/Δ
F
SLC25A3Δ
ns
G
APOE
TBP
mRNA Counts (Mutant / Wild Type)
SLC31A1:
COX17:
BCS:
H
SLC25A1Δ
SLC25A3Δ
SLC25A4Δ
SLC25A20Δ
NDUFS3Δ
NDUFAF7Δ
PARK2Δ
SLC25A50Δ
SLC25A1Δ/Δ
COX17Δ/Δ
OCR (Mutant / Wild Type)
I
SLC25A1Δ/Δ
SLC25A1Δ
NDUFAF7Δ
APOE Secretion (Mutant/Wild Type)
R=-0.62
p=0.022
Basal OCR (Mutant/Wild Type)

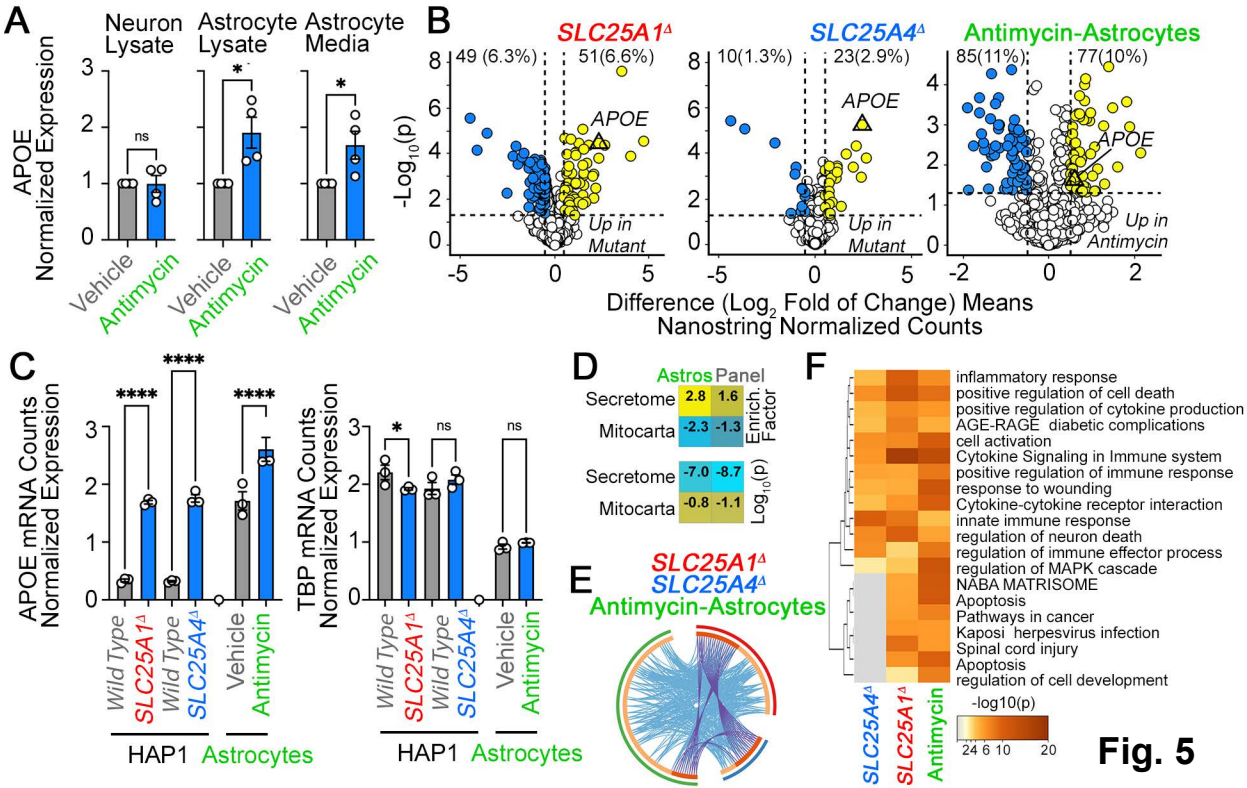

A
Neuron Lysate
Astrocyte Lysate
Astrocyte Media
APOE Normalized Expression
ns
*
*
Vehicle
Antimycin
B
SLC25A1Δ
SLC25A4Δ
Antimycin-Astrocytes
49 (6.3%)
51(6.6%)
10(1.3%)
23(2.9%)
85(11%)
77(10%)
APOE
Up in Mutant
Up in Antimycin
-Log10(p)
Difference (Log2 Fold of Change) Means
Nanostring Normalized Counts
C
APOE mRNA Counts Normalized Expression
TBP mRNA Counts Normalized Expression
****
*
ns
Wild Type
SLC25A1Δ
SLC25A4Δ
Vehicle
Antimycin
HAP1
Astrocytes
D
Astros Panel
Secretome
Mitocarta
2.8
1.6
-2.3
-1.3
Enrich Factor
-7.0
-8.7
-0.8
-1.1
Log10(p)
E
SLC25A1Δ
SLC25A4Δ
Antimycin-Astrocytes
F
inflammatory response
positive regulation of cell death
positive regulation of cytokine production
AGE-RAGE diabetic complications
cell activation
Cytokine Signaling in Immune system
positive regulation of immune response
response to wounding
Cytokine-cytokine receptor interaction
innate immune response
regulation of neuron death
regulation of immune effector process
regulation of MAPK cascade
NABA MATRISOME
Apoptosis
Pathways in cancer
Kaposi herpesvirus infection
Spinal cord injury
Apoptosis
regulation of cell development
-log10(p)
24 6 10 20
SLC25A4Δ
SLC25A1Δ
Antimycin

