The CRISPR-Cas9 crGART HeLa transcriptome: A novel cell model for de novo purine synthesis deficiency

Randall C Mazzarino[a,b,c,d], Veronika Baresova[e], Marie Zikánová[e], Nathan Duval[a,b,c], Terry G. Wilkinson II[a,b], David Patterson[a,b,d], Guido N. Vacano[a,b]

Affiliations :
[a] Knoebel Institute for Healthy Aging, University of Denver, 2155 E. Wesley Avenue, Denver, CO 80210, USA
[b] Eleanor Roosevelt Institute, University of Denver, Denver, CO 80210, USA
[c] Department of Biological Sciences, University of Denver, Denver, CO 80210, USA
[d] Molecular and Cellular Biophysics Program, University of Denver, Denver, CO 80210, USA
[e] Research Unit for Rare Diseases, Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic.

Corresponding author: Guido Vacano, Ph.D.
Email: guido.vacano@du.edu
Address: University of Denver, 2155 E. Wesley Avenue Room 543, Denver, CO 80210

ABSTRACT

In humans, GART [phosphoribosylglycinamide formyltransferase (EC 2.1.2.2) / phosphoribosylglycinamide synthetase (EC 6.3.4.13) / phosphoribosylaminoimidazole synthetase (EC 6.3.3.1)] is a trifunctional protein which catalyzes the second, third, and fifth reactions of the ten step de novo purine synthesis (DNPS) pathway. The second step of DNPS is conversion of phosphoribosylamine (5-PRA) to glycineamide ribonucleotide (GAR). 5-PRA is extremely unstable under physiological conditions and is unlikely to accumulate in the absence of GART activity. Recently, a HeLa cell line null mutant for GART was constructed via CRISPR-Cas9 mutagenesis. This cell line, crGART, is likely an important cellular model for DNPS inactivation that does not accumulate DNPS pathway intermediates. In the current study, we characterize the crGART versus HeLa transcriptomes in purine-supplemented and purine-depleted growth conditions. We observed multiple transcriptome changes and discuss pathways and ontologies particularly relevant to Alzheimer disease and Down syndrome.

Keywords: RNA-seq, Purine synthesis, Alzheimer's disease, Down syndrome, Development.

Abbreviations: adenine phosphoribosyltransferase (APRT), adenosine monophosphate (AMP), adenosine triphosphate (ATP), adenylosuccinate lyase (ADSL), Alzheimer disease (AD), 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP), 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase / inosine monophosphate cyclohydrolase (ATIC), AMP deaminase (AMPD), de novo purine synthesis (DNPS), differentially expressed gene (DEG), Down syndrome (DS), false discovery rate (FDR), fetal calf macroserum (FCM), fetal calf serum (FCS), gamma-amino butyric acid (GABA), gene ontology (GO), glycineamide ribonucleotide

(GAR), guanosine monophosphate (GMP), inosine monophosphate (IMP), phosphoribosylamine (5-PRA), phosphoribosylaminoimidazole carboxylase / phosphoribosylaminoimidazole succinocarboxamide synthetase (PAICS), phosphoribosylglycinamide formyltransferase / phosphoribosylglycinamide synthetase / phosphoribosylaminoimidazole synthetase (GART), phosphoribosylformylglycinamidine synthase (PFAS), phosphoribosyl pyrophosphate (PRPP), pyrophosphate amidotransferase (PRAT), xanthine monophosphate (XMP)

1. Introduction

De novo purine biosynthesis (DNPS) is one of the oldest and most fundamental biochemical pathways [1]. In mammals, starting with phosphoribosyl pyrophosphate (PRPP), the six enzyme, ten step pathway produces inosine monophosphate (IMP), which is subsequently converted to guanosine monophosphate (GMP) or adenosine monophosphate (AMP) via two more enzymatic reactions. Purines are critical as building blocks and carriers of genetic information in the form of RNA and DNA, intra and intercellular signaling molecules, energy currency, and substrates and co-enzymes. While salvage pathways can produce purine nucleoside monophosphates from free purine bases and PRPP, ultimately, all purines are produced by DNPS. DNPS is upregulated at the G1/S phase and is critical during cellular division [2], most likely to supply purines for DNA replication and elevated RNA transcription. Given its importance in cellular division, and that supply/transport of free purine bases across placental

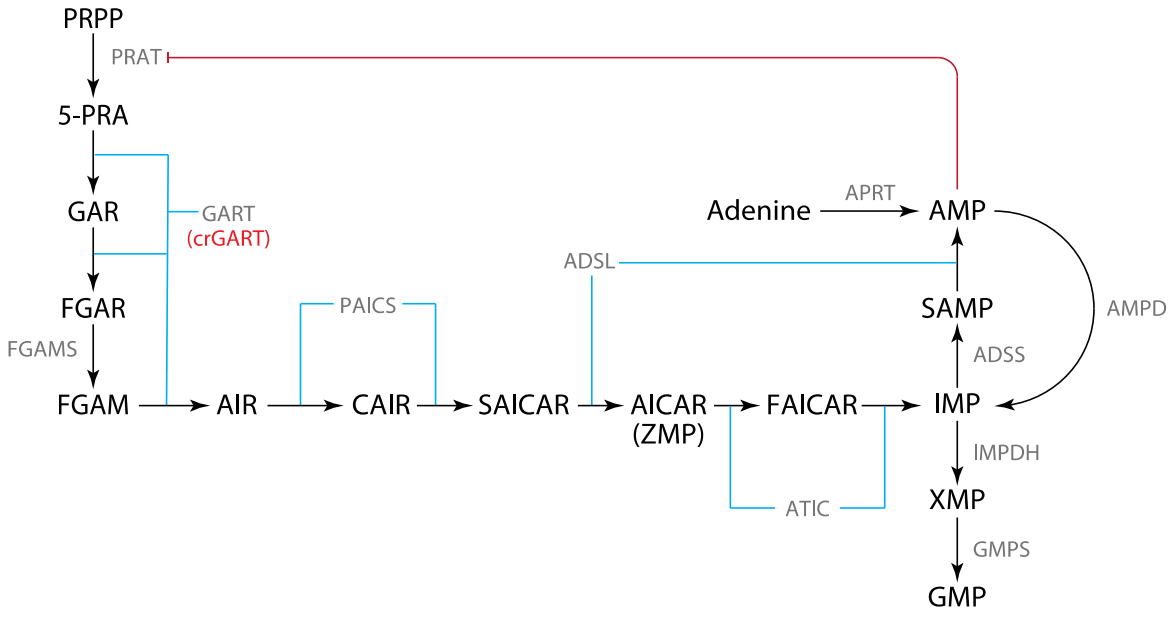

Figure 1. De novo purine synthesis pathway. DNPS mediates the conversion of PRPP to IMP. IMP is subsequently converted to AMP or GMP. The HeLa GART KO, crGART, is indicated.

membranes is inefficient [3,4], DNPS is critical in mammalian development, including embryonic development. In mammals, the trifunctional GART enzyme catalyzes steps 2, 3, and 5 of DNPS (Figure 1). The human gene is located on Hsa21 and is therefore present in three copies in Down syndrome (DS, Trisomy 21), the most common genetic cause of intellectual disability in humans. Triplication of the GART gene has been hypothesized to be related to the pathology

associated with DS [5]. Characterization of the crGART transcriptome should allow identification of pathways in which GART plays a regulatory role. These would then be logical candidates to investigate for relevance to DS.