Fig. 5

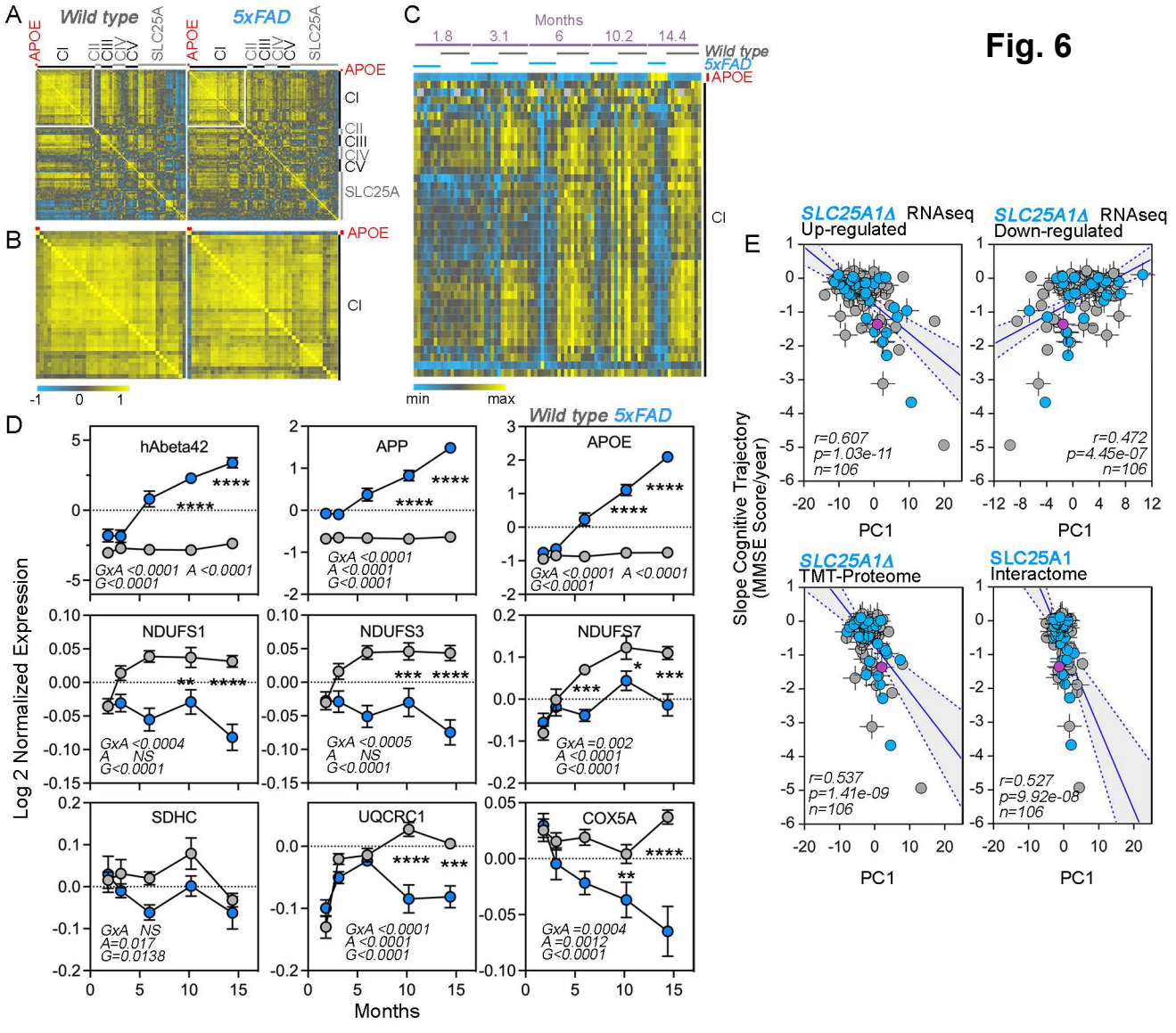
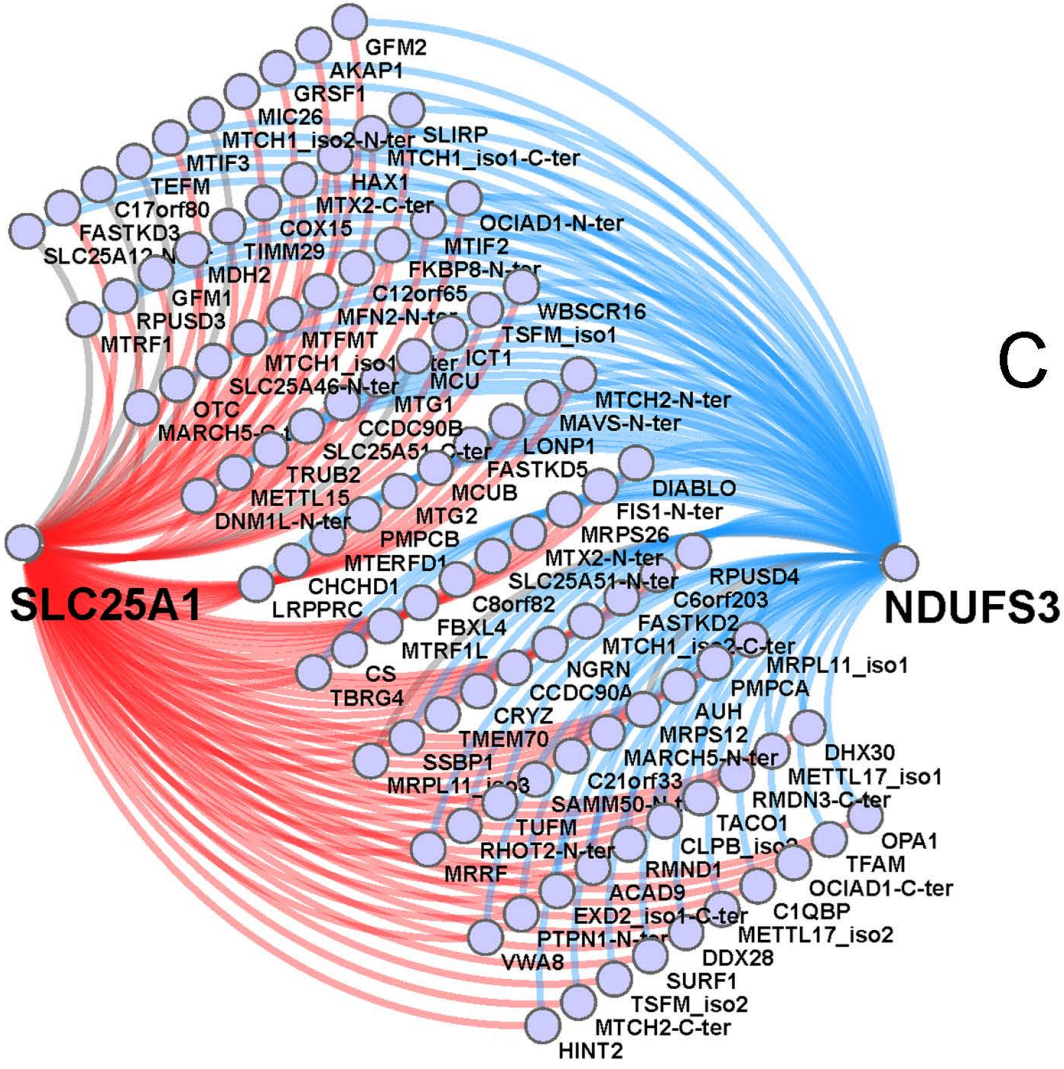