Functional mutations in DNPS genes are extremely rare in humans. To date, fewer than 100 patients have been identified with adenylosuccinate lyase (ADSL) deficiency, one patient has been identified with AICA-ribosiduria (ATIC deficiency), and two patients (siblings) have been identified with PAICS deficiency [6]. ADSL deficiency is a spectrum disorder with three generalized classes: neonatal fatal, severe, or mild to moderate. Features of ADSL deficiency include seizures, psychomotor retardation, respiratory failure, and craniofacial abnormalities [7]. AICAribosiduria is characterized by mental retardation, blindness, epilepsy, and craniofacial and body dysmorphic features [8]. The siblings identified with PAICS deficiency died within three days of birth and exhibited craniofacial abnormalities and body dysmorphic features [6]. The majority of these mutations are amino acid point mutations resulting in decreased enzymatic activity. Thus far, no functional mutations in GART have been reported. Taken together, these data suggest that defects in DNPS are usually embryonic fatal.

DNPS nulls for ADSL, ATIC, and GART were recently generated in HeLa cells via CRISPR-Cas9 induced mutagenesis [9]. The ADSL and ATIC mutants (crADSL and crATIC) accumulate DNPS pathway intermediates when cultured in purine free media. These intermediates, SAICAR and ZMP respectively, are regulators of transcription, and we have presented evidence for transcriptional regulation via DNPS deficiency and intermediate accumulation ([10] and [11]). We were unable to detect intermediate accumulation in the GART mutant (crGART). This is expected since the initial substrate for GART is phosphoribosylamine (5-PRA), which is extremely unstable under physiological conditions [12]. We hypothesize that changes in transcription due to GART knockout in this cell line are due to the deficit in DNPS, and not intermediate accumulation. To evaluate the crGART transcriptome, we employed RNA-seq to compare the crGART and HeLa transcriptomes in adenine-supplemented and adenine-depleted conditions. Our results indicate that GART may have an important role in embryogenesis, neural development, and perhaps special relevance to Alzheimer's disease and Down syndrome.

2. Materials and Methods

Methods were performed as previously described ([10] and [11]), except as noted.

2.1. Data processing

RNA-seq sequence (FASTQ format by the Genomics and Microarray Core Facility at the University of Colorado, Denver) was aligned to the Ensembl Homo_sapiens.GRCh38.98 transcriptome using salmon version 1.0.0, then differential gene expression analysis was performed using DESeq2 version 1.26.0 with independent hypothesis weighting (IHW) [13] and approximate posterior estimation for GLM coefficients (apeglm) [14], R version 3.5.3.

2.2. ClueGO analysis

The Cytoscape (version 3.7.2) app ClueGO (version 2.5.5) was employed for ontology and pathway analysis of lists of differentially expressed genes (DEGs). A list of 300 DEGs with the top 150 most positive log2 and 150 most negative log2 values was used to query gene ontology (GO) databases (UniProt-GOA_08.01.2020 for Biological process, Cellular component, and Molecular function) and Reactome databases (Pathway_08.01.2020 and Reactions_08.01.2020).

2.3. Panther analysis

Panther Overrepresentation Test analysis via the Gene Ontology Consortium portal (https://www.geneontology.com) was performed to confirm and augment the ClueGO results (Supplementary Figures 1-5). Unlike ClueGO, Panther is capable of using large gene lists. The complete list of significant DEGs was used to query Gene Ontology biological process, cellular component, and molecular function. The "Test Type" option was set for "Fisher's Exact" and "Correction" was set to "Calculate False Discovery Rate". The GO ontology database release date was 2020-02-21.

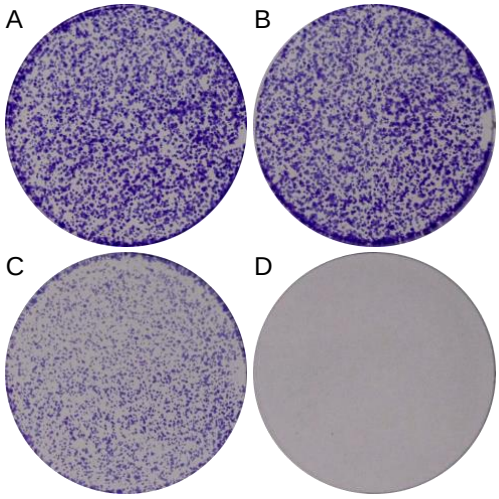

Figure 2: crGART requires adenine for growth. HeLa (A,B) and crGART (C,D) cells were plated and cultured in adenine-supplemented (A,C) or adenine-depleted (B,D) media. Plates were stained with crystal violet.

3. Results

3.1. Adenine is required for proliferative growth of crGART

Given that GART catalyzes three of the ten steps in the DNPS pathway, we hypothesized that crGART cells would exhibit proliferative arrest in adenine-deprived conditions. HeLa and crGART cells were cultured in complete-serum media overnight and then subsequently cultured in media supplemented with dialyzed-serum (FCM) with or without supplemental adenine. HeLa cells showed proliferative growth in both media conditions while crGART showed proliferative growth in only adenine-supplemented conditions (Figure 2).

3.2. GART substrate was not detected in adenine starved crGART cells

Next, we assessed whether the GART substrate 5-PRA accumulates in the crGART cell line. We demonstrated previously that crADSL [10] and crATIC [11] cells accumulate substrate when cultured in adenine free media. crGART cells were cultured as previously described and metabolites extracted. HPLC-EC analysis did not indicate accumulation of 5-PRA (data not shown). This is consistent with previous work demonstrating that 5-PRA is highly unstable and breaks down in approximately five seconds under cellular conditions [12,14]. Since it apparently

does not accumulate substrate, this suggests that crGART is likely useful as a model of general DNPS deficiency.

3.3. Transcriptome analysis of crGART versus HeLa identified differentially expressed genes

To investigate the effect of GART KO on the transcriptome, we compared the crGART and HeLa transcriptomes after culture in adenine-supplemented and adenine-depleted conditions.

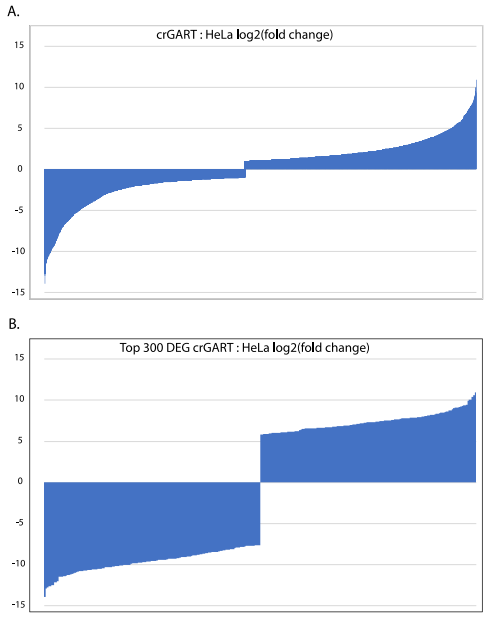

Figure 3: Plot of log(2) values crGART : HeLa DEG fold change. A. All significant DEGs B. Significant DEGs in the "top 300" list (see Methods).

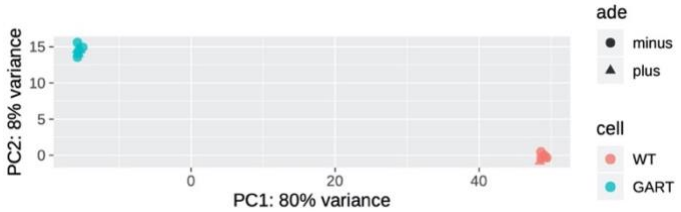

Figure 4: Principle component analysis of crGART and HeLa replicates. PCA shows a robust difference by cell type.

4218 genes were significantly differentially expressed by cell type. This represents a log(2)counts crGART : HeLa fold change range of -13.94 to 10.93 (Figure 3A). In order to assess what processes or pathways are affected, we performed GO and Reactome functional analysis of DEGs. For ClueGO analysis the top 300 most DEG list used represents a log(2)counts fold change range of -13.74 to -7.67 and 5.83 to 10.93 (Figure 3B). Positive values indicate enrichment in crGART and negative log2 values indicate enrichment in HeLa. Principal component analysis shows clustering by cell type and supplementation (Figure 4).

3.4. ClueGO analysis: Functional enrichment analysis employing Gene Ontology and Reactome databases

We queried the Gene Ontology database (Biological process, Cellular component, and Molecular function) as well as the Reactome knowledgebase (Pathways and Reactions) for this analysis. We obtained a very large number of terms but will focus only on terms of special interest to our laboratory (Figure 5). All data is available in supplementary material.

For the GO terms associated with Biological process, 70 GO groups were identified with 548 GO terms. Related GO groups were organized into neural function, development, muscle function, fatty acids, cardiac function, G-protein coupled activity, oxides, and lipids categories. Within neural function, associated terms include neuron migration, dendrites, axon, hippocampus, neuroblast proliferation, synapse, GABA, and catecholamines. DNPS is known to be important for neural function and diseases associated with DNPS exhibit strong neurological phenotypes. Interestingly, another category identified was development. Development includes disparate terms, ranging from organ morphogenesis related terms such as renal, pancreas, and prostate

development, to mesenchyme morphogenesis, stem cell differentiation, alkaline phosphatase activity, blood vessel morphogenesis, epithelial cell differentiation, bone development, endoderm, etc. Placental development included labyrinthine development and female pregnancy. For muscle function terms, we obtained smooth muscle function, myotubes and

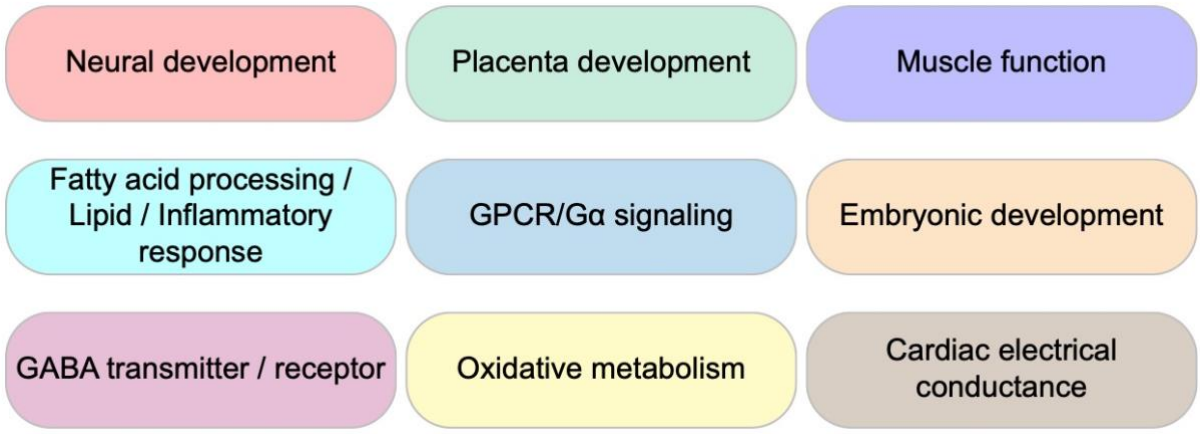

Figure 5: Prominent GO groups from ClueGO analysis

sarcomere organization, vasoconstriction, actin filament movement, and striated and smooth muscle contraction. Cardiac function was also enriched, including sinoatrial and atrioventricular node function terms, as well as atria and ventricle development. Oxidation related terms such as nitric oxide and superoxide metabolism were found. Lipid terms included fatty acids, phospholipase activity, phosphatidyl metabolic process, PI3K, and inflammatory response centered around IL-1 and TNFα. Given the importance of purines in development and neurobiology, the observed enrichment in related terms was expected. However, enrichment in cardiac terms involving electrical conduction was unexpected and may suggest a novel line of inquiry. The enrichment for placental development terms was also unexpected and suggests a purine requirement early in development and that DNPS may play an important role in blastocyst formation.

For the GO terms associated with Cellular component, 12 groups were identified with 26 terms. Terms include voltage gated potassium channels, I-band and intercalated disc, gap junction, GABA synapse and various other synaptic membrane terms.

For the GO terms associated with Molecular function, 25 groups were identified with 54 terms. Term categories included neuronal and ion transmembrane function, development, phospholipid activity, cytokine, etc. In the neuronal category, neuropeptide, acetylcholine receptor, potassium ion gate channels, and neurotransmitter transmembrane transporter terms were prominent. For phospholipids, notable terms included lipase activity, phospholipase (A2, 1, and C) activity, phosphatidylcholine acylhydrolase activity, phosphatidylinositol 3 kinase, and nitric oxide synthase. Enrichment for Syndecan protein binding and G-protein coupled peptide receptor terms is consistent with the G-protein coupled activity category in the Biological process results above.

Reactome Pathways knowledgebase showed 14 groupings of 29 terms. Categories include diseases associated with O-glycosylation of proteins, peptide hormone metabolism, rhodopsin-like receptors, and GPCR and G-alpha signaling. Neuronal and development terms were identified, as well as TFAP-2 which is heavily involved in neural-crest development. Consistent with the GABA terms we obtained in Biological process, we identified GABA receptor and GABA B receptor terms. This suggests that neural deficits associated with DNPS or GART deficiency may be associated with alteration of GABAergic signaling.

Reactome Reactions knowledgebase showed 10 groupings of 24 terms. Term categories included proteoglycans and GPI anchors, GPCR, GEF, and Gi activated ligands. Keratin filaments was also noted.

ClueGO networks, pie charts, and ontology histograms are shown in Supplementary Figures 1-5.