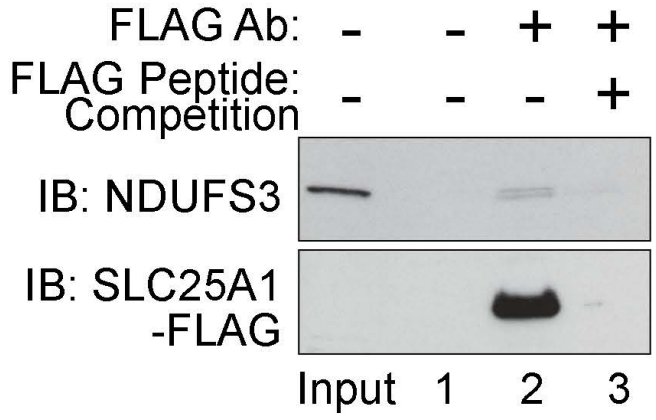
Fig. 6
A
Wild type
5xFAD
APOE
CI
CII
CIII
CIV
CV
SLC25A
B
C
Months
1.8
3.1
6
10.2
14.4
Wild type
5xFAD
min
max
D
Wild type 5xFAD
hAbeta42
APP
APOE
NDUFS1
NDUFS3
NDUFS7
SDHC
UQCRC1
COX5A
GxA <0.0001 A <0.0001 G<0.0001
GxA <0.0004 A NS G<0.0001
GxA <0.0005 A NS G<0.0001
GxA =0.002 A <0.0001 G<0.0001
GxA NS A=0.017 G=0.0138
GxA =0.0004 A =0.0012 G<0.0001
Log 2 Normalized Expression
Months
E
SLC25A1Δ RNAseq Up-regulated
SLC25A1Δ RNAseq Down-regulated
SLC25A1Δ TMT-Proteome
SLC25A1 Interactome
r=0.607 p=1.03e-11 n=106
r=0.472 p=4.45e-07 n=106
r=0.537 p=1.41e-09 n=106
r=0.527 p=9.92e-08 n=106
Slope Cognitive Trajectory (MMSE Score/year)
PC1

Fig. S1

A


SLC25A1
NDUFS3
GFM2
AKAP1
GRSF1
MIC26
MTCH1_iso2-N-ter
SLIRP
MTCH1_iso1-C-ter
MTIF3
TEFM
HAX1
C17orf80
MTX2-C-ter
FASTKD3
COX15
OCIAD1-N-ter
TIMM29
MTIF2
MDH2
FKBP8-N-ter
GFM1
C12orf65
RPUSD3
WBSCR16
MTRF1
MFN2-N-ter
TSFM_iso1
MTFMT
MTCH1_iso1
ICT1
SLC25A46-N-ter
MCU
OTC
MTG1
MTCH2-N-ter
MARCH5-C-ter
CCDC90B
MAVS-N-ter
SLC25A51-C-ter
LONP1
TRUB2
FASTKD5
METTL15
MCUB
DIABLO
DNM1L-N-ter
MTG2
FIS1-N-ter
PMPCB
MRPS26
MTERFD1
MTX2-N-ter
CHCHD1
SLC25A51-N-ter
RPUSD4
LRPPRC
C8orf82
C6orf203
FBXL4
FASTKD2
MTRF1L
MTCH1_iso2-C-ter
CS
NGRN
MRPL11_iso1
TBRG4
CCDC90A
PMPCA
CRYZ
AUH
TMEM70
MRPS12
SSBP1
MARCH5-N-ter
DHX30
MRPL11_iso3
C21orf33
METTL17_iso1
SAMM50-N-ter
RMDN3-C-ter
TUFM
TACO1
OPA1
RHOT2-N-ter
CLPB_iso2
TFAM
MRRF
RMND1
OCIAD1-C-ter
ACAD9
C1QBP
EXD2_iso1-C-ter
METTL17_iso2
PTPN1-N-ter
DDX28
VWA8
SURF1
TSFM_iso2
MTCH2-C-ter
HINT2

B


FLAG Ab: - - + +
FLAG Peptide: Competition - - - +
IB: NDUFS3
IB: SLC25A1 -FLAG
Input 1 2 3

C

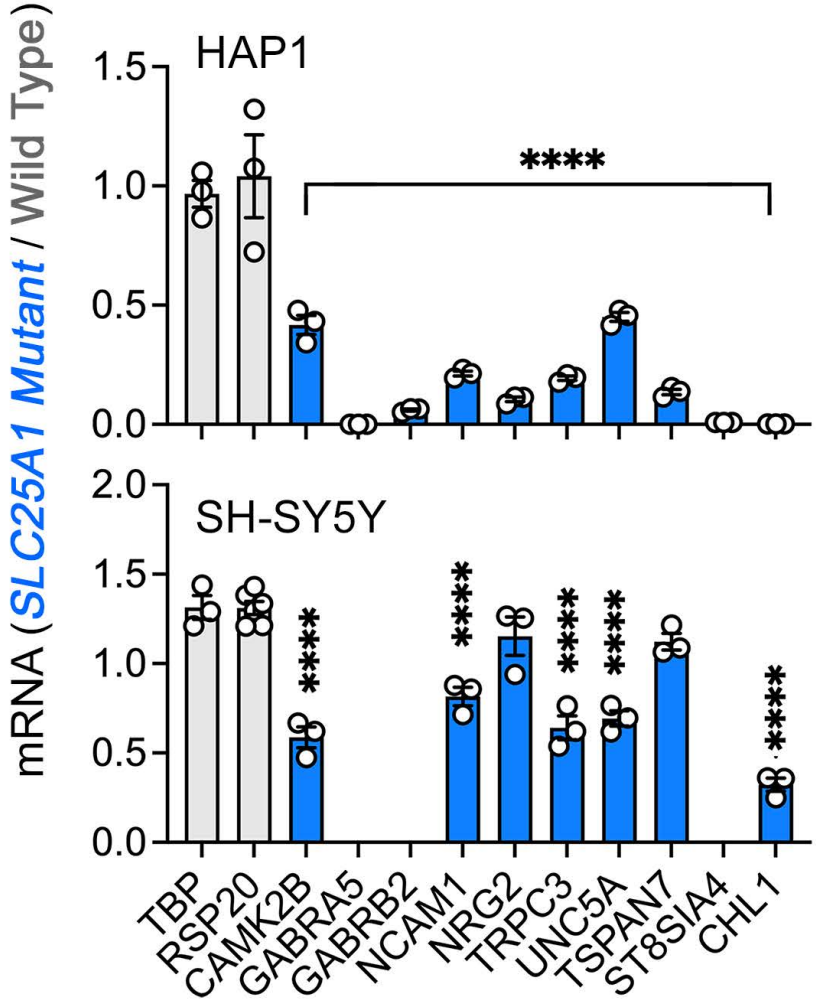

HAP1
SH-SY5Y
mRNA (SLC25A1 Mutant / Wild Type)
1.5
1.0
0.5
0.0
2.0
****
TBP
RSP20
CAMK2B
GABRA5
GABRB2
NCAM1
NRG2
TRPC3
UNC5A
TSPAN7
ST8SIA4
CHL1