3.5. Corroboration of Gene Ontology analysis via PANTHER analysis

PANTHER analysis with the complete list of significant DEGs was consistent with the ClueGO results and revealed new ontology terms such as DNA and RNA binding, polymerase, and transcription regulation (Supplementary Tables 1).

3.6. Comparison of crATIC, crADSL and crGART DEGs

To get a better sense of which DEGs are due to DNPS deficiency rather than intermediate accumulation, we identified the significant DEGs unique to the crGART vs HeLa comparison. There were 1282 genes that changed significantly in the crGART vs HeLa comparison but did not change in the crADSL vs HeLa [10] and crATIC vs HeLa comparisons [11]. The list of genes was used to query PANTHER via the Gene Ontology web portal (www.geneontology.org). We obtained Biological process ontologies related to RNA splicing and mitochondrion organization (Supplementary Tables 2). It is unclear why these genes are significantly changed only in the crGART cell line. It is possible that the CRISPR Cas9 mutagenesis mediated an off-target change in the genome. Or, the absence of GART may have an effect unrelated, or peripherally related, to DNPS that affects the transcription of these genes.

There are 7745 genes that change significantly in all the mutants vs HeLa. These are likely due to deficient DNPS rather than accumulation of metabolic intermediates. PANTHER functional enrichment analysis returned numerous Biological process ontologies, including regulation of protein localization to plasma membrane, regulation of osteoblast differentiation, positive regulation of I-kappaB kinase/NF-kappaB signaling, heart morphogenesis, establishment of vesicle localization, Ras protein signal transduction, embryonic organ morphogenesis, and many others (Supplementary Tables 3). This result illustrates the importance of DNPS in multiple aspects of cell development and function.

4. Discussion

In this study, we evaluated the purine dietary requirement for crGART and performed HPLC-EC to detect accumulation of metabolites during purine starvation. We compared the crGART and HeLa transcriptomes via RNA-seq analysis in adenine-supplemented and adenine-depleted conditions.

One of the purposes of adenine supplementation is to shut down DNPS. Adenine phosphoribosyltransferase (APRT) catalyzes the conversion of adenine and phosphoribosyl pyrophosphate (PRPP) to AMP and pyrophosphate (PPi). APRT is present in all mammalian tissues and is uniquely responsible for metabolic adenine salvage from dietary sources [15]. Adenine supplementation effectively shuts down DNPS [16,17]. Finally, AMP can be converted to IMP by AMP deaminase (AMPD), and then converted to GMP by IMP dehydrogenase (IMPDH, converts IMP into XMP) and GMP synthase (converts XMP into GMP, see Figure 1) [18].

GART catalyzes three non-sequential reactions in DNPS, steps 2, 3, and 5. The conversion of 5-PRA to GAR is the first reaction catalyzed by GART (specifically the GARS domain of the trifunctional protein [16]). As discussed previously, 5-PRA is extremely unstable under physiological conditions, and is unlikely to accumulate. Our results show that crGART requires purine (adenine) supplementation for proliferative growth, but apparently does not accumulate pathway intermediates during purine starvation. In the crADSL and crATIC DNPS-KO models, metabolic substrates (SAICAR and ZMP respectively) readily accumulate during purine starvation. There is strong evidence that both SAICAR and ZMP alter cellular processes [19-21]. Since the crGART line does not accumulate detectable metabolic intermediates of DNPS during purine starvation, it is likely a useful model of DNPS deficiency in the absence of substrate accumulation.

While the GART gene encodes a trifunctional protein, it also encodes a monofunctional GARS protein via alternative transcription. This alternative transcript includes an intronic polyadenylation signal located in the intron separating the last GARS exon from the first AIRS exon. In human, mouse and Drosophila, this transcript has an in-frame TAA stop codon which is part of the 5' donor splice site. The transcript encodes a GARS protein with sequence identical to the GARS domain in the trifunctional protein. The biological significance of the monofunctional protein has not yet been elucidated. It is possible that GARS overexpression may play a role in elevated purine levels in DS. Monofunctional GARS protein may have a role in release of metabolites from the purine pathway (or purinosome) so they can be used in other metabolic pathways. Or, it may interact directly with PRAT, the first enzyme in the de novo pathway, to facilitate transfer and prevent degradation of (highly unstable) 5-PRA [5]. The crGART cell line should be invaluable for investigating the role of the monofunctional GARS protein.

We considered the possibility of using a PRAT KO cell line as an alternative model of DNPS deficiency. However, to our knowledge, a PRAT KO HeLa cell line is not currently available: the

DNPS null HeLa cell lines include knocked-out GART, PFAS (aka FGAMS), PAICS, ADSL and ATIC. Even if a PRAT KO cell line was available, there are other caveats that would render it an inappropriate model for DNPS deficiency. In humans, the PRAT gene is upstream of the PAICS gene and separated from it by less than 150 nucleotides [based on the coordinates of PRAT (ENSG00000128059) and PAICS (ENSG00000128050), Ensembl release GRCh38.p13]. This suggests that CRISPR-Cas9 mediated mutagenesis of PRAT would likely disrupt transcriptional regulation of the PAICS gene. The substrate for PRAT is PRPP, which participates in several enzymatic reactions, including phosphorylation of nucleosides in purine salvage, pyrimidine synthesis, histidine and tryptophan biosynthesis, NAD biosynthesis, and others [22]. PRAT KO would remove one pathway for catalysis of PRPP and would therefore likely disrupt substrate levels for the other enzymatic reactions involving PRPP. We had previously isolated and reported on a mutant that does not complement PRAT or FGARAT (aka FGAMS) mutants, and still produces 5-PRA using NH_4Cl instead of glutamine as nitrogen donor [23]. This suggests that PRAT KO might be rescued by a compensatory mutation or enzyme activity. Finally, PRAT enzyme activity is reduced by increased AMP concentration [16]. Increased AMP favors the (inactive) dimer form of the enzyme rather than the (active) tetramer form [24].

Our RNA-seq analysis led to identification of numerous DEGs and gene ontologies from ClueGO functional enrichment analysis. Many of these were consistent with our previous analyses of the crADSL and crATIC mutants and are likely to be due to DNPS deficiency. These results support the hypothesis that DNPS is essential in development and that alterations in DNPS and intermediate metabolite accumulation both regulate the transcriptome. This is consistent with previous work demonstrating that DNPS is upregulated in the G1/S phase cell cycle interface [3,4], is critical in embryogenesis which is marked by rapid cellular division, and that purines are essential in development [25] especially in neural development [26]. It is possible that inborn errors of metabolism due to mutation in ADSL, ATIC, and PAICS resulting in decreased enzymatic activity may cause *in utero* defects that lead to cognitive or body dysmorphic phenotypes.

During the course of our analysis, we noted DEGs and gene ontologies potentially relevant to DS. This is perhaps unsurprising given that 1) the GART gene is located on Hsa21 and is triplicated in DS, 2) GART expression is dysregulated in DS and 3) purine levels are also dysregulated in DS [27]. In addition, although GART is triplicated in DS and the current work involves a GART null model, it is likely that pathways affected by absence of GART greatly overlap those affected by increased GART. These ontologies include terms relevant to placental development, neural development and cognition, cardiac development, and Alzheimer disease. We further discuss prominent DEGs and gene ontologies below.