Fig. S2

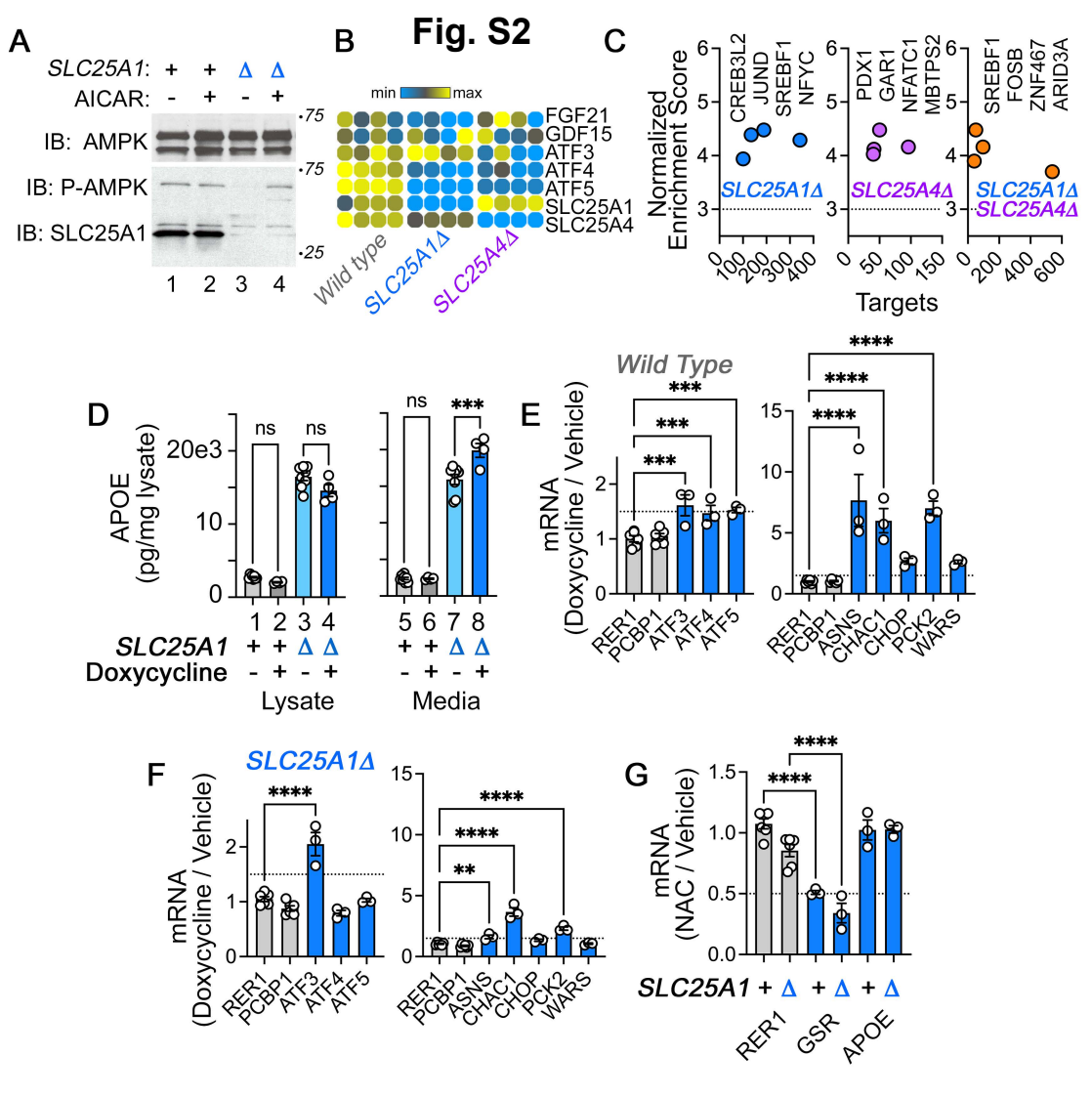
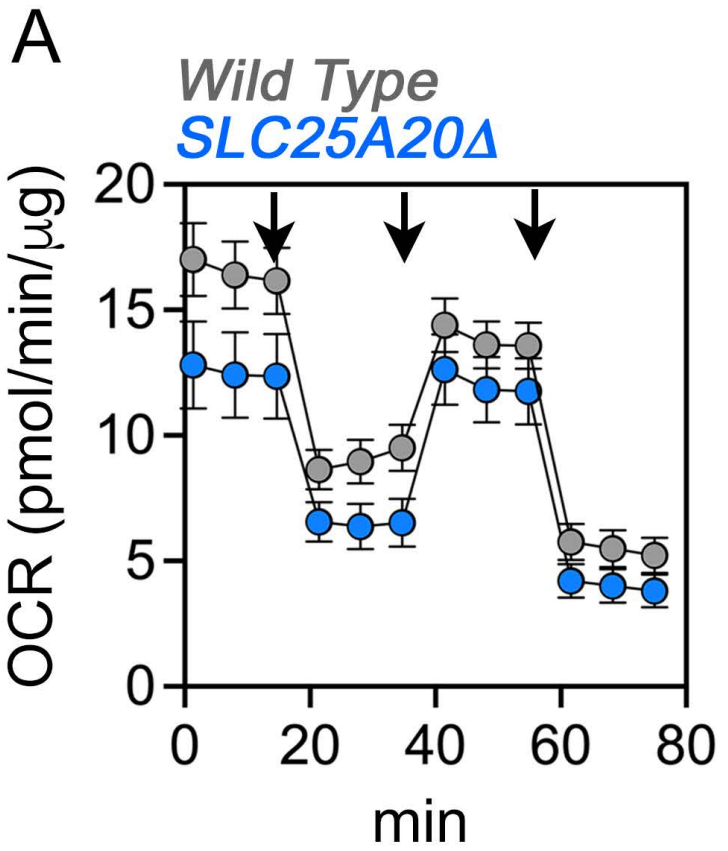