People with DS are at elevated risk for developmental as well as aging related disorders. In addition to intellectual disability, common disorders include hypotonia [28], congenital heart malformation, disease of pulmonary circulation, cardiac arrest, hypotension, infantile spasm, epilepsy, OSA (sleeping disorders), dementia, hypothyroidism, and obesity [29]. Our transcriptomic analysis revealed multiple terms potentially relating GART and DNPS to these disorders. Ontological groupings show enrichment in terms associated with organismal

development, cardiac electrophysiology, neural function/transmission and GABA, placenta, alkaline phosphatase activity, phospholipase activity, Amyloid β, and muscle function.

The placenta is the first and largest fetal organ [30], is derived from fetal and maternal components and is essential for *in utero* development [31]. It is responsible for hormone signaling and transfer of nutrients, gases, and waste between the embryo and the mother, supporting normal fetal growth and development [32]. Placental malformations are common in embryonic lethal mouse lines, which exhibit abnormalities in cardiac, neural, and vascular systems, indicating a strong link between placentation and cardiac and neural development [33].

In trisomy 21 placenta, villus abnormalities, hypovascularity and placental hypoplasia [34,35] have been observed. Labyrinth layers in trisomy 21 placenta were noted to weigh less and exhibit signs of delayed development [36]. About half of newborns with DS typically exhibit congenital heart defects (CHD). These CHDs typically manifest as impaired septum fusion, causing leakage between cardiac chambers [37]. This may be due to impaired placentation [38,39]. Owing to the term enrichment associated with placental formation and function, GART poses a potential lens to investigate embryonic contribution to neurological and cardiac defects associated with DS.

DS is most commonly associated with intellectual disability [29]. Previous work has demonstrated that defects in purine metabolism and/or purine concentration in the developing brain are related to intellectual disabilities [5,25]. DS brains show decreased neuronal density by mass and volume in various brain regions (e.g. cortex, hippocampus, cerebellum), which occurs during development, possibly during gestation [40]. Purinergic signaling regulates axon guidance and growth as well as establishment of correct synaptic contacts [25] and is crucial for CNS development [41]. The GART enzyme exhibits altered spatiotemporal expression in the developing nervous system in DS. Typically, levels of GART are highly expressed in the developing cerebellum and decrease precipitously post-partum. However, in DS, GART levels persist and decrease later in development [5].

Our ClueGO results include terms related to alkaline phosphatase function, specifically through differential expression of ALPP, ALPI, ALPG, and ALPL. ALPP, ALPI, and ALPG show elevated expression in crGART. ALPL, which encodes tissue non-specific alkaline phosphatase (TNAP), is elevated in HeLa. TNAP is a membrane bound extracellular enzyme present in mineralizing bones, renal tissue, and the central nervous system (CNS) [42]. In studies of murine central nervous system (CNS) development TNAP was found to be associated with the neural tube [43] and is highest in early embryonic development associating with neural precursor and progenitor cells [44]. TNAP expression has also been observed during synaptic formation and maturation [45], promoting axonal growth [46]. TNAP also has a role in the hydrolysis of extracellular nucleotides [25,47]. Extracellular ATP was found to induce migration of neural progenitor cells [48], indicating that TNAP may play a role in regulating extracellular ATP pools for migratory events. Another TNAP function is found in the metabolism of Vitamin B6, which is a cofactor for enzymes involved in neurotransmitter (e.g. GABA) synthesis [49]. Not much is

known about the direct role of TNAP in proliferation and differentiation. However, given the function and spatiotemporal expression of TNAP, it is likely that TNAP is directly involved in purinergic signaling or regulation of the extracellular purine pool. Hence, altered TNAP levels may potentially be deleterious during CNS development.

Gamma-amino butyric acid (GABA) is the main inhibitory neurotransmitter in healthy adult brains and has been of particular interest in DS [40,50]. Analysis of fetal brain tissue has shown a smaller hippocampus and decreased GABA neurotransmitters in DS which suggests impaired neurogenesis or migration of GABAergic interneurons [51]. DS patients exhibit an increased incidence of epileptic seizures [52], children exhibit sleep disturbances [53] and hyperactivity [54]. These conditions may be partially due to abnormal GABA signaling. Studies employing DS murine models have shown that altered GABA signaling results in synaptic excitatory/inhibitory signal imbalance, impaired synaptic plasticity [40,50,55], and learning and memory deficits [56,57]. The enrichment for GABA-related terms within our analysis suggests that GART and DNPS may play a role in these processes.

Alzheimer's disease (AD) is a form of dementia characterized by accumulation of neural amyloid β plaques, Tau neurofibrillary tangles, and chronic neural inflammation. Recent work supports the hypothesis that inflammation plays a critical role in AD, and therapeutic intervention designed to reduce neural inflammation shows promise. Inflammation is typically mediated by cytokine and chemokine secretion as well as fatty acid metabolism [58]. Chemokines and cytokines (such as IL-1β and TNFα) are secreted in response to injury and act as proinflammatory signals, resulting in clearance cell recruitment to the damaged tissue [58]. Fatty acid derivatives, specifically lipoxins (derived from ω-6 via phospholipaseA2 and lipoxygenase catalysis of arachidonic acid) [59] and ω-3 metabolites such as maresins, resolvins, and protectins [60] are all potent anti-inflammation mediators. ω-6 fatty acid is typically stored in the phospholipid bilayer as arachidonic acid, which is then cleaved by phospholipase activity and then metabolized via secondary enzymes to eicosanoids and lipoxins [61]. Coincidentally, extracellular ATP signaling plays a crucial role in inflammation through the purinergic P2 and P1 receptors [62]. DNPS and GART levels may play important roles in these processes.

The peroxisome proliferator activated receptor gamma (PPARG, encodes PPARγ) and cluster of differentiation 36 (CD36) genes were highly expressed in the three DNPS-KO transcriptomes that we have characterized (crADSL, crATIC, and crGART), with average log2(fold change) values of approximately 4.5 and 8.5, respectively. PPARγ is an inducible transcription factor and is considered a master regulator in lipid metabolism, with roles in fat, carbohydrate, and general energy metabolism, insulin sensitivity, cell proliferation and differentiation, inflammation, and cancer [63]. CD36 is involved in fatty acid uptake and metabolism [64], inflammation cascade [65], angiogenesis, apoptosis, thrombosis, atherosclerosis, Alzheimer's disease, and insulin resistance [66]. CD36 expression can be regulated by ω3 and ω6 class fatty acids, which increase or decrease CD36 mRNA expression depending on cell type [67,68]. Interestingly, a CD36-PPARγ axis exists, which regulates fatty acid storage, triglyceride synthesis, glucose uptake, and fatty acid metabolism [69]. It is unclear as why crADSL, crATIC, and crGART would

all be robustly expressing these genes, however the results reported here and previously [10,11] suggest a link between DNPS and the CD36-PPARγ axis.