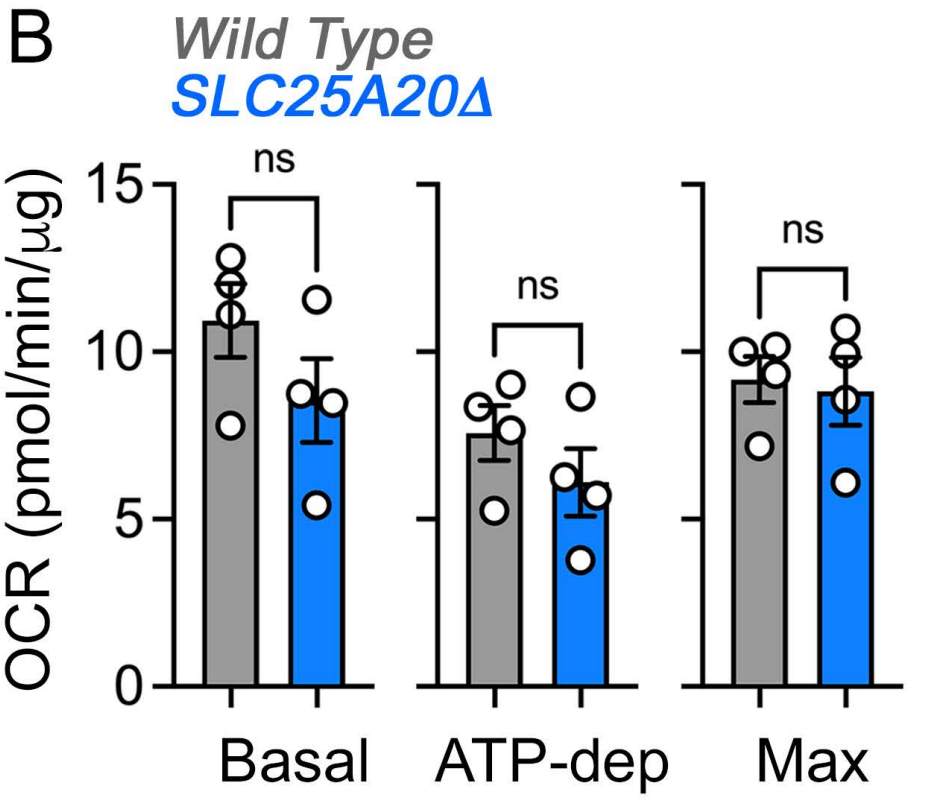