In conclusion, our results indicate that DNPS deficiency affects the cellular transcriptome, significantly altering the expression of over 4000 genes. Our functional enrichment analysis identified ontologies and pathways related to placental development, neural development, cardiac function and inflammation. Many of these ontologies are relevant to DS and AD. This is perhaps unsurprising, since the GART gene is trisomic in DS, purine levels are significantly altered in DS, and almost all people with DS exhibit AD neuropathology by age 40 [28]. We believe that the crGART cell line is an attractive model for DNPS deficiency in the absence of substrate accumulation and will be a valuable tool for investigating the role of DNPS dysregulation in DS and other disorders.

Acknowledgements

The Cancer Center and the Genomics (Microarray) Shared Resource are supported in part by the Cancer Center Support Grant #P30-CA046934 from the National Cancer Institute.

MZ and VB were supported by Charles University [programmes PRIMUS/17/MED/6 and PROGRES Q26/LF1] and by the Ministry of Education, Youth and Sports of CR [LQ1604 National Sustainability Programme II].

Funding

This work was funded by The HBB Foundation, the Sam and Frieda Davis Trust, The Butler Family Fund of the Denver Foundation, and the Lowe Fund of the Denver Foundation.

Conflicts of interests

The authors have no conflicts of interest.

References:

[1] G. Caetano-Anollés, L.S. Yafremava, H. Gee, D. Caetano-Anollés, H.S. Kim, J.E. Mittenthal, The origin and evolution of modern metabolism, Int. J. Biochem. Cell Biol. 41 (2009) 285–297. doi:10.1016/j.biocel.2008.08.022.

[2] A. Fridman, A. Saha, A. Chan, D.E. Casteel, R.B. Pilz, G.R. Boss, Cell cycle regulation of purine synthesis by phosphoribosyl pyrophosphate and inorganic phosphate, Biochem J. 454 (2013) 91–99. doi:10.1042/BJ20130153.

[3] C.Y. Chan, H. Zhao, R.J. Pugh, A.M. Pedley, J. French, S.A. Jones, et al., Purinosome formation as a function of the cell cycle, Proceedings of the National Academy of Sciences. (2015). doi:10.1073/pnas.1423009112.

[4] H. Zhao, C.R. Chiaro, L. Zhang, P.B. Smith, C.Y. Chan, A.M. Pedley, et al., Quantitative Analysis of Purine Nucleotides Indicates Purinosomes Increase de Novo Purine Biosynthesis, Journal of Biological Chemistry. (2015). doi:10.1074/jbc.M114.628701.

[5] G. Brodsky, T. Barnes, J. Bleskan, L. Becker, M. Cox, D. Patterson, The human GARS-AIRS-GART gene encodes two proteins which are differentially expressed during human brain development and temporally overexpressed in cerebellum of individuals with Down syndrome, Hum Mol Genet. 6 (1997) 2043–2050.

[6] A. Pelet, V. Skopova, U. Steuerwald, V. Baresova, M. Zarhrate, J.-M. Plaza, et al., PAICS deficiency, a new defect of de novo purine synthesis resulting in multiple congenital anomalies and fatal outcome, (2019) 1–30. doi:10.1093/hmg/ddz237/5584440.

[7] G. Van Den Berghe, J. Jaeken, Adenylosuccinase deficiency, Adv. Exp. Med. Biol. 195 Pt A (1986) 27–33. doi:10.1007/978-1-4684-5104-7_4.

[8] S. Marie, B. Heron, P. Bitoun, T. Timmerman, G. Van Den Berghe, M.-F. Vincent, AICA-ribosiduria: a novel, neurologically devastating inborn error of purine biosynthesis caused by mutation of ATIC, Am J Hum Genet. 74 (2004) 1276–1281. doi:10.1086/421475.

[9] V. Baresova, M. Krijt, V. Skopova, O. Souckova, S. Kmoch, M. Zikánová, CRISPR-Cas9 induced mutations along de novo purine synthesis in HeLa cells result in accumulation of individual enzyme substrates and affect purinosome formation, Mol Genet Metab. 119 (2016) 270–277. doi:10.1016/j.ymgme.2016.08.004.

[10] R.C. Mazzarino, V. Baresova, M. Zikánová, N. Duval, T.G. Wilkinson, D. Patterson, et al., The CRISPR-Cas9 crADSL HeLa transcriptome: A first step in establishing a model for ADSL deficiency and SAICAR accumulation, Mol Genet Metab Rep. 21 (2019) 100512. doi:10.1016/j.ymgmr.2019.100512.

[11] R.C. Mazzarino, V. Baresova, M. Zikánová, N. Duval, T.G. Wilkinson, D. Patterson, et al., The CRISPR-Cas9 crATIC HeLa transcriptome: Characterization of a novel cellular model for ATIC deficiency and ZMP accumulation, bioRxiv. 8 (2020) 2020.04.28.067066. doi:10.1101/2020.04.28.067066.

[12] J. Rudolph, J. Stubbe, Investigation of the mechanism of phosphoribosylamine transfer from glutamine phosphoribosylpyrophosphate amidotransferase to glycinamide ribonucleotide synthetase, Biochemistry. 34 (1995) 2241–2250. doi:10.1021/bi00007a019.

[13] N. Ignatiadis, B. Klaus, J.B. Zaugg, W. Huber, Data-driven hypothesis weighting increases detection power in genome-scale multiple testing, Nat. Methods. 13 (2016) 577–580. doi:10.1038/nmeth.3885.

[14] A. Zhu, J.G. Ibrahim, M.I. Love, Heavy-tailed prior distributions for sequence count data: removing the noise and preserving large differences, Bioinformatics. 35 (2019) 2084–2092. doi:10.1093/bioinformatics/bty895.

[15] C.H.T.P. Silva, M. Silva, J. Iulek, O.H. Thiemann, Structural complexes of human adenine phosphoribosyltransferase reveal novel features of the APRT catalytic mechanism, J. Biomol. Struct. Dyn. 25 (2008) 589–597. doi:10.1080/07391102.2008.10507205.

[16] E.W. Holmes, J.A. McDonald, J.M. McCord, J.B. Wyngaarden, W.N. Kelley, Human glutamine phosphoribosylpyrophosphate amidotransferase. Kinetic and regulatory properties, J Biol Chem. 248 (1973) 144–150.

[17] A.S. Tu, D. Patterson, Characterization of a guanine-sensitive mutant defective in adenylo-succinate synthetase activity, J. Cell. Physiol. 96 (1978) 123–132. doi:10.1002/jcp.1040960115.
[18] R.W. Watts, Molecular variation in relation to purine metabolism, J Clin Pathol Suppl (R Coll Pathol). 8 (1974) 48–63.
[19] K.E. Keller, Z.M. Doctor, Z.W. Dwyer, Y.-S. Lee, SAICAR induces protein kinase activity of PKM2 that is necessary for sustained proliferative signaling of cancer cells, Mol Cell. 53 (2014) 700–709. doi:10.1016/j.molcel.2014.02.015.
[20] J.M. Corton, J.G. Gillespie, S.A. Hawley, D.G. Hardie, 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur. J. Biochem. 229 (1995) 558–565. doi:10.1111/j.1432-1033.1995.tb20498.x.
[21] G.P. Meares, H. Qin, Y. Liu, A.T. Holdbrooks, E.N. Benveniste, AMP-activated protein kinase restricts IFN-γ signaling, J Immunol. 190 (2013) 372–380. doi:10.4049/jimmunol.1202390.
[22] B. Hove-Jensen, K.R. Andersen, M. Kilstrup, J. Martinussen, R.L. Switzer, M. Willemoës, Phosphoribosyl Diphosphate (PRPP): Biosynthesis, Enzymology, Utilization, and Metabolic Significance, Microbiol. Mol. Biol. Rev. 81 (2017). doi:10.1128/MMBR.00040-16.
[23] D.C. Oates, D. Vannais, D. Patterson, A mutant of CHO-K1 cells deficient in two nonsequential steps of de novo purine biosynthesis, Cell. 20 (1980) 797–805. doi:10.1016/0092-8674(80)90326-8.
[24] E.W. Holmes, J.B. Wyngaarden, W.N. Kelley, Human glutamine phosphoribosylpyrophosphate amidotransferase. Two molecular forms interconvertible by purine ribonucleotides and phosphoribosylpyrophosphate, J Biol Chem. 248 (1973) 6035–6040.
[25] M. Fumagalli, D. Lecca, M.P. Abbracchio, S. Ceruti, Pathophysiological Role of Purines and Pyrimidines in Neurodevelopment: Unveiling New Pharmacological Approaches to Congenital Brain Diseases, Front. Pharmacol. 8 (2017) 941. doi:10.3389/fphar.2017.00941.
[26] R.J. Rodrigues, J.M. Marques, R.A. Cunha, Purinergic signalling and brain development, Seminars in Cell & Developmental Biology. 95 (2019) 34–41. doi:10.1016/j.semcdb.2018.12.001.
[27] D. Patterson, Molecular genetic analysis of Down syndrome, Hum Genet. 126 (2009) 195–214. doi:10.1007/s00439-009-0696-8.
[28] I.T. Lott, Neurological phenotypes for Down syndrome across the life span, Prog. Brain Res. 197 (2012) 101–121. doi:10.1016/B978-0-444-54299-1.00006-6.
[29] M. Alexander, H. Petri, Y. Ding, C. Wandel, O. Khwaja, N. Foskett, Morbidity and medication in a large population of individuals with Down syndrome compared to the general population, Dev Med Child Neurol. 58 (2016) 246–254. doi:10.1111/dmcn.12868.
[30] M.Y. Turco, A. Moffett, Development of the human placenta, Development. 146 (2019). doi:10.1242/dev.163428.
[31] G.J. Burton, E. Jauniaux, What is the placenta? Am J Obstet Gynecol. 213 (2015) S6.e1–S6–8. doi:10.1016/j.ajog.2015.07.050.

[32] N.M. Gude, C.T. Roberts, B. Kalionis, R.G. King, Growth and function of the normal human placenta, Thromb. Res. 114 (2004) 397–407. doi:10.1016/j.thromres.2004.06.038.

[33] V. Perez-Garcia, E. Fineberg, R. Wilson, A. Murray, C.I. Mazzeo, C. Tudor, et al., Placentation defects are highly prevalent in embryonic lethal mouse mutants, Nature. 555 (2018) 463–468. doi:10.1038/nature26002.

[34] F. Debieve, A. Moiset, K. Thomas, S. Pampfer, C. Hubinont, Vascular endothelial growth factor and placenta growth factor concentrations in Down's syndrome and control pregnancies, Mol. Hum. Reprod. 7 (2001) 765–770. doi:10.1093/molehr/7.8.765.

[35] F. Qureshi, S.M. Jacques, M.P. Johnson, R.F. Hume, R.L. Kramer, Y. Yaron, et al., Trisomy 21 placentas: histopathological and immunohistochemical findings using proliferating cell nuclear antigen, Fetal. Diagn. Ther. 12 (1997) 210–215. doi:10.1159/000264470.

[36] A.D. Adams, F. Guedj, D.W. Bianchi, Placental development and function in trisomy 21 and mouse models of Down syndrome: Clues for studying mechanisms underlying atypical development, Placenta. 89 (2020) 58–66. doi:10.1016/j.placenta.2019.10.002.

[37] S.B. Freeman, L.F. Taft, K.J. Dooley, K. Allran, S.L. Sherman, T.J. Hassold, et al., Population-based study of congenital heart defects in Down syndrome, Am. J. Med. Genet. 80 (1998) 213–217.

[38] C.L. Maslen, Recent Advances in Placenta-Heart Interactions, Front Physiol. 9 (2018) 735. doi:10.3389/fphys.2018.00735.

[39] U. Radhakrishna, S. Albayrak, R. Zafra, A. Baraa, S. Vishweswaraiah, A.M. Veerappa, et al., Placental epigenetics for evaluation of fetal congenital heart defects: Ventricular Septal Defect (VSD), PLoS ONE. 14 (2019) e0200229. doi:10.1371/journal.pone.0200229.

[40] A. Contestabile, S. Magara, L. Cancedda, The GABAergic Hypothesis for Cognitive Disabilities in Down Syndrome, Front Cell Neurosci. 11 (2017) 54. doi:10.3389/fncel.2017.00054.

[41] H.A. Jinnah, R.L. Sabina, G. Van Den Berghe, Metabolic disorders of purine metabolism affecting the nervous system, Handb Clin Neurol. 113 (2013) 1827–1836. doi:10.1016/B978-0-444-59565-2.00052-6.

[42] Á. Sebastián-Serrano, L. de Diego-García, C. Martínez-Frailes, J. Avila, H. Zimmermann, J.L. Millán, et al., Tissue-nonspecific Alkaline Phosphatase Regulates Purinergic Transmission in the Central Nervous System During Development and Disease, Comput Struct Biotechnol J. 13 (2015) 95–100. doi:10.1016/j.csbj.2014.12.004.

[43] S. Narisawa, H. Hasegawa, K. Watanabe, J.L. Millán, Stage-specific expression of alkaline phosphatase during neural development in the mouse, Dev Dyn. 201 (1994) 227–235. doi:10.1002/aja.1002010306.

[44] D. Langer, Y. Ikehara, H. Takebayashi, R. Hawkes, H. Zimmermann, The ectonucleotidases alkaline phosphatase and nucleoside triphosphate diphosphohydrolase 2 are associated with subsets of progenitor cell populations in the mouse embryonic, postnatal and adult neurogenic zones, Neuroscience. 150 (2007) 863–879. doi:10.1016/j.neuroscience.2007.07.064.