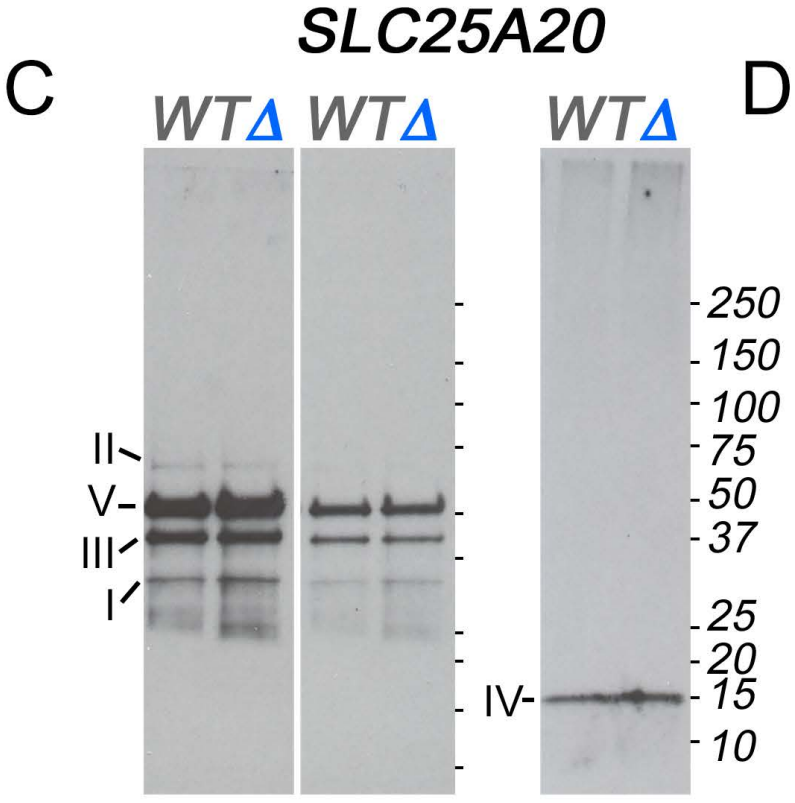
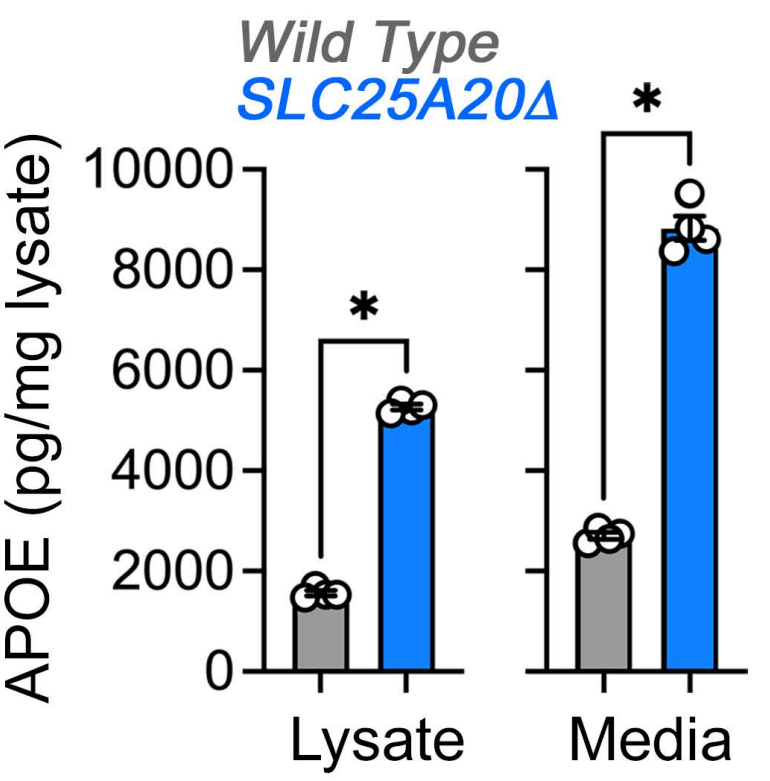

A
SLC25A1: + + Δ Δ
AICAR: - + - +
IB: AMPK
IB: P-AMPK
IB: SLC25A1
75
75
25
1 2 3 4
B
min max
FGF21
GDF15
ATF3
ATF4
ATF5
SLC25A1
SLC25A4
Wild type
SLC25A1Δ
SLC25A4Δ
C
Normalized Enrichment Score
CREB3L2
JUND
SREBF1
NFYC
PDX1
GAR1
NFATC1
MBTPS2
SREBF1
FOSB
ZNF467
ARID3A
SLC25A1Δ
SLC25A4Δ
SLC25A1Δ
SLC25A4Δ
Targets
D
APOE (pg/mg lysate)
ns
ns
ns
***
1 2 3 4
5 6 7 8
SLC25A1 + + Δ Δ + + Δ Δ
Doxycycline - + - + - + - +
Lysate
Media
E
Wild Type
mRNA (Doxycycline / Vehicle)
***
***
***
****
****
****
RER1
PCBP1
ATF3
ATF4
ATF5
ASNS
CHAC1
CHOP
PCK2
WARS
F
SLC25A1Δ
mRNA (Doxycycline / Vehicle)
****
****
****
**
RER1
PCBP1
ATF3
ATF4
ATF5
ASNS
CHAC1
CHOP
PCK2
WARS
G
mRNA (NAC / Vehicle)
****
****
SLC25A1 + Δ + Δ + Δ
RER1
GSR
APOE

Fig. S3


A
Wild Type
SLC25A20Δ
OCR (pmol/min/μg)
20
15
10
5
0
0
20
40
60
80
min


B
Wild Type
SLC25A20Δ
OCR (pmol/min/μg)
15
10
5
0
ns
ns
ns
Basal
ATP-dep
Max


SLC25A20
C
WTΔ
WTΔ
WTΔ
II
V
III
I
IV
250
150
100
75
50
37
25
20
15
10


D
Wild Type
SLC25A20Δ
APOE (pg/mg lysate)
10000
8000
6000
4000
2000
0
*
*
Lysate
Media