[45] C. Fonta, L. Negyessy, L. Renaud, P. Barone, Postnatal development of alkaline phosphatase activity correlates with the maturation of neurotransmission in the cerebral cortex, J. Comp. Neurol. 486 (2005) 179–196. doi:10.1002/cne.20524.
[46] M. Díez-Zaera, J.I. Díaz-Hernández, E. Hernández-Álvarez, H. Zimmermann, M. Díaz-Hernández, M.T. Miras-Portugal, Tissue-nonspecific alkaline phosphatase promotes axonal growth of hippocampal neurons, Mol. Biol. Cell. 22 (2011) 1014–1024. doi:10.1091/mbc.E10-09-0740.
[47] H. Zimmermann, M. Zebisch, N. Sträter, Cellular function and molecular structure of ecto-nucleotidases, Purinergic Signal. 8 (2012) 437–502. doi:10.1007/s11302-012-9309-4.
[48] K. Striedinger, P. Meda, E. Scemes, Exocytosis of ATP from astrocyte progenitors modulates spontaneous Ca2+ oscillations and cell migration, Glia. 55 (2007) 652–662. doi:10.1002/glia.20494.
[49] A. Amadasi, M. Bertoldi, R. Contestabile, S. Bettati, B. Cellini, M.L. di Salvo, et al., Pyridoxal 5'-phosphate enzymes as targets for therapeutic agents, Curr. Med. Chem. 14 (2007) 1291–1324. doi:10.2174/092986707780597899.
[50] G. Deidda, I.F. Bozarth, L. Cancedda, Modulation of GABAergic transmission in development and neurodevelopmental disorders: investigating physiology and pathology to gain therapeutic perspectives, Front Cell Neurosci. 8 (2014) 119. doi:10.3389/fncel.2014.00119.
[51] H.-Q. Huo, Z.-Y. Qu, F. Yuan, L. Ma, L. Yao, M. Xu, et al., Modeling Down Syndrome with Patient iPSCs Reveals Cellular and Migration Deficits of GABAergic Neurons, Stem Cell Reports. 10 (2018) 1251–1266. doi:10.1016/j.stemcr.2018.02.001.
[52] I.T. Lott, M. Dierssen, Cognitive deficits and associated neurological complications in individuals with Down's syndrome, Lancet Neurol. 9 (2010) 623–633. doi:10.1016/S1474-4422(10)70112-5.
[53] M. Carter, E. McCaughey, D. Annaz, C.M. Hill, Sleep problems in a Down syndrome population, Arch. Dis. Child. 94 (2009) 308–310. doi:10.1136/adc.2008.146845.
[54] S.M. Pueschel, J.C. Bernier, J.C. Pezzullo, Behavioural observations in children with Down's syndrome, Journal of Intellectual Disability Research. 35 (2008) 502–511. doi:10.1111/j.1365-2788.1991.tb00447.x.
[55] T. Begenisic, L. Baroncelli, G. Sansevero, M. Milanese, T. Bonifacino, G. Bonanno, et al., Fluoxetine in adulthood normalizes GABA release and rescues hippocampal synaptic plasticity and spatial memory in a mouse model of Down syndrome, Neurobiol Dis. 63 (2014) 12–19. doi:10.1016/j.nbd.2013.11.010.
[56] A.C.S. Costa, M.J. Grybko, Deficits in hippocampal CA1 LTP induced by TBS but not HFS in the Ts65Dn mouse: a model of Down syndrome, Neurosci Lett. 382 (2005) 317–322. doi:10.1016/j.neulet.2005.03.031.
[57] A.M. Kleschevnikov, P.V. Belichenko, A.J. Villar, C.J. Epstein, R.C. Malenka, W.C. Mobley, Hippocampal long-term potentiation suppressed by increased inhibition in the Ts65Dn mouse, a genetic model of Down syndrome, Journal of Neuroscience. 24 (2004) 8153–8160. doi:10.1523/JNEUROSCI.1766-04.2004.

[58] L. Chen, H. Deng, H. Cui, J. Fang, Z. Zuo, J. Deng, et al., Inflammatory responses and inflammation-associated diseases in organs, Oncotarget. 9 (2018) 7204–7218. doi:10.18632/oncotarget.23208.
[59] M.A. Sugimoto, L.P. Sousa, V. Pinho, M. Perretti, M.M. Teixeira, Resolution of Inflammation: What Controls Its Onset? Front Immunol. 7 (2016) 160. doi:10.3389/fimmu.2016.00160.
[60] C.N. Serhan, N. Chiang, J. Dalli, B.D. Levy, Lipid mediators in the resolution of inflammation, Cold Spring Harbor Perspectives in Biology. 7 (2014) a016311. doi:10.1101/cshperspect.a016311.
[61] V.S. Hanna, E.A.A. Hafez, Synopsis of arachidonic acid metabolism: A review, J Adv Res. 11 (2018) 23–32. doi:10.1016/j.jare.2018.03.005.
[62] D.J. Kominsky, E.L. Campbell, S.P. Colgan, Metabolic shifts in immunity and inflammation, J Immunol. 184 (2010) 4062–4068. doi:10.4049/jimmunol.0903002.
[63] A.J. Kroker, J.B. Bruning, Review of the Structural and Dynamic Mechanisms of PPARγ Partial Agonism, PPAR Res. 2015 (2015) 816856. doi:10.1155/2015/816856.
[64] M.Y. Pepino, O. Kuda, D. Samovski, N.A. Abumrad, Structure-function of CD36 and importance of fatty acid signal transduction in fat metabolism, Annu. Rev. Nutr. 34 (2014) 281–303. doi:10.1146/annurev-nutr-071812-161220.
[65] O. Kuda, C.M. Jenkins, J.R. Skinner, S.H. Moon, X. Su, R.W. Gross, et al., CD36 protein is involved in store-operated calcium flux, phospholipase A2 activation, and production of prostaglandin E2, Journal of Biological Chemistry. 286 (2011) 17785–17795. doi:10.1074/jbc.M111.232975.
[66] R.L. Silverstein, M. Febbraio, CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior, Sci Signal. 2 (2009) re3. doi:10.1126/scisignal.272re3.
[67] J.-C. Vallvé, K. Uliaque, J. Girona, A. Cabré, J. Ribalta, M. Heras, et al., Unsaturated fatty acids and their oxidation products stimulate CD36 gene expression in human macrophages, Atherosclerosis. 164 (2002) 45–56. doi:10.1016/s0021-9150(02)00046-1.
[68] A. Pietsch, W. Erl, R.L. Lorenz, Lovastatin reduces expression of the combined adhesion and scavenger receptor CD36 in human monocytic cells, Biochem. Pharmacol. 52 (1996) 433–439. doi:10.1016/0006-2952(96)00245-6.
[69] L. Maréchal, M. Laviolette, A. Rodrigue-Way, B. Sow, M. Brochu, V. Caron, et al., The CD36-PPARγ Pathway in Metabolic Disorders, Int J Mol Sci. 19 (2018). doi:10.3390/ijms19051529.

Author Contributions

R.M., D.P., and G.V. designed the experiments. R.M. performed the ClueGO analyses, and was primary author of the manuscript. V.B. and M.Z. created and provided the CRISPR-Cas9 GART KO HeLa cell line, crGART. N.D. performed HPLC-EC analysis to detect DNPS metabolites and intermediates. T.G.W. provided sample preparation expertise. G.V. performed salmon and DESeq2 processing of fastq sequence and provided informatics support. G.V. and D.P. directed and supervised the team members and manuscript preparation. All authors reviewed and edited the manuscript.

Competing interests

The author(s) declare no competing interests